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Modelling the airway
epithelium *in vitro* as a tool
for understanding pulmonary
innate defence mechanisms.

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Thesis submitted for the degree of Doctor of Philosophy

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December 2015

Abstract

The airway epithelium forms a continuous barrier from the nose to the alveoli and serves a variety of functions. Multiple functionally distinct cell types are involved in these processes. The innate defence functions require a patent airway epithelium, with infections often associated with epithelial defects and phenotypic alterations that are themselves associated with multiple lung diseases. Non-typeable *Haemophilus influenzae* (NTHi) and respiratory syncytial virus (RSV) are frequently identified in the airways in a range of respiratory diseases. These pathogens often trigger exacerbations and worsening symptoms that often result in hospitalisation. This is particularly true in paediatric populations. Although mortality for NTHi and RSV infections alone are themselves low it remains unclear what role these infections play in mortality rates in complex chronic respiratory infections. These studies aimed to establish NTHi and RSV infections within airway epithelium models, and use them as tools to study pulmonary innate defence mechanisms in order to understand the role of these infections in respiratory disease.

In vitro airway models were established using lung derived cell lines, undifferentiated primary human bronchial epithelial (uHBE) cells and air-liquid interface (ALI) differentiated uHBE cell cultures. Following establishment of differentiation we validated ALI cultures using a number of markers, including for the putative innate defence PLUNC family proteins, gel-forming mucins and tubulin. These markers are representative of different epithelial cell types within the cultures. Cultures were infected with NTHi or RSV for periods of time ranging from 1 hour to 7 days with a view to establishing chronic infections and allowing biofilm formation. Monolayer cultures showed an enhanced susceptibility to both infections. Cytokine array profiling showed enhanced pro-inflammatory cytokine profiles in response to NTHi and RSV infections in ALI cells resulting in an ability to manage infections compared to monolayer cultures. Expression analysis indicated that both infections altered the transcription of a number of pro-inflammatory genes. Neutrophil products and trypsin were shown to degrade PLUNC proteins in ALI cell secretions. NTHi also appeared to cause degradation of PLUNC proteins suggesting that infection may impair the innate defence shield of the airway epithelium. Our data showed that differential ALI cultures of human airway cells are a useful model for the study of respiratory pathogens.

Acknowledgements

First I would like to thank my supervisors Dr Colin Bingle, Dr Lynne Bingle and Professor Mark Everard for giving me the opportunity to undertake this PhD. I am glad that I had the opportunity to work with you and wish to thank you for all the support and guidance you have provided.

I would also like to thank my friends and colleagues in the Department of Infection and Immunity, who created a friendly work environment and offered help when necessary. I offer particular thanks to the technical staff, especially Vanessa and Karen for your expertise, advice and friendship. And thank you to Jamil, Pran and Selina, my fellow PhD students, for the laughs, banter and cake when they were needed the most.

Thank you to my family who, despite not understanding much about what I do, were still on hand to offer encouragement when needed.

And most importantly, thank you to my wonderful husband Andy for your patience, support and encouragement and motivating me when I needed to get things done. I wouldn't have gotten to where I am today if it hadn't been for your support and for that I am extremely grateful.

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Publications and Conference Abstracts

Publications arising from work presented in this thesis

Bingle, C. D., **Wilson, K**, Lunn, H, Barnes, F.A., High A.S., Wallace, W.A., Rassl,D, Campos, M.A., Ribeiro, M, Bingle, L. (2010). Human LPLUNC1 is a secreted product of goblet cells and minor glands of the respiratory and upper aerodigestive tracts. *Histochem Cell Biol.* 133 (5): 505-515

Ugonna, K., Bingle, C.D., Plant, K, **Wilson, K**, Everard M.L. (2014). Macrophages are required for dendritic cell uptake of respiratory syncytial virus from an infected epithelium. *PLos One.* 9 (3): e91855.

Conference Abstracts

Wilson, K, Bingle, L, Bingle, C.D., Everard, M.L. Differential responses of monolayer and differentiated airway epithelial cell cultures to NTHi infection. *Eur Respir J* 2012; 40: Suppl. 56, P4116. Vienna, Austria.

Abbreviations

AcP	Accessory Protein
ALI	Air-Liquid Interface
AMP	Adenosine monophosphate
AP-1	Activator Protein-1
BAL	Bronchoalveolar Lavage
BEGM	Bronchial Epithelial Growth Medium
BHI	Brain Heart Infusion
BPI	Bactericidal Permeability Increasing
BPIF	BPI-Fold Containing
CAM	Cellular Adhesion Molecules
CARD	Caspase Recruitment Domain
CCSP	Clara Cell Secretory Protein
cDNA	Complementary Deoxyribonucleic Acid
CF	Cystic Fibrosis
cfu	Colony Forming Units
CMV	Cytomegalovirus
COPD	Chronic Obstructive Pulmonary Disease
CREB	Cyclic AMP Responsive Element Binding Protein
CREBBP	CREB Binding Protein
DAMP	Damage Associated Molecular Pathogen
DAPI	4',6-diamidino-2-phenylindole
DD	Death Domain
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
dsRNA	Double Stranded RNA
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EPS	Extracellular Polysaccharide
ERK	Extracellular Signal Regulated Kinase
FBS	Foetal Bovine Serum
FoxJ1	Forkhead Box Protein J1
GAS	IFN γ Associated Sequence
GFP	Green Fluorescent Protein
HAAC	Heparin Agarose Affinity Chromatography
Hap	Haemophilus Adherence and Penetration
HBE	Human Bronchial Epithelial
Hia	<i>Haemophilus influenzae</i> Adhesin
Hib	<i>Haemophilus influenzae</i> Type b
HMW	High Molecular Weight
HPV	Human Papilloma Virus
HRP	Horse-Radish Peroxidase
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
IKK	I κ B Kinase
IL	Interleukin
IL-1R	Interleukin-1 Receptor
IMS	Industrial Methylated Spirits
IP	IFN- γ Induced Protein
IRAK	IL-1R-Associated Kinases

IRF	IFN Regulatory Factors
IRSE	IFN Stimulated Response Elements
ISG	IFN Stimulated Genes
JEM	Japanese Encephalitis Virus
JNK	Jun N-terminal Kinase
LF	Lactoferrin
LIF	Leukocyte Inhibitory Factor
LOS	Lipooligosaccharide
LPLUNC1	Long Palate Lung and Nasal Epithelium Clone 1
LPS	Lipopolysaccharide
LYS	Lysozyme
MAP1B	Microtubule-Associated Protein 1B
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK/MAP3K	Mitogen Activated Protein Kinase Kinase Kinase
MCP	Macrophage Chemotactic Protein
MDA	Melanoma Differentiation-Associated Gene
MMP	Matrix Metalloproteinase
MOI	Multiplicity of Infection
mRNA	Messenger RNA
mTECs	Murine Tracheal Epithelial Cells
Muc	Mucin
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NDV	Newcastle Disease Virus
NET	Neutrophil Extracellular Trap
NF	Nuclear Factor
NIK	NF- κ B Inducing Kinase
NK	Natural Killer
NTHi	Non-Typeable <i>Haemophilus influenzae</i>
OMP	Outer Membrane Protein
ORF	Open Reading Frame
PAF	Platelet Activating Factor
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
pfu	Plaque Forming Units
PLUNC	Palate, Lung and Nasal Epithelium Clone
PMN	Polymorphonuclear
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidene Fluoride
RANTES	<u>R</u> egulated upon <u>A</u> ctivation in <u>N</u> ormal <u>T</u> -cell, <u>E</u> xpressed and <u>S</u> ecreted
RD	Repressor Domain
RHD	Rel-Homology Domain
RHIM	RIP Homotypic Interaction Motif
RIG	Retinoic Acid Inducible Gene
RIP	Receptor Interacting Protein
RNA	Ribonucleic Acid
ROCK	Rho Kinase
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial Virus

RV	Rhinovirus
SAPK	Stress-Activated Protein Kinase
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SeV	Sendai Virus
SLPI	Secretory Leukoprotease Inhibitor
SPLUNC1	Short Palate Lung and Nasal Epithelium Clone 1
ssRNA	Single Stranded RNA
STAT	Signal Transducer and Activator of Transcription
SV40	Simian Virus 40
TAB	TAK1-Binding Protein
TACE	TNF- α Converting Enzyme
TAD	Transactivation Domain
TAE	Tris-Acetate-EDTA
TAK	TGF β -Activated Kinase
TBK1	TANK Binding Kinase 1
TBS	Tris Buffered Saline
TGFβ	Transforming Growth Factor β
TIMP	Tissue Inhibitor of Matrix Metalloproteinases
TIR	TLR/IL-1R
TLR	Toll-Like Receptor
TNF	Tissue Necrosis Factor
TNFR	TNF Receptor
TRADD	TNF-Receptor-Associated Protein with a Death Domain
TRAF	TNF Receptor-Associated Factor
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR Domain-Containing Adaptor Inducing IFN- β
uHBE	undifferentiated Human Bronchial Epithelial Cells
VSV	Vesicular Stomatitis Virus
β-NAD	β -Nicotinamide Adenine Dinucleotide

1. Introduction

1.1. The Human Airway Epithelium

The human airway epithelium forms a continuous lining of the entire respiratory tract from the cartilaginous trachea to the gas exchanging alveoli. From the trachea, through the bronchi, the respiratory bronchioles and finally the alveolar ducts, the airways gradually decreasing in diameter (Empey 1978). The cellular structure of the airway at these differing regions changes according to the function of the particular section. The airway epithelium is an integral part of the airway system, with defence mechanisms to protect the respiratory surface from invading pathogens or foreign particles, which we inhale constantly. Patients with a 'diseased' airway are often more susceptible to infections from these inhaled pathogens than 'normal' airways due to the effect of alterations of cellular structure on the innate defence mechanisms.

1.1.1. Structure of the Airway Epithelium

Predominantly the airway epithelium is a ciliated, pseudostratified, columnar epithelium broadly consisting of ciliated, basal and secretory cell types in the proximal conducting epithelium (Spina 1998). The epithelium is deemed pseudostratified as the multi-level arrangement of the cells' nuclei makes it appear stratified despite all cells being in contact with the basement membrane. As the airway narrows so does the thickness of this epithelium until it is a single layer of roughly cuboidal cells in the distal gas exchanging epithelium (Empey 1978) (See Figure 1.1).

1.1.1.1. Basal Cells

Basal cells are characteristically the smallest cells of the airway epithelium, with a large nucleus to cytoplasmic ratio and a densely staining nucleus. There are few cytoplasmic organelles, secretory granules or cilia; there are, however bundles of low molecular weight cytokeratin (Hicks, Hall et al. 1997) which function in cell adhesion to the basal lamina and other cells. Basal cell morphology differs depending on the height of the epithelium. In a tall epithelium, the basal cells have higher levels of keratin filaments than shorter epithelium. The primary role of basal cells is to interact with the basal lamina and columnar epithelium to adhere the cells of the airway epithelium together.

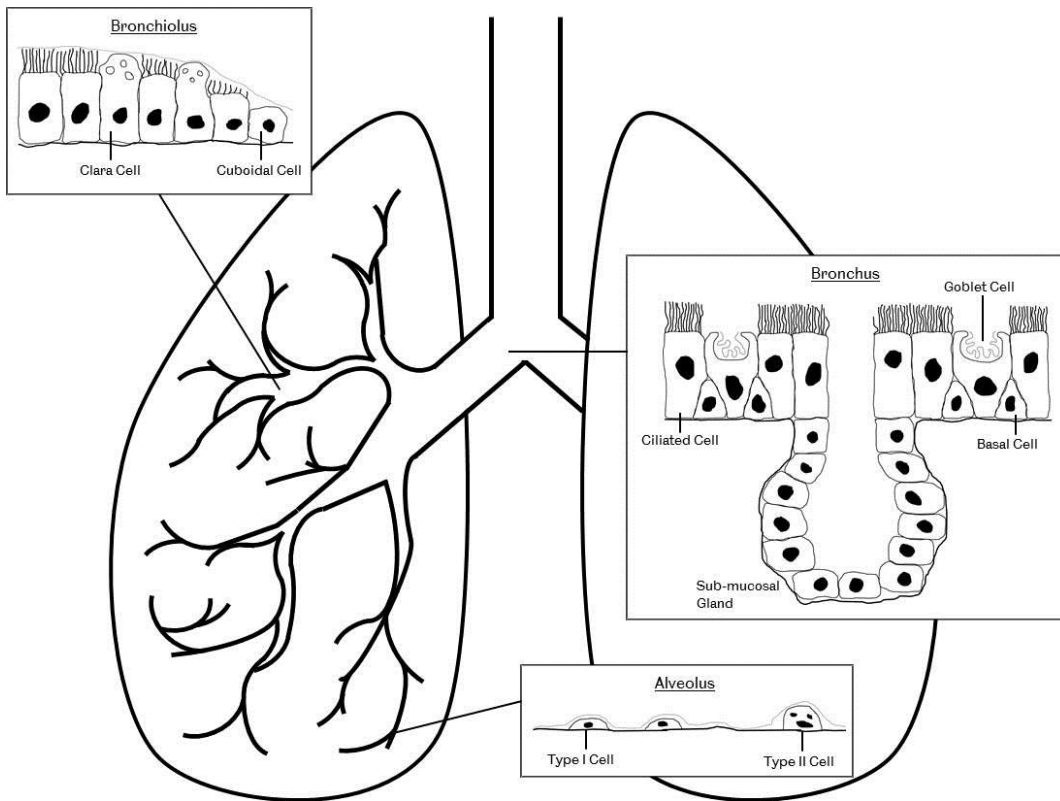


Figure 1.1: The cell types of the airway epithelium

The variety of cell types present in the airway decreases with size. The larger bronchus contains basal, goblet and ciliated cells and is also the location of most sub-mucosal glands. The smaller bronchiolus consists mainly of ciliated and Clara cells, eventually becoming a single layer of cuboidal cells in the very small airways (respiratory bronchioles). Finally, the respiratory surface of the alveolus consists of Type I and Type II alveolar cells.

This interaction occurs via hemidesmosomal attachments that use anchoring filaments and fibrils to form bonds with keratin in the basement membrane (Green and Jones 1996), acting as a tether, a feature only found on basal cells in the airway epithelium (Evans, Cox et al. 1990). These attachments are strong, as the basal cells are the only cell type that firmly attaches to the basal lamina. They are also responsible for the binding of all other cell types; however these do not bind to the basal lamina so the basal cell is an important mediator. Tracheal columnar cells bind to the basal cells as well as neighbouring cells using desmosomal attachments; a trans-membrane complex belonging to the cadherin family consisting of cell adhesion and linking proteins that tether intracellular keratin to the cell surface adhesion proteins allowing cells to bind to each other (Green and Jones 1996). Desmosomal attachment structure numbers may be

related to the level of physical stress on the epithelium as well as its adhesiveness; as it appears that there are the same amounts of hemidesmosomal attachment structures per cell (Evans, Cox et al. 1990). Therefore basal cells are important to the integrity of the airway epithelium as they differentiate with respect to the height of the epithelium, as well as the number of desmosomal attachments and the density of the keratin filaments. Basal cells are also thought to secrete various cytokines, 15-lipoxygenase and neutral endopeptidase as well as being the primary cell type from which goblet and ciliated cells originate (Boers, Ambergen et al. 1998; Knight and Holgate 2003).

1.1.1.2. Secretory Cells

The secretory epithelial cell types consist of mucous (goblet) cells, serous cells and Clara (Club) cells. The main characteristic of the goblet cell is membrane-bound, electron-lucent, acidic, mucin (Muc) granules that are secreted throughout the airway. Goblet cells are the predominant source of mucin secretion in the non-cartilaginous airways alongside Clara cells, which have been shown to produce mucins in low levels in mice (Evans, Williams et al. 2004; Zhu, Ehre et al. 2008). Sub-mucosal glands found in the cartilaginous airways also secrete mucus (Empey 1978). Mucins are the major component of mucus, which is necessary for the trapping of invading pathogens and foreign particles for removal before they can cause damage to the airway epithelium and preventing tissues from drying out. Goblet cells are involved in regulating the amount of mucins secreted as well as the acidity and viscosity of the mucus, all important mediators in generating functional mucus. Normal human airways have approximately 6800 mucus secreting cells per mm² of epithelial surface (Knight and Holgate 2003), which change in shape and size depending on the proliferation of other epithelial cells (Rogers 2003). In some circumstances goblet cells are thought to be progenitors for ciliated cells (Patel, Brody et al. 2011) and can self-renew.

Another type of cell classed as secretory, is the serous cell. These have a similar morphology to goblet cells but their granules are electron-dense rather than electron-lucent (Rogers 2003). Serous secretions act as a lubricant to reduce friction generated by muscle movement in the lungs.

The final major type of secretory cell type is the Clara cell. Clara cells are the predominant secretory cell type in the bronchioles (Thompson, Robbins et al. 1995). They contain electron-dense granules of approximately 500-600nm and are thought to

secrete mostly bronchiolar surfactant and Clara Cell Secretory Protein (CCSP) rather than mucins (Rogers 2003; Evans, Williams et al. 2004).

1.1.1.3. Ciliated Cells

The most predominant cell type throughout the airway epithelium is the ciliated columnar epithelial cell, which arises from basal cells. Each cell has up to 300 cilia (Knight and Holgate 2003), which beat in a synchronized way at more than 100 beats per minute (Empey 1978), to sweep mucus away from the peripheral airways and towards the trachea as part of the mucociliary clearance process. It has been suggested in various studies that ciliated and goblet cells share a common lineage and will trans-differentiate between the cell types, thus remodelling the airway epithelium. A recent study conducted by Turner et al (2011) showed trans-differentiation of ciliated cells to goblet cells by fluorescent tagging of the forkhead box protein J1 (FoxJ1) gene, a ciliated cell specific protein required for motile cilia (Yu, Ng et al. 2008). Using lentiviral constructs, they inserted a Cre recombinase under the control of the FoxJ1 promoter and floxed the stop sequence to prevent expression of the inserted green fluorescent protein (GFP) under the control of a cytomegalovirus (CMV) promoter. FoxJ1 is expressed in ciliated cells, so upon differentiation and expression of FoxJ1, Cre was also expressed, which irreversibly removed the stop sequence to allow expression of the GFP tag, therefore labelling the ciliated cell lineage green (Patel, Brody et al. 2011; Turner, Roger et al. 2011). It had previously been shown that cells cultured in the presence of interleukin (IL)-13 developed more goblet cells (Tyner, Kim et al. 2006), so after this period of differentiation, cells were exposed to IL-13 to change the proportions of cell types during a further differentiation period. Turner et al (2011) found expression of green fluorescence in goblet cells that also stained positively for MUC5AC, showing trans-differentiation of FoxJ1 expressing ciliated cells to goblet cells.

1.1.2. Function of the Airway Epithelium

The major function associated with the airway epithelium is that of protection, it is the first line of defence against foreign invading particles. The junctional mechanisms holding the various cell types together mean that the airway epithelium also forms a protective barrier. Junctional mechanisms comprise 3 aspects; zonula adherens which are involved in adhesion and recognition through use of cellular adhesion molecules (CAM), desmosomes which as previously mentioned are involved in conserving the integrity of the epithelium and finally tight junctions which provide an effective

physical barrier against invading pathogens (Thompson, Robbins et al. 1995). The mucociliary phenotype of the airway epithelium protects the lower/smaller airways from infection by trapping foreign particles (including bacteria and viruses) in mucus secreted by the goblet cells and sub-mucosal glands. The mucus, complete with trapped particles, is then transported up towards the trachea, and the oropharynx by the beating of the cilia, where it is either swallowed or expectorated. The epithelium also secretes various antimicrobials (including lactoferrin (LF), defensins and cathelicidin), anti-proteases (including secretory leukoprotease inhibitor, elafin and alpha-1-antitrypsin) and antioxidants as additional protective mechanism that aid in the trapping and clearance of foreign particles (Thompson, Robbins et al. 1995).

1.1.2.1. Airway Lining Fluid

The airway lining fluid (mucus) is crucial for maintaining hydration and protection of the airway epithelium. It broadly consists of mucins, immunoglobulins, anti-microbial enzymes and a variety of other proteins and inorganic salts and water.

The major contributor to mucus composition are mucins, with gel-forming mucins MUC5AC and MUC5B being the most highly expressed. Mucins are high molecular mass, highly glycosylated proteins that function as components of mucosal secretions where they are involved as lubricants and also function as part of the host defensive shield. Other mucins detected in low amounts include MUC2, MUC7 and MUC16. MUC7 is expressed in the serous cells of the sub-mucosal glands (Sharma, Dudus et al. 1998). MUC2 is expressed by goblet and serous cells of the sub-mucosal gland and the epithelium. MUC16 is expressed on the surface of serous and goblet cells, both on the surface of the airway epithelium and the sub-mucosal gland, and becomes trapped within the ciliary layer as part of the periciliary fluid (Davies, Kirkham et al. 2007). There are many more mucins than are mentioned here; some are membrane-tethered and some are secreted and these are reviewed in Linden, Sutton et al. (2008) and Kreda, Davis et al. (2012). As outlined above the gel forming mucins, MUC5AC and MUC5B are the major airway mucins and are described in more detail below.

1.1.2.1.1. MUC5AC

Initial identification of MUC5 identified 3 proteins, MUC5A, MUC5B and MUC5C, however MUC5A and MUC5C were shown to be encoded by the same gene, designated *Muc5AC* which is localised to chromosome 11p15 (Nguyen, Aubert et al. 1990).

Characterisation of this gene identified a 24bp tandem repeat domain and a consensus cysteine rich domain consisting of 10 cysteine residues repeated multiple times in the protein (Guyonnet Duperat, Audie et al. 1995). The gene contains at least 49 exons and encodes a peptide approximately 5700 amino acids in length (Guo, Zheng et al. 2014). The protein is highly glycosylated and this makes the protein form a huge complex that is measured in the mega Dalton range. MUC5AC is secreted in high quantities from goblet cells (Hovenberg, Davies et al. 1996) in the respiratory and digestive tracts. In the airways of asthmatics, levels of MUC5AC are significantly increased in comparison to normal airways (Ordonez, Khashayar et al. 2001; Takeyama, Fahy et al. 2001; Rose and Voynow 2006; Woodruff, Modrek et al. 2009; Fahy and Dickey 2010).

1.1.2.1.2. MUC5B

The gene encoding MUC5B is also localised to chromosome 11p15, but is separated from the *Muc5AC* gene by CpG islands (Nguyen, Aubert et al. 1990; Guyonnet Duperat, Audie et al. 1995). The *Muc5B* gene has a similar sequence to that of *Muc5AC*, and encodes a peptide that is approximately 5662 amino acids long (Desseyn, Buisine et al. 1998). Like MUC5AC, MUC5B is also very highly glycosylated. MUC5B is expressed predominantly in the mucous cells of the sub-mucosal gland (Sharma, Dudus et al. 1998). A recent paper identified MUC5B as the dominant gel-forming mucin in the normal distal airway epithelium which consists of basal and mucous cells, and expression of both MUC5B and MUC5AC are increased in idiopathic pulmonary fibrosis/ usual interstitial pneumonia, associating them with respiratory disease (Seibold, Smith et al. 2013).

As both MUC5 proteins are secreted predominantly by goblet cells and from submucosal glands, it is perhaps not unsurprising to find that both proteins are significantly elevated in a number of chronic lung diseases. Excessive production of MUC5B and MUC5AC in chronic inflammatory lung diseases is a direct result of goblet cell hyperplasia and metaplasia. This increase in cell number directly correlates with increased MUC5B and MUC5AC levels (Hauber, Foley et al. 2006). It is also clear that the proteins can have alterations in their glycosylation associated with lung disease (Schulz, Sloane et al. 2005).

1.1.2.1.3. PLUNCs

A key family of proteins secreted into the mucus are the ‘bactericidal permeability increasing (BPI) fold containing’ (BPIF) family (also known as the Palate, Lung and Nasal Epithelium Clone (PLUNC) family), which are localised to the goblet cells and sub-mucosal glands of the upper respiratory tract, though not all are mucus of respiratory associated. The genes for the PLUNC family members are located within a 300kb region on human chromosome 20q11 (Bingle and Craven 2002). Family members have very little sequence homology, initially making them difficult to identify. They are termed ‘short’ or ‘long’ depending on the number of BPI-like domains they contain and currently short PLUNCs contain a single domain whereas the LPLUNCs have two domains. In humans, there are four short proteins (including one pseudogene) and seven long proteins (including two pseudogenes) (Bingle, Bingle et al. 2011).

The best characterised family member, SPLUNC1 (BPIFA1) is encoded by the *plunc* gene is a leucine-rich protein of 256 amino acids (Bingle and Bingle 2000). SPLUNC1 is found in some non-ciliated upper airway epithelial cells, but is predominantly found in the airway sub-mucosal glands (Bingle, Cross et al. 2005). SPLUNC1 has been identified in a range of cancers in the lung and airways (Acton, Dahlberg et al. 1996; He, Xie et al. 2000; Iwao, Watanabe et al. 2001; Bingle, Cross et al. 2005), salivary gland (Vargas, Speight et al. 2008) and the stomach (Sentani, Oue et al. 2008), and an increase in SPLUNC1 expression has been associated with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) (Bingle, Barnes et al. 2007).

LPLUNC1 (BPIFB1), another well characterised family member, is a 484 amino acid protein that is post-translationally modified to form two differentially glycosylated isoforms. It is expressed in goblet cells of the upper airways and in the sub-mucosal glands (Bingle, Wilson et al. 2010). These proteins are rarely co-localised. The true functions of these proteins are unknown but based on structural comparisons it is thought that they may play a role in innate defence (Bingle and Craven 2004). There is increasing evidence that these proteins play a role in host defence against a range of respiratory and oral pathogens.

Studies have shown that SPLUNC1 functions in the maintenance of homeostasis in the upper airway (McGillivray and Bakaletz 2010), and has shown anti-biofilm activity as a surfactant (Gakhar, Bartlett et al. 2010) and anti-microbial functions against a number

of pathogens, including *Pseudomonas aeruginosa* (Lukinskiene, Liu et al. 2011) and *Mycoplasma pneumoniae* (Chu, Thaikoottathil et al. 2007). Ghafouri, Kihlstrom et al. (2004) has shown PLUNC in human nasal lavage fluid exhibits its anti-microbial effects by binding to lipopolysaccharide (LPS). More relevant to this thesis, SPLUNC1 has also been shown to be involved in NTHi infection. Jiang, Wenzel et al. (2013) showed that SPLUNC1 was degraded by human neutrophil elastase, increasing the airways susceptibility to NTHi infections, that is a known cause of acute exacerbations in COPD, Data on functions of LPLUNC1 in the lungs is more limited but it has been shown the protein is also differentially expressed in CF (Bingle, Wilson et al. 2012) and IPF lung tissues.

1.1.2.1.4. Anti-microbial agents

As well as secreting high volumes of mucus, the sub-mucosal glands are responsible for secreting some anti-microbial agents; three major agents are lysozyme (LYS), LF and secretory leukoprotease inhibitor (SLPI). Lysozyme is a hydrolysing enzyme that cleaves the $\beta 1 \rightarrow 4$ glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid residues in peptidoglycan of the bacterial cell wall. It can easily target the peptidoglycan of Gram-positive bacteria; however it isn't as effective against Gram-negative bacteria resulting in the need for a co-factor such as LF to aid its access to the peptidoglycan layer. LF binds to LPS, with the oxidised iron parts of LF forming peroxides to destabilise membrane permeability and break down bacterial cell walls for lysozyme to access peptidoglycan (Ellison, Giehl et al. 1988; Ellison and Giehl 1991; Farnaud and Evans 2003). LF is an 80kDa non-haem iron-binding glycoprotein belonging to the transferrin family that plays a major role in the maintenance of cellular iron levels as well as possessing anti-microbial activity. It is thought to exhibit its anti-microbial activity by two mechanisms; one of which is direct interaction with the agent causing infection, the other is the sequestering of iron in infection sites therefore depriving the infecting micro-organism of nutrients (Gonzalez-Chavez, Arevalo-Gallegos et al. 2009). SLPI is an endogenous serine protease inhibitor secreted by the serous cells of the sub-mucosal gland as well as Clara and goblet cells in the epithelium. It provides protection from serine proteases released by the neutrophil during inflammation. The N-terminal domain has been shown to exhibit some antibacterial activity (Hiemstra, Maassen et al. 1996). SLPI has also been shown to exhibit antiviral activity, particularly against HIV (McNeely, Dealy et al. 1995). Other key anti-

microbial agents that interact with the airway mucus and common pathogens include β -defensins and LL-37 (Zuyderduyn, Ninaber et al. 2011).

1.1.2.2. Airway Cilia Beating

Structurally the axoneme of the respiratory cilia consists of nine outer microtubule doublets surrounding two single central tubules. Microtubules are made up of α - and β -tubulin heterodimers and provide a useful marker of ciliated cells. The wave motion exhibited by cilia is driven by inner and outer dynein arms, nexin links and radial spoke apparatus (Figure 1.2). The axoneme terminates at a cytoplasmic basal body in the epithelial cell (You, Huang et al. 2004). FoxJ1, the specific ciliated cell marker expressed in the later stages of ciliogenesis, traffics the cytoplasmic basal bodies to positions directly below the cells apical surface to complete axoneme development (You, Huang et al. 2004).

The periciliary layer/brush is required for fluid movement of cilia. This layer is approximately 7 μ m in depth (cilia are approximately 6 μ m in length) and is composed of macromolecules forming a mesh that act as a tether for mucins in the mucus layer. The periciliary brush acts as an intermediate between the periciliary space and the mucus layer to prevent penetration of mucus into the much more fluid periciliary layer (Button, Cai et al. 2012). Any particles that survive this process and reach the epithelial cells, whether due to abnormalities in cilia function, inadequate mucus or increased pathogen survival, may produce airway infections triggering the immune system to generate an inflammatory response.

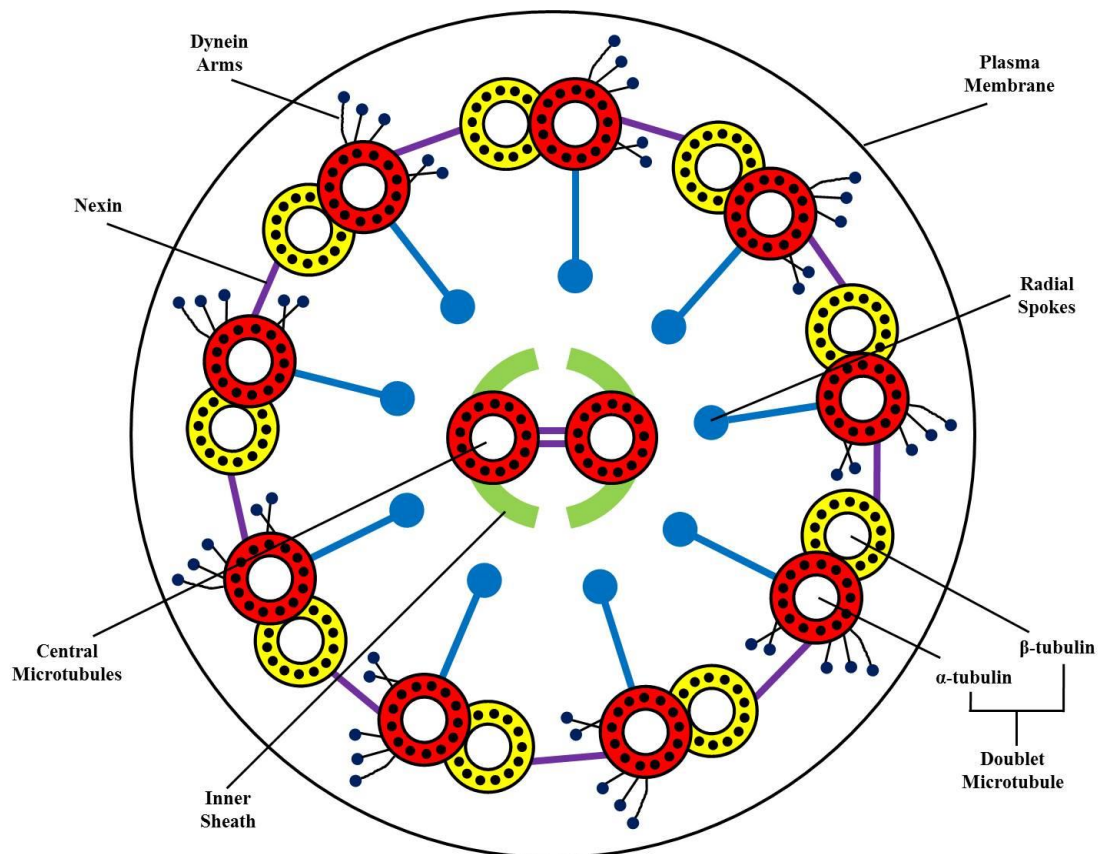


Figure 1.2: The structure of cilia

The ciliary axoneme consists of two single central tubules surrounded by nine microtubule doublets (that consist of α - and β -tubules). The wave motion exhibited by cilia is driven by inner and outer dynein arms, nexin links and radial spoke

1.1.3. Diseases of the Human Airway Epithelium

Given the importance of the respiratory epithelium in providing the respiratory tract with much of its function, it is not surprising that many lung diseases are associated with epithelial abnormalities. The airways of patients suffering from diseases such as asthma, CF and COPD undergo cellular remodelling producing differing phenotypes in comparison to in the “normal” airways. In airway diseases such as asthma, CF and COPD the airways tend to have increased numbers of goblet cells and fewer ciliated cells; they also tend to have an increased susceptibility to structural damage (Parker, Sarlang et al. 2010). Many of the morphological change seen in these different diseases are actually quite similar and lend support to the view that multiple drivers underpin the remodelling process. The following section gives a brief overview of some of the changes that are seen in the airway epithelium during the development of a number of chronic respiratory diseases.

1.1.3.1. Asthma

Asthma is an inflammatory disease that is characterised by variable airflow obstruction, airway inflammation and airway hyper-responsiveness (predominantly caused by eosinophils) (Sont, Willems et al. 1999). Symptoms include broncho-constriction, hypersensitive airways, breathlessness and wheezing. Relief from symptoms is achieved through the regular use of corticosteroids and β -2-agonists to relax the smooth muscle surrounding the narrowed airway. According to the World Health Organisation (WHO) asthma is the most common chronic respiratory disease with prevalence on the increase. Exacerbations of symptoms may be caused by a variety of environmental factors such as allergen exposure (Djukanovic, Feather et al. 1996), pollution (Taggart, Custovic et al. 1996; van der Zee, Hoek et al. 1999) and cigarette smoke (Chilmonczyk, Salmun et al. 1993), as well as upper respiratory pathogens. The most frequent cause of exacerbations is viral infection, such as rhinovirus (RV) which is the major viral cause, and respiratory syncytial virus (RSV) (Nicholson, Kent et al. 1993; Johnston, Pattermore et al. 1995). The airway cells of asthmatics are deficient in interferon (IFN)- β which leads to an inadequate immune response, facilitating viral replication and further infection of cells (Wark, Johnston et al. 2005). RSV capitalises on a deficiency of IFN- α exhibited by asthmatics (Gehlhar, Bilitewski et al. 2006) which up regulates IL-10 expression to suppress eosinophil influx (Grissell, Powell et al. 2005).

Bacterial pathogens have also been shown to be involved in exacerbations of asthma (Cunningham, Johnston et al. 1998; Lieberman, Lieberman et al. 2003). A recent study of the microbiota of asthmatic airways shows that pathogenic Proteobacteria, especially *Haemophilus* species, were identified much more frequently in the airways of asthmatics and exhibited a disturbed microbiota when compared to non-asthmatic controls (Hilty, Burke et al. 2010).

Most asthma patients have increased levels of the pro-inflammatory Th2 cytokines IL-4 and IL-13 in the airway (Grunig, Warnock et al. 1998; Wills-Karp, Luyimbazi et al. 1998). Th2 cytokines appear to be activated non-specifically as a result of defective epithelium. It is thought that the epithelial-mesenchymal trophic unit responsible for maintaining the tissue micro-environment of the epithelium becomes dysregulated, leaving the epithelium susceptible to damage (Holgate, Roberts et al. 2009). A murine model of mucous cell metaplasia showed no change in the number of cells after epithelial injury, despite a 75% decrease in Clara cells (as a result of Clara cell

metaplasia) and a 25% decrease in ciliated cells, leading to an approximate mucous cell population of 70% of total cells (Reader, Tepper et al. 2003). This makes mucous cells the predominant cell type whereas in normal airways ciliated cells are the predominant cell type accounting for approximately 50% of total cells (Spina 1998). The increase in mucous cell numbers leads to hyper-secretion of mucus, resulting in ineffective clearance of pathogens by the cilia and obstruction of airflow. Epithelial cells identify the pathogens left behind following the ineffective clearance through pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) leading to activation of the inflammatory response and a prolonged exacerbation.

1.1.3.2. Cystic Fibrosis (CF)

Cystic fibrosis (CF) is an autosomal recessive genetic disorder causing mutations in the chloride ion channel cystic fibrosis trans-membrane conductance regulator (CFTR), giving rise to abnormal transport of chloride and sodium across the epithelium (Trapnell, Chu et al. 1991). As a result of this impaired function the surface is dehydrated causing the mucus layer to compress the periciliary brush and cilia to slow down and eventually halt mucociliary clearance (mucus stasis) (Button, Cai et al. 2012). As is the case in asthma, the chronic and unresolved pro-inflammatory environment that exists in the lungs of patients with CF is associated with a proliferation of airway goblet cells. This directly leads to the over secretion of mucus and the resulting increased thickness of mucus prevents the effective host defence action of secreted and the cellular immune response as movement of these products are inhibited. Mucus stasis allows invading pathogens trapped in the immobile mucus layer to overcome/evade the immune system and initiate chronic bacterial infection of the airways, particularly anaerobic pathogens. The airways of CF patients can be colonised with multiple pathogens, some existing in biofilms which leads to increased frequency of exacerbations and infections from multiple bacterial species at the same time.

1.1.3.3. Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a life threatening lung disease characterised by irreversible lung damage and airway obstruction causing limitations in airflow. It is predominantly caused by smoking and develops over a number of years. The WHO estimated that in 2004 there were 64 million people worldwide suffering from COPD, with 3 million deaths. They predict that by 2030 it will be the third leading cause of death worldwide. Symptoms include a persistent cough with increased sputum

production, increasing breathlessness, wheezing and frequent chest infections. Sufferers endure frequent exacerbations of symptoms, eliciting an inflammatory response, which is inherently linked to the progression of the disease.

The key aspect of COPD progression is tissue damage and the inflammation that directly results from this. Inflammation causes the recruitment of cells to the airways, causing the lumen to become filled with mucus exudates and inflammatory cells. Investigations into the nature of the cellular inflammatory response, through analysis of bronchoalveolar lavage (BAL) fluid, in the peripheral airways have identified CD8+ T-cells as the major contributor, as well as identifying neutrophils, macrophages and CD4+ T-cells (Saetta, Di Stefano et al. 1998; Hogg, Chu et al. 2004) all with their own range of cytotoxic agents to contribute to destruction of the airway epithelium. The immune response is also associated with thickening of the airway epithelium, resulting in airflow limitation. This remodelling process is more strongly associated with disease progression than the extent and severity of the inflammatory response (Hogg, Chu et al. 2004). This thickening of the airway wall, combined with a malfunction of the mucociliary process, the expansion of goblet cells within the airway epithelium and increase in mucosal tissue thickness, results in the accumulation of mucus in the lumen of the airway. Unlike asthma and CF, COPD also affects the lower airways, with destruction of the alveoli and development of emphysema resulting in reduction in oxygen levels and increased shortness of breath.

It is thought that persistence of micro-organisms in the airway is a cause for disease progression. A bacterial profiling study conducted using bronchoscopy in the distal airways of COPD patients showed colonisation with a range of bacterial species, some with the potential to be pathogenic (Cabello, Torres et al. 1997). The most abundantly identified organisms were non-pathogenic microorganisms, but of the potentially pathogenic the most abundant was *Haemophilus influenzae* (Cabello, Torres et al. 1997). The presence potentially pathogenic microorganisms is significantly associated with higher numbers of neutrophils and a higher concentration of tissue necrosis factor (TNF)- α in BAL fluid suggesting that these pathogens are responsible for enhancing airway inflammation, linking them to disease progression (Soler, Ewig et al. 1999).

1.2. Infection of the Respiratory Tract

1.2.1. Bacterial Infection

It is now accepted that the human airway is not sterile but is host to a variety of commensal bacterial species that remain in the airways without causing any harm to the host. However, when the microbiota is altered, potentially by an imbalance of a particular species or defence measure or infection with a new bacterium, infection occurs with some infections resulting in colonisation of the epithelium in the form of a biofilm. The airways are also home to a wide variety of bacterial species that make up the commensal microbiota. A study published by Hilty et al (2010) examined the microbial communities of the airways of asthmatic and COPD patients alongside healthy controls and found the airway microbiota was disturbed in asthmatic airways. They identified the seven most frequent genera as *Corynebacterium*, *Prevotella*, *Staphylococcus*, *Streptococcus*, *Veilonella*, *Haemophilus* and *Neisseria*.

1.2.1.1. Biofilms

A bacterial biofilm is a complex of aggregated cells, enclosed in extracellular polysaccharide (EPS) matrix complete with water channels for the transport of organic compounds and nutrients to the bacteria deep within the biofilm structure (Woodworth, Tamashiro et al. 2008). Studies performed on *Pseudomonas aeruginosa* biofilms have indicated a developmental sequence for single species biofilm development consisting of five stages; attachment, irreversible binding, two maturation steps and a cell dispersion step (Sauer, Camper et al. 2002). The attachment step is probably triggered within seconds of the activation of an environmental signal. This may be changes in levels of nutrients, pH, temperature, iron levels or oxygen concentration. Attachment is reversible, however rough surfaces are more susceptible. It has also been found in laboratory situations that bacteria attach more readily to hydrophobic surfaces than other surfaces. The second step, occurring minutes after the attachment step, is irreversible attachment/binding. Chemical signals emitted by the bacteria are used to 'inter-communicate' and once the signals have reached a certain threshold, genetic mechanisms of EPS production are activated in order to 'trap' planktonic bacteria in order to form cell aggregates as well as nutrients to feed the bacteria. Steps three and four are maturation steps. Step three maturation is complete when the cell aggregates have reached a thickness of 10µm, which causes a significant reduction in bacterial motility. Step four is complete when the cell aggregates have reached 100µm. The final step is the cell dispersion step. Several days after the completion of step four, some

bacteria develop a planktonic phenotype; they are released from the biofilm into the airway in order to potentially infect another area and form another biofilm (Sauer, Camper et al. 2002; Aparna and Yadav 2008). Studies into the structure of the *P. aeruginosa* biofilm, a common biofilm forming infecting bacterium in the CF airway, shows that the biofilm structure enables the bacteria to resist the effects of antibiotics, >1000 fold, and to evade host defence factors much more efficiently (Woodworth, Tamashiro et al. 2008). Bacteria in the centre of the biofilm are thought to enter a 'dormant' state, requiring fewer nutrients and slowing down growth in order to increase chances of survival.

The development, structural integrity and viability of biofilms relies on signals transcribed in response to stress and other signalling through quorum sensing. Quorum sensing is a system that detects signals and responds in order to control the density of the bacterial population. This 'communication' between bacteria is widespread within non-pathogenic and pathogenic bacteria species (Aparna and Yadav 2008). Both Gram positive and negative bacteria share the same principles but the signal molecules and molecular mechanisms for activating this signalling differ. Quorum sensing plays an important role in diseases that were caused by opportunistic pathogens (Bjarnsholt and Givskov 2007). Targeted blocking of the small molecules involved in quorum sensing could be used as a method to control infections.

1.2.2. Viral Infection

The respiratory tract is also a target of viral infections, rhinovirus (RV), influenza and respiratory syncytial virus (RSV) being the most common. These pathogens frequently affect 'normal' airways, as seen by the seasonal increases in the frequency of 'colds'. However, the implications are more serious for patients with a diseased airway phenotype, as infections often trigger exacerbations of established diseases, including COPD, CF and asthma. The key viral pathogen used in this thesis is RSV.

1.2.3. Impact and Treatment of Respiratory Tract Infections

NTHi is a major cause of mucosal infection, including sinusitis, conjunctivitis otitis media and also triggers COPD exacerbations. This predominantly affects children under the age of 2 years old, the elderly and the immuno-compromised. The incidence of NTHi infections has increased steadily over the last 20 years or so. Symptoms may persist for several weeks without treatment. Treatment usually comprises of

antibiotics, including ampicillin, cefcapene, clarithromycin, azithromycin and fluoroquinolones such as levofloxacin (Sekiya, Eguchi et al. 2008) for several days. Any treatment with antibiotics increases the risk of bacterial resistance, especially in patients with chronic respiratory disease that have experienced repeated infections with multiple pathogens due to treatment with multiple antibiotics. This success of this treatment can be limited, resulting in colonisation of the airway with multiple pathogens and biofilm formation.

Similarly RSV predominantly affects children, the elderly and the immunocompromised, with most children having an RSV infection by the time they are 3 years old. Usually RSV is not treated and left to 'go away' on its own. Treatment to manage symptoms usually occurs if complications arise, such as development of bronchiolitis and pneumonia, with bronchodilators and antibiotics, and in rare cases anti-viral drugs such as ribavarin. Ribavarin use is limited due to the risk of toxicity and variable efficacy (Olszewska and Openshaw 2009). Pavlizumab is a passive monoclonal antibody therapy that can be administered during the RSV season. It acts by preventing spread of RSV infection to the lower airways to prevent bronchiolitis (Olszewska and Openshaw 2009). Once infected with RSV, infections re-occur seasonally, impacting the subject throughout life.

In normal airways these infections are not usually an issue, and recovery occurs fairly quickly and rarely requires hospitalisation. They are an issue for COPD, CF and asthma sufferers, who are more likely to require hospitalisation as the infections trigger exacerbations and worsening symptoms. The nature of the 'diseased' epithelium with increased mucus production often makes these infections difficult to treat. While child mortality for NTHi and RSV infections is low, it is unclear what role these infections play in mortality rates in complex chronic infections.

1.3. *Haemophilus influenzae*

In the asthmatic/COPD and control airways, a lot of the bacterial species identified belonged to the *Haemophilus* family, more specifically *Haemophilus influenzae* (Hilty, Burke et al. 2010). *H. influenzae* is divided into two specific types; encapsulate and non-encapsulate strains. Encapsulate strains are serotyped a-f, with serotype b being the most prevalent, and commonly causing systemic infections. The type b polysaccharide capsule acts a virulence factor, and has been shown to promote intravascular survival

(Moxon and Vaughn 1981). It is also known to cause bacteraemia, pneumonia, meningitis and septicaemia and is frequently identified as a cause of respiratory infections. However, the development and routine use of the *Haemophilus influenzae* type b (Hib) vaccine has reduced the number of invasive Hib infections. Strains categorised as non-encapsulate lack a polysaccharide capsule, and are therefore known as non-typeable *Haemophilus influenzae* (NTHi). They are frequently the causative agent of localised infections of the respiratory tract (Gilsdorf, Chang et al. 1992).

1.3.1. NTHi

NTHi, one of the many causes of COPD and CF exacerbations (Sethi, Evans et al. 2002; Cardines, Giufre et al. 2012), is a gram-negative pleomorphic bacillus commonly identified as a cause of a variety of airway infections as well as being a major cause of otitis media. Furthermore it is routinely found to be a coloniser of the airways of asthmatic and bronchiectasis subjects as well as COPD and CF sufferers. A recent review states that *Haemophilus influenzae* is responsible for 20-30% of exacerbations in COPD (Sethi 2011), and is a major pathogen in stable disease. As NTHi is part of the normal human airway flora, infections can and do occur in people not suffering from an airway disease, however colonisation is rarely seen in these subjects.

Rarely causing diseases resulting from systemic infection, such as meningitis, NTHi is able to adhere to the epithelium, causing respiratory infections and otitis media as mentioned above. It is thought that NTHi can enter the epithelial cell, remaining dormant until the immune response has cleared before re-emerging and causing an infection. NTHi have many surface antigens that aid them in establishing an infection and evading host defence mechanisms, including lipooligosaccharide (LOS), pili/fimbriae and outer membrane proteins like HMW1, HMW2, Hap and Hia. Not all NTHi are biofilm forming and genetic profiling using multiple strains of NTHi show high heterogeneity with strains having different combinations of these surface antigens, suggesting that there is no single defining feature responsible for infection (Clemans, Bauer et al. 2000).

1.3.1.1. LOS

NTHi do not possess the typical lipopolysaccharide capsule of the encapsulate strains; its major surface antigen is LOS. There is a considerable amount of antigenic diversity between the oligosaccharide epitopes of LOS between NTHi strains (Patrick, Kimura et

al. 1987). NTHi can release highly active LOS, regardless of bacterial viability, potentially prolonging inflammation, or gain easier access to target areas as a smaller molecule than the bacterium (Gu, Tsai et al. 1995). LOS plays a large role in the development of infection and stimulation of an immune response, though is not the only feature to induce proinflammatory cytokines (Clemans, Bauer et al. 2000). NTHi has been shown to adhere to and invade epithelial cells through interaction with the platelet-activating factor (PAF) receptor (Swords, Buscher et al. 2000) which then initiates signalling in the host cell (Swords, Ketterer et al. 2001).

1.3.1.2. Haemagglutinating Pili

Haemagglutinating pili, initially identified in Hib (Guerina, Langermann et al. 1982; Mason, Kaplan et al. 1985) and later NTHi strains, are encoded by the *hif* locus, which consists of five genes, *hifA-hifE* (van Ham, van Alphen et al. 1994). Characterisation studies of Hib pili show them to be approximately 5nm in diameter and up to 450nm in length (Stull, Mendelman et al. 1984). Many early functional studies implicated pili in the promotion and enhancement of adherence of Hib to human erythrocytes expressing the AnWj (formerly Anton) antigen (van Alphen, Poole et al. 1987), human airway tissues (Loeb, Connor et al. 1988; Farley, Stephens et al. 1990; Read, Wilson et al. 1991), as well as human mucins (Kubiet, Ramphal et al. 2000). HifA (also known as pilin), the major structural pilus subunit, is encoded by the *hifA* gene. The shaft of the pilus consists of repeating HifA subunits, appearing to have a double-stranded structure similar in morphology to filamentous actin. The pilus tip fibrillum contains HifD and HifE subunits (St Geme, Pinkner et al. 1996) and it is suggested the HifE mediates pilus adherence, after antiserum was found to block pilus-mediated haemagglutination (McCrea, Watson et al. 1997). Assembly of the pilus structure is mediated by HifB, which functions in the periplasm acting as a chaperone for the pilus subunits as they move from the inner membrane to the outer membrane (St Geme, Pinkner et al. 1996). HifC, located in the outer membrane, functions as an usher, in order to facilitate the incorporation of the transported subunits into the pilus structure (Watson, Gilsdorf et al. 1994). Analysis of NTHi isolates identified the *hif* locus and pili expression in approximately 15% of strains; these strains express the whole locus or nothing at all and are thought to be important early in colonisation (Geluk, Eijk et al. 1998; Krasan, Cutter et al. 1999).

1.3.1.3. Outer Membrane Proteins

Haemophilus influenzae strains have been shown to possess many outer membrane proteins (OMPs); key proteins include P1, P2, P5 and P6. OMP P1 is encoded by the gene *ompP1* which produces a heat-modifiable protein (Gonzales, Leachman et al. 1987) approximately 47 kDa in size (Munson and Grass 1988). Studies performed with OMP P1 from Hib in rat models showed P1 induced protection against bacteraemia (Loeb 1987; Munson and Hunt 1989). Characterisation studies performed on Hib P1 have identified some surfaces expose immunodominant epitopes (Proulx, Munson et al. 1991; Proulx, Hamel et al. 1992; Panezutti, James et al. 1993). P1 has been shown to be present in outer membrane vesicles (OMVs) (Sharpe, Kuehn et al. 2011), with recent work investigating OMVs as potential vaccine targets showing some cross-protective immunity between strains in a murine model (Roier, Leitner et al. 2012).

The most abundant outer membrane protein expressed by *Haemophilus influenzae* is the porin, P2, which is highly variable in size (ranging from 32kDa to 42kDa) and between strains (Loeb and Smith 1980), providing the basis for a subtyping system (Murphy, Dudas et al. 1983). This variability has been pin-pointed primarily to four large hypervariable regions in the gene that encode eight surface-exposed loops, with molecular mass correlating with size differences in these regions (Sikkema and Murphy 1992). Persistence of the same strain in a patient with COPD showed immunological changes in the surface of P2 (van Alphen, Eijk et al. 1991), which may aid bacterial survival against the immune response and potential vaccines that may use P2 as a target. The P2 protein expresses highly strain-specific immunodominant epitopes on the surface of the bacteria (Haase, Campagnari et al. 1991), in addition to being a target for human serum bactericidal antibody (Murphy and Bartos 1988). P2 is considered a potentially useful target for a vaccine as some of the surface-exposed loops are relatively conserved allowing antibody generation against multiple strains. Loop 5 contains potentially immunodominant epitopes (Yi and Murphy 1994) which have been shown to be a target for a prominent antibody response (Yi and Murphy 1997) and antibodies raised against loop 6 have shown bactericidal activity against multiple strains (Neary, Yi et al. 2001). P2 has also been identified in OMVs elicited by NTHi (Sharpe, Kuehn et al. 2011), providing another potential vaccine target alongside continuing work against the protein itself (Ostberg, Russell et al. 2009; Roier, Leitner et al. 2012).

Another major outer membrane protein in *Haemophilus influenzae* is the P5, a 35kDa heat modifiable protein, displaying antigenic variability between strains (Munson and Granoff 1985). It has been shown to form fimbriae, so is frequently referred to as P5 fimbria. The P5-fimbriae have a diameter of approximately 2.4nm-3.6nm and are long and flexible (Bakaletz, Tallan et al. 1988; Bakaletz, Ahmed et al. 1992). They have been implicated in attachment to mucus and respiratory cells (Bakaletz, Tallan et al. 1988; Bakaletz, Tallan et al. 1989; Miyamoto and Bakaletz 1996; Reddy, Bernstein et al. 1996), as well as cells infected with RSV (Jiang, Nagata et al. 1999). Studies performed using a chinchilla animal model showed the immune response to outer membrane and fimbrial proteins resulted in reduced occurrence of otitis media and nasopharyngeal colonisation (Bakaletz, Tallan et al. 1989; Sirakova, Kolattukudy et al. 1994). NTHi has been shown to bind to the epithelium through interaction of P5 with intracellular adhesion molecule (ICAM)-1 (Avadhanula, Rodriguez et al. 2006).

P6 is a highly conserved, surface exposed, 16kDa peptidoglycan-associated lipoprotein (Kyd, Dunkley et al. 1995). It has been shown to have a protective effect, enhancing respiratory clearance in a rat (Kyd, Dunkley et al. 1995) and chinchilla model (DeMaria, Murwin et al. 1996). P6 is highly conserved between species and has been shown to elicit specific immune responses so has the potential to be utilised as a vaccination target (Hotomi, Yamanaka et al. 2002). A later study identified an induction of specific IgA and Th2 immune responses to P6 in a murine model (Kodama, Suenaga et al. 2000). More recently, P6 was identified as an inducer of a variety of proinflammatory cytokines, its most potent and selective effect was seen on macrophage proinflammatory cytokines, specifically IL-8 and TNF- α (Berenson, Murphy et al. 2005). Further study conducted by this group using alveolar and blood macrophages from COPD patients, ex-smokers without COPD and non-smokers showed COPD patients had reduced IL-8, TNF- α and IL-1 β in alveolar macrophages but not blood macrophages when stimulated with P6, showing a specific effect on alveolar macrophages and that the defective responses exhibited by COPD patients may allow for colonisation in COPD patients (Berenson, Wrona et al. 2006).

1.3.1.4. HMW Proteins

Whilst working to identify major targets of human serum antibody responses to *Haemophilus influenzae*, Barenkamp and Bodor (1990) initially identified a group of high molecular weight (HMW) proteins. Further work identified two related HMW

proteins from a prototypic NTHi strain; these proteins were termed HMW1 and HMW2 (Barenkamp and Leininger 1992). HMW1 is encoded by the *hmw1* gene which transcribes into a 160kDa polypeptide. HMW2 is encoded by the *hmw2* gene which gives a 155kDa polypeptide. Whilst in the periplasm, the cleavage of a 441 amino acid N-terminal fragment gives mature proteins of 125kDa and 120kDa respectively (St Geme and Grass 1998). Localised to the surface of NTHi these proteins are found in approximately 70-75% of NTHi isolates, and are thought to be involved in bacterial attachment to the epithelium (St Geme and Falkow 1990; St Geme, Falkow et al. 1993; Noel, Love et al. 1994). Despite this localisation, further study has shown it is unlikely they are responsible for eliciting the proinflammatory response (Clemans, Bauer et al. 2000). The HMW1 adhesin has been shown to recognise sialylated glycoprotein receptors on epithelial cells (St Geme 1994).

1.3.1.5. Hap

Another factor implicated in the binding of NTHi to epithelial cells is a gene identified as *hap* for Haemophilus adherence and penetration, that encodes a mature protein 155kDa in length (St Geme, de la Morena et al. 1994). Expression of the Hap gene results in three proteins: an extracellular protein of 110kDa that represents the N-terminal, and a 45kDa outer membrane protein which represents the C-terminal, and the mature 155kDa protein which is a serine protease that undergoes autoproteolytic cleavage to provide the smaller proteins (St Geme, de la Morena et al. 1994; Hendrixson, de la Morena et al. 1997). Initial sequence comparisons with proteins of known function suggested similarity to IgA1 protease of *Neisseria gonorrhoeae* and *Haemophilus influenzae* and also to pertactin, a protein found in *Bordetella pertussis* that promotes attachment to cells suggesting a function in attachment that has potential protease action (St Geme, de la Morena et al. 1994). Further work found that Hap does facilitate attachment of bacteria to epithelial cells; however, it is unable to cleave human IgA1.

The 110kDa protein acts as a monomeric autotransporter, facilitating the binding of NTHi to the extracellular matrix proteins, such as laminin, fibronectin and collagen IV (Fink, Green et al. 2002) as well as epithelial cells. It is not clear what receptor on epithelial cells the C-terminus interacts with, but it is thought that a region within it enhances autoaggregation of bacteria and formation of micro-colonies. Hap-mediated adherence is increased when its serine protease activity is inhibited, showing that this

domain is retained on the surface of the bacterium rather than released extracellularly (Fink, Green et al. 2002; Fink, Buscher et al. 2003).

1.3.1.6. Hia

Further work conducted by Barenkamp and St Geme III (1996) identified another gene for a predicted outer membrane protein/adhesin that they termed *hia* for *Haemophilus influenzae* adhesin. This gene codes for a protein of 114kDa and appears to be expressed in NTHi strains that do not express HMW1/HMW2 proteins; 95% of NTHi strains express either HMW1/HMW2 or *hia* (St Geme, Kumar et al. 1998). Hia functions as a trimeric autotransporter that remains fully associated with the cell as there is no capacity for C-terminal cleavage as in Hap (St Geme and Cutter 2000). Two binding domains are responsible for the binding activity of Hia (HiaBD1 and HiaBD2) both of which interact with the same host, however HiaBD1 has a higher affinity than HiaBD2 (Laarmann, Cutter et al. 2002; Yeo, Cotter et al. 2004). To achieve full adhesive activity and stability, these binding domains trimerise to give three identical binding pockets (Cotter, Surana et al. 2006).

1.3.1.7. IgA1 Protease

IgA1 proteases are present in many different bacterial species known to cause infections on the human mucosal surfaces. They specifically cleave in the hinge region of the α -chain of IgA, releasing intact Fab and Fc fragments (Male 1979), rendering the IgA unable to bind antigens so hampering the immune response. Studies examining a number of *Haemophilus* species and *Streptococcus pneumoniae* for synthesis of IgA1 protease identified both capsulate and non-typeable strains of *H. influenzae* (Kilian, Mestecky et al. 1979; Male 1979). IgA protease was later identified in approximately 98% of *H. influenzae* strains tested, with commensal strains appearing to be the small percentage that don't synthesise it (Mulks, Kornfeld et al. 1980). Further research conducted by this group showed that *H. influenzae* strains produce distinct types of IgA protease that seem to correlate with specific serotypes; type 1 target the prolyl-seryl bond at position 231-232 and is produced mostly by the A, B, D and F serotypes, type 2 targets the prolyl-threonyl bond at position 235-236 and is produced exclusively by C and E serotypes, and type 3, with no specific bond target identified, exhibits a unique double cleavage pattern, producing two fragments that differ in molecular weight (Mulks, Kornfeld et al. 1982). The type 3 activity was later identified as simultaneous action of type 1 and type 2 cleavage by strains that simultaneously secrete two

serologically different proteases, each with one type 1 or type 2 cleavage activity (Kilian and Thomsen 1983). Gene studies show that IgA1 protease is encoded by a gene designated *iga*. Use of hybrid type 1 and type 2 *iga* genes localised the exact site of substrate cleavage to a 370 base-pair (bp) region near the amino-terminal coding region, termed the cleavage specificity determinant (CSD). The CSD regions exhibit large sequence differences between the two types of *iga* genes (Grundy, Plaut et al. 1990). The structure of IgA1 protease is stabilised by binding to the Fc region allowing the hinge peptide access to the active site (Johnson, Qiu et al. 2009). The length of the CSD region is altered by its variability, which could determine which specific bond is accessible (Lomholt, Poulsen et al. 1995; Mistry and Stockley 2011). There is significant sequence homology between IgA1 and Hap protein (St Geme, de la Morena et al. 1994), showing IgA1 function as an autotransporter and is involved in the mediation of bacterial aggregation to form micro-colonies (Plaut, Qiu et al. 1992). This bacterial aggregation activity implicates IgA1 protease in biofilm formation, and has subsequently been identified in NTHi biofilms, where immuno-labelling and electron microscopy showed that the majority of labelling appeared to be associated with the cell membrane in the top region of the biofilm (Webster, Wu et al. 2006). Most recently, Murphy et al (2011) showed that NTHi that express the two different types of *iga* genes (*igaA* for type 1 protease and *igaB* for type 2) are adapted for infection and colonisation of the COPD airway.

1.3.2. NTHi and Biofilms

Recently studies have been conducted into the biofilm forming capabilities and properties of *H. influenzae* in the airways. This process wasn't initially thought to occur (Moxon, Sweetman et al. 2008) despite evidence showing its ability to form biofilms in a chinchilla model of otitis media, the model most commonly used for studying NTHi biofilms (Ehrlich, Veeh et al. 2002). Unlike biofilms for other bacterial species, NTHi is thought to switch gene expression during the attachment phase to form a glue-like structured matrix, composed of multiple molecules and macromolecules including proteins, deoxyribonucleic acid (DNA) and EPS (Moxon, Sweetman et al. 2008).

Murphy and Kirkham (2002) found that P2, P5 and P6 and haemagglutinating pili are needed for biofilm formation, however earlier work by Gilsdorf et al (1996) suggests that haemagglutinating pili are not required for internalisation or adherence but help promote colonisation. This bacterium invades the airway by interacting with microvilli

that are elongated from the airway surface. This results in the formation of lamellipodia, in process which is halted by Cytochalasin D (Murphy and Kirkham 2002). Successful invasion of the airway occurs when the bacterium has entered the vacuoles of a specific but unidentified non-ciliated cell type (Ketterer, Shao et al. 1999). A study by Starner et al (2006) implicated NTHi in CF when bacterial isolates identified in BAL fluid were found to form biofilms on the Calu-3 cell line. They also showed that, like *P. aeruginosa*, NTHi has an increased resistance to antibiotics. Biofilm formation by NTHi also elicited an inflammatory response (Starner, Zhang et al. 2006).

Utilisation of a chinchilla model of otitis media has shown that NTHi in a biofilm structure is able to survive neutrophil extracellular trap (NET)-mediated clearance, resulting in persistence of the bacterial community (Hong, Juneau et al. 2009). Further study showed that NTHi LOS is involved in stimulating NET formation, with toll-like receptor (TLR)-4 deficient murine neutrophils exhibiting decreased levels of NET formation, implicating signalling through the immune mediator MyD88 as necessary for NET formation. Survival of NTHi in NETs and resistance to killing by further recruited neutrophils was partly conferred by LOS moieties on the surface of the bacterium (Juneau, Pang et al. 2011). An inability to kill and/or control the bacterium results in a continual influx of neutrophils to the infection site, resulting in an increase in the number of dead neutrophils. This further stimulates recruitment of inflammatory cytokines, prolonging or worsening exacerbations of respiratory diseases or otitis media. It has also been suggested that a significant amount of the nucleic acid content within the extracellular matrix of an NTHi biofilm is derived from host cells such as neutrophils, with co-localisation of staining for NTHi and neutrophil elastase seen by Hong, Juneau et al. (2009) suggesting that persistence in NET structures is another method of the resistance to bacterial clearance. One study also suggested that NTHi induces neutrophil necrosis (Naylor, Bakstad et al. 2007), which could provide nucleic acids to the bacteria for use in biofilm formation. The NET may be implicated in the antibiotic resistance a biofilm structure infers to the bacterium, as the increased biomass related to the NET may provide an effective barrier against the actions of antibiotics (Juneau, Pang et al. 2011).

1.3.3. Immune Response and NTHi

NTHi predominantly stimulates the immune response through activation of TLR2 to activate MyD88 and both the canonical and non-canonical NF- κ B signalling pathways

(Shuto, Xu et al. 2001). NTHi clearance studies performed in mouse lungs identified MyD88-dependent signalling through TLR4 as important (Wieland, Florquin et al. 2005), the same group however ruled out TLR9 importance in host defence against NTHi infection (Wieland, Florquin et al. 2010). Many studies have utilised the disease otitis media, which as previously mentioned NTHi is the major causative agent, to study the immune responses (Hernandez, Leichtle et al. 2008; Leichtle, Hernandez et al. 2009). Activation of TLR2 by NTHi triggers both the MyD88 dependent and independent pathways, utilising canonical and non-canonical NF- κ B signalling, particularly through activation of p38 MAPK to activate a plethora of pro-inflammatory cytokines (Jono, Xu et al. 2003; Mikami, Gu et al. 2005; Mikami, Lim et al. 2006; Lee, Takeshita et al. 2008; Xu, Xu et al. 2008). NTHi has also been shown to up-regulate expression of some mucins, including MUC5AC (Wang, Lim et al. 2002; Jono, Xu et al. 2003; Chen, Lim et al. 2004; Shen, Yoshida et al. 2008; Jono, Lim et al. 2012) and MUC1, which has also been shown to have an anti-inflammatory role (Kyo, Kato et al. 2012).

A major stimulator of the immune response is lipooligosaccharide (LOS). It stimulates early expression of IL-1 α , IL-1 β , TNF- α , IL-6, IL-8 and macrophage chemotactic protein (MCP)-1, leading to secretion of TNF- α , IL-6, IL-8 and MCP-1 in 9HTEo tracheal epithelial cells (Clemans, Bauer et al. 2000). Inhibition of LOS using polymyxin B showed that other factors contribute to immune response, not just LOS; however it was also shown that the surface proteins implicated in adherence previously mentioned, haemagglutinating pili, HMW1, HMW2, Hia and Hap proteins, do not stimulate the release of inflammatory cytokines (Clemans, Bauer et al. 2000). Studies conducted using the ALI human cell culture model and whole NTHi organisms found increased levels of the inflammatory cytokines IL-1 β , IL-6, TNF- α , GM-CSF and IL-8 in a long term culture with NTHi (Ren, Nelson et al. 2012).

TNF- α expression is induced earlier than IL-6 and IL-8 which Clemans (2000) suggested may be used to sustain IL-6 and IL-8 levels and activate immune cells. This finding was supported by Foxwell (1998) using tracheal infection in rats with formalin killed NTHi who found that TNF- α has disappeared 12-15h post infection. It was suggested that TNF- α had a priming effect to recruit immune cells quicker to the site of infection, to prevent progression to a more severe IL-5 mediated inflammatory response (Foxwell, Kyd et al. 1998). However, TNF- α causes damage to epithelial cells, and

NTHi preferentially adhere to damaged cells, so levels of expression are not maintained for an extended period of time.

1.4. RSV

RSV is a non-segmented, enveloped anti-sense ribonucleic acid (RNA) virus belonging to the *Paramyxoviridae* family, more specifically a subfamily called the *Pneumovirinae* (Zhang, Peeples et al. 2002). It is the major cause of viral respiratory infections in children, and is frequently associated with an increased risk of developing otitis media (Sagai, Suetake et al. 2004). RSV causes significant damage to the airway epithelium, and preferentially targets the ciliated cells, causing loss of cilia, ciliostasis and thus reduced function of mucociliary clearance mechanisms (Zhang, Peeples et al. 2002; Sajjan, Wang et al. 2008).

1.4.1. The RSV virion

The genome of RSV, 15.2kb, has ten messenger RNAs (mRNAs) that code for eleven proteins, four of these are for the nucleocapsid-polymerase complex and three others are virion surface proteins. The nucleocapsid protein (N), phosphoprotein (P), large polymerase protein (L) and transcription anti-termination factor (M2-1) are the proteins involved in the nucleocapsid-polymerase complex. The heavily glycosylated attachment (G) protein, fusion (F) protein and another small hydrophobic protein (SH) makes up the three virion surface proteins (Zhang, Peeples et al. 2002). Other proteins include a matrix protein (M), the RSV transcription and replication inhibitor M2-2 and two non-structural (NS) proteins, NS1 and NS2.

1.4.1.1. M2 Proteins

The two proteins transcribed from the overlapping open reading frames (ORFs) of the M2 gene, M2-1 and M2-2 (Collins, Hill et al. 1990), are involved in the viral RNA synthesis process. M2-1 has transcriptional processivity and anti-termination functions which are critical to RSV replication. It is associated with transcription elongation, potentially inhibiting the polymerase from terminating at gene end signals to allow transcription of downstream genes (Collins, Hill et al. 1996; Fearn and Collins 1999).

M2-2 inhibits transcription and replication of RSV in a mini-genome model system however this protein has been shown to be unnecessary for RSV replication but may have a role later in the infection in virion morphogenesis (Collins, Hill et al. 1996) and

it is also suggested that M2-2 has a regulatory role in the balance between viral RNA transcription and replication (Bermingham and Collins 1999). Studies performed in rodents using a deletion mutant showed attenuation of the virus despite formation of larger syncytia than wild-type viral strains, providing the potential for a live attenuated vaccine (Jin, Cheng et al. 2000). Overproduction of M2-2 protein resulted in an inhibition of viral replication (Cheng, Park et al. 2005).

1.4.1.2. M Protein

The M protein is detected early in the infection process in the nucleus of infected cells (Ghildyal, Baulch-Brown et al. 2003). Further research conducted by this group identified that nuclear transport of M protein is mediated by importin β 1 nuclear import receptor and Ran, a guanine nucleotide-binding protein (Ghildyal, Ho et al. 2005); and later in the infection process is exported from the nucleus by a Crm-1-dependent factor (Ghildyal, Ho et al. 2009). Whilst in the nucleus, M protein affects cell replication. Cell cycle analysis showed that accumulation of p53, a cell stress response factor, coincided with a maximal effect of M protein induced G1 and G2/M phase arrest of the cell cycle in primary human bronchial epithelial (HBE) cells (Bian, Gibbs et al. 2012).

1.4.1.3. P, N and L Proteins

The P, N and L proteins are essential components of the viral RNA polymerase, and are all required for viral RNA replication (Grosfeld, Hill et al. 1995), they are also associated with the viral RNA genome in the form of nucleocapsids (Jin, Cheng et al. 2000). P protein is approximately 241 amino-acids long and constitutively phosphorylated within the virion core and infected cells, a process which is required for P protein oligomerisation to form tetramers (Asenjo and Villanueva 2000) and for efficient virus replication (Lu, Ma et al. 2002). Alongside its essential function on viral RNA replication and transcription, N protein is a major structural protein involved in encapsidation of the RNA genome. It has also been identified as one of the major target antigens of cytotoxic T lymphocytes in mice and humans (Bangham, Openshaw et al. 1986). A recent study showed that N protein allows continued translation of cellular and viral proteins by limiting phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α), preventing it from inhibiting translation of cellular and viral proteins (Groskreutz, Babor et al. 2010). N, P and M2-1 have been identified as components of cytoplasmic inclusion bodies which have been suggested to be the site of RSV replication (Garcia, Garcia-Barreno et al. 1993). N and P protein have been show to

interact, with 20 amino-acids at the carboxy-terminal of P protein identified as the key binding site (Slack and Easton 1998). More recent work identified 6 amino-acids near to the C-terminal of N protein as essential for binding ability to P protein and replication functions (Stokes, Easton et al. 2003).

The large (L) protein is an RNA-dependent RNA polymerase, and contains multiple enzyme activities required for replication of RSV. The L protein is approximately 2165 amino acids long, and consists of a single major ORF (Stec, Hill et al. 1991). The sequence composition was found to be similar to other negative-strand RNA viruses including Sendai virus, measles virus and Newcastle disease virus (Stec, Hill et al. 1991). Six conserved regions in the L protein have been identified across the family of non-segmented negative-sense RNA viruses and have been associated with individual enzymatic activities (Tiong-Yip, Aschenbrenner et al. 2014). Most enzymatic activities required for RSV RNA replication are associated with the L protein, making it a target for drug development, most commonly L protein inhibitors to prevent viral replication (Liuzzi, Mason et al. 2005; Tiong-Yip, Aschenbrenner et al. 2014).

1.4.1.4. G, F and SH Fusion Proteins

RSV G protein is heavily glycosylated and expressed in two forms; a complete membrane bound (mG) form and an N-terminally truncated secreted (sG) form (Teng, Whitehead et al. 2001). It is thought that sG is chiefly responsible for protection against innate immune responses, allowing the virus to replicate. Early studies suggested that G protein suppresses the release of the pro-inflammatory cytokines IL-10, TNF- α , IL-12 and IL-8 from peripheral blood mononuclear cells (PBMCs) (Konig, Krusat et al. 1996; Konig, Streckert et al. 1996). Later studies using an RSV strain deficient in sG showed increased expression of ICAM-1, IL-8 and Regulated upon Activation in Normal T-cell, Expressed and Secreted (RANTES) from the alveolar cell line A549. An increased binding activity of the epithelial cells to NF- κ B was also observed in the mutant strain confirming suggestions of G proteins involvement in modification of the inflammatory response to promote viral replication (Arnold, Konig et al. 2004). A more recent study suggest that G protein acts as a decoy antigen to reduce the antibody-mediated neutralisation/clearance of RSV by inhibiting the effects of leukocytes that have the Fc receptor, and that this process involves the pulmonary macrophages and complement (Bukreyev, Yang et al. 2012). RSV has recently been shown to modify microRNAs that are known to affect the host antiviral response, and it is suggested G protein expression

is a partial mediator of this (Bakre, Mitchell et al. 2012). G protein has become a target for therapeutic use through two mechanisms: (1) direct antiviral activity or (2) immunomodulatory activity to reduce viral load and improve the dysfunctional immune response G-protein mediates (Kauvar, Harcourt et al. 2010).

F protein is initially produced as a 68 kDa precursor in infected cells, which is activated by cleavage in either the trans-Golgi cisternae or trans-Golgi network into two disulphide-linked subunits; F₁ is approximately 48kDa and F₂ around 20kDa (Ferne, Dapolito et al. 1985; Gruber and Levine 1985). This cleavage is required for surface expression (Bolt, Pedersen et al. 2000) and it has been determined that the cytoplasmic domain is not essential for fusion (Branigan, Day et al. 2006). The F₁ sub-unit has been shown to interact with LF, and the F₂ sub-unit has been shown to interact with surfactant protein-A (SP-A) to modulate RSV infection (Sano, Nagai et al. 2003). A study conducted using RSV deficient in G and SH protein showed F protein to be associated with lipid rafts on the surface of cells to cause infection (Fleming, Kolokoltsov et al. 2006). It has also been shown that the F₂ subunit determines the specificity of RSV infection, and not G protein as initially thought (Schlender, Zimmer et al. 2003). F protein has also been shown to trigger p53-dependent cell death, causing loss of epithelial integrity by cell shedding, obstruction of the airways, secondary necrosis and inflammation as a result of these actions. Apoptosis was reduced when a monoclonal antibody to F protein was administered post infection, suggesting this may be a useful target for therapies after infection (Eckardt-Michel, Lorek et al. 2008).

The SH protein occurs in three forms: two unglycosylated species of approximately 7.5kDa (SH₀), a form that represents translational initiation (SH_t) of approximately 4.5kDa and an N-glycosylated form of SH₀ approximately 13-15kDa (SH_g). The multiple forms means that SH accumulates in both its glycosylated and unglycosylated forms and all forms are present as oligomers (Collins and Mottet 1993). Studies conducted in a bid to elucidate function found similarities to Parainfluenza virus 5, which also encodes an SH protein, in its ability to inhibit TNF- α signalling (Fuentes, Tran et al. 2007). Studies conducted on the transmembrane domain of SH in the presence of lipid bilayers show that SH forms pentameric alpha-helical bundles forming cation-selective ion channels (Gan, Ng et al. 2008). Further studies performed by this group suggested that SH forms viroporins, which are short viral proteins that form oligomers of a specific size which act as ion channels and enhance host membrane

permeability. They found that SH forms a pentameric ion channel, however the biological function of this is still unknown (Gan, Tan et al. 2012).

An analysis of the role of all three proteins in fusion found that when F, G and SH were expressed together when fusion occurred. They also found that co-expression of F and SH genes elicited a high level of fusion but less than when all three genes were expressed (Heminway, Yu et al. 1994). Using heparin agarose affinity chromatography (HAAC) Feldman et al (2001) identified two high molecular weight complexes of 175kDa and 210 kDa that reacted with antibodies to all three proteins, suggesting that all three surface proteins form an oligomeric complex within infected cells; whereas ultracentrifugation analysis and immunoprecipitation showed F and G proteins exist on the cell surface as a protein complex suggesting that multiple glycoprotein complexes may exist in the envelope of RSV (Low, Tan et al. 2008). To facilitate attachment of RSV to the host cell, F and G proteins bind to cellular glycosaminoglycans (Hallak, Collins et al. 2000). It is thought that RSV infects through binding of F protein to TLR4 with G protein can acting as a fractalkine receptor agonist to mediate immune cell chemotaxis (Tripp, Jones et al. 2001).

1.4.1.5. Non-Structural Proteins

The non-structural proteins, NS1 and NS2, are both type 1 IFN antagonists (Bossert and Conzelmann 2002) that can form homo- and heteromers (Swedan, Andrews et al. 2011). NS1, localised to the nuclei when expressed alone and in the mitochondria when expressed with NS2 (Swedan, Andrews et al. 2011), can act as an E3 ligase by forming a complex with E3 ligase components. This is crucial for RSVs ability to degrade signal transducer and activator of transcription (STAT)2 and evade the type 1 IFN immune response; a process for which NS2 is also required (Elliott, Lynch et al. 2007) NS2, a multifunction IFN antagonist localised to the mitochondria when expressed alone (Swedan, Andrews et al. 2011), has been identified as an important virulence determinant as deletion of the NS2 gene attenuates RSV (Wright, Karron et al. 2006). Expressed early in the infection process, NS1 and NS2 have been shown to delay apoptosis of cells in order to allow more viral replication. This was achieved through an IFN and epidermal growth factor receptor (EGFR) dependent pathway (Bitko, Shulyayeva et al. 2007). They have also been implicated in suppression of dendritic cell maturation, resulting in a weak or incomplete immune response as there would be less T-lymphocyte activation and antigen presentation (Munir, Le Nouen et al. 2008). In

their roles as an IFN antagonists, NS1 and NS2 have been shown to decrease levels of TRAF3, a mediator of multiple IFN signals, although NS1 achieved this much more efficiently. NS1, specifically the N-terminal (Swedan, Andrews et al. 2011), was also shown to reduce I κ B Kinase (IKK) ϵ , a specific activator of IFN Regulatory Factors (IRF)-3 (Swedan, Musiyenko et al. 2009). NS1 has also been shown to disrupt the association of IRF-3 with its transcriptional co-activator CBP to reduce IRF-3 binding to the IFN- β promoter without blocking phosphorylation, nuclear translocation and dimerisation of virally induced IRF-3 (Ren, Liu et al. 2011). NS2 binds to the N terminal of caspase recruitment domains (CARD) therefore inhibiting induction of IFN transcription from both the retinoic acid inducible gene (RIG)-I and TLR3 pathways (Ling, Tran et al. 2009). Swedan et al (2011) showed specific interactions with host microtubule-associated protein 1B (MAP1B) for both NS1 and NS2.

1.4.2. Immune Response to RSV

The pathogenesis of RSV is largely driven by the presence of immune cells recruited to the site of infection. Neutrophils are the predominant infiltrating cell type identified in BAL fluid and tissues (Villenave, Shields et al. 2013). Monocytes, macrophages and lymphocytes are also frequently identified in debris in the lumen of arterioles and airways, alongside the neutrophils (Villenave, Shields et al. 2013).

The activation of the immune response and recruitment of inflammatory leukocytes in response to RSV is initiated by activation of a number of TLRs, including TLR2 (Murawski, Bowen et al. 2009), TLR3 (Groskreutz, Monick et al. 2006; Huang, Wei et al. 2009), TLR4 (Haeberle, Takizawa et al. 2002; Tulic, Hurrelbrink et al. 2007; Wheeler, Chase et al. 2009) and TLR7 (Huang, Wei et al. 2009). This results in activation of the MyD88 (Rudd, Schaller et al. 2007) and RIG-I signalling pathways (Ling, Tran et al. 2009). Activation of these pathways results in up-regulation of IFNs, predominantly type III (Okabayashi, Kojima et al. 2011), IL-6, IL-8, IFN- γ -induced protein 10 (IP-10), RANTES, ICAM-1 and TNF- α (Tulic, Hurrelbrink et al. 2007; Lotz and Peebles 2012; Smith, Kulkarni et al. 2013; Villenave, Shields et al. 2013).

1.5. Modelling the Airway Epithelium

Models are a key tool in understanding the fundamentals of many of the essential functions and processes of the human body, including normal and inflammatory

responses, disease pathogenesis and the action of potential therapies. A number of *in vivo* and *in vitro* models are currently used to study the airway epithelium.

In vivo is defined as the use of/or taking place in the whole organism. Small mammals, such as mice and rats, are the most common tool for *in vivo* studies, which may include functional studies or investigations into disease pathogenesis. Genetic modification of animals can be used to label genes/proteins of interest, introduce mutations or knock-down/up-regulate production to investigate function.

In the cases of genetic diseases like CF, genetic mutations can be introduced into animals to achieve the disease phenotype. A frequently used model for CF is the β ENaC-Transgenic mouse, in which β ENaC expression is up-regulated to mimic the increased Na⁺ absorption seen in CF patients (Mall, Grubb et al. 2004). The most common murine model of allergic inflammation of the airway is the OVA model, in which the mouse inhales doses of ovalbumin to trigger the immune response (Renz, Smith et al. 1992). Administration of lipopolysaccharide (LPS) is used induce a disease state that resembles COPD.

In vitro study involves the use of components of an organism that have been isolated from their usual biological niche. This most commonly involves the use of cells, either primary or immortalised cell lines, to study a particular tissue. There are various cell culture methods available for studying the human airway epithelium using immortalised cell lines or primary cells. Cells usually have a finite number of replications before they undergo cell death, however it is possible to immortalise these by addition of DNA tumour virus genes that code for regions with multiple functions, such as the simian virus 40 (SV40) large T protein, human papilloma virus (HPV) genes, or even the entire genome of some viruses, most commonly the Epstein-Barr virus (Reddel 1995). Examples of the most commonly used cell lines for work in the airways are Beas-2-B, A549 and NCI-H292 cells, of which A549s and NCI-H292s are derived from lung cancers.

The alternative is the use of primary cells, obtained either directly from a patient/cadaver or from commercial companies. Primary cells, particularly bronchial epithelial or nasal cells are easily obtained by bronchial or nasal brushings. It is also possible to extract cells from explants from cadavers if processed in an appropriate time

frame. Cells purchased from a commercial company are usually obtained via resected tissue and are screened for infections.

Cell lines are easy to obtain and work with, whereas primary cells are moderately easy to obtain if from a commercial company but are surrounded by ethical implications if taken from patients. Also, there is a high level of inter-donor variability to consider. Primary cells are also more difficult to maintain as they are more prone to infection, particularly those obtained from local brushings. They also have a finite lifespan and cannot be cultured through repeated passages. Cells obtained from explants or brushings may also have the added complication of contaminating cell types. Whilst both types of culture technique use airway cells, there are issues surrounding accurate representation of the human airway. It is thought that cell lines do not accurately represent the diverse cell types present in the airway as well as the normal airways ability to senesce and renew. Primary cells can be cultured using standard submerged culture methods, but still do not represent the varying cell types present in a normal airway. However, culture at an air-liquid interface (ALI) allows cells to differentiate into the cell types seen in the airway in representative proportions, thus forming a model for various studies relating to the human airways (Ross, Dailey et al. 2007).

1.5.1. Primary Airway Epithelial Cell Culture at the Air-Liquid Interface (ALI)

The ALI culture technique was first established as a method for differentiating cells by Whitcutt et al (1988). After using the technique on cells from multiple species, in comparison to submerged culture, they achieved cell differentiation whilst using the ALI technique for culture of guinea pig epithelial cells. This cellular differentiation resembled the mucociliary structure, secreted mucus substances and had a similar epithelial polarity to that in the *in vivo* epithelium (Whitcutt, Adler et al. 1988). In humans the ALI culture technique had been used for growth of keratinocytes from skin and squamous carcinoma cells in 1988 (Regnier, Desbas et al. 1988), and was first described for use with tracheobronchial epithelial cells in 1990 (Wu, Martin et al. 1990). Culturing primary cells, particularly primary human tracheobronchial epithelial cells, at the ALI provides an *in vitro* model of airway differentiation. This allows for various studies including the response of the airway to damage and its repair mechanisms, as well as manifestations of bacterial/viral infections in the airway of both healthy people and patients with a chronic airway disease such as COPD, CF and asthma. Recent studies have concluded that this method of primary cell culture is important to

recapitulate the transcriptional profile of the airway and provides a good representation of the airway transcriptome *in vivo* (Dvorak, Tilley et al. 2011; Pezzulo, Starner et al. 2011).

There are several published methods for culturing airway cells at ALI, on different platforms using differing periods of time for differentiation. The most common method appears to be the use of polycarbonate transwells, with a porous membrane surface on which cells form a confluent epithelial layer, with nutrients provided from the basolateral compartment. The cells are allowed to differentiate for a set period of time, usually between 14 and 28 days. The growth medium contains many growth factors, including; bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, epinephrine, transferrin and retinoic acid (Kettle, Simmons et al. 2010). One of the most important growth factors for differentiation is retinoic acid as it has been shown to be important for normal development and maturation of the lung by regulating proliferation, differentiation and morphogenesis of cells through activation of retinoid X receptors to activate and suppress many different genes. Cells cultured in the absence of retinoic acid resulted in differentiation of a stratified squamous epithelium (Koo, Yoon et al. 1999). The mucous phenotype can be restored to squamously differentiated cells through the addition of retinoic acid to culture medium. Collagen coated transwells have also been used as a platform (Nlend, Bookman et al. 2002; Ross, Dailey et al. 2007; Parker, Sarlang et al. 2010), as collagen also affects the differentiation of the secretory cell type but is thought to be most important for ciliated cell differentiation (Ross, Dailey et al. 2007).

1.5.2. Modulation of Differentiation with Growth Factors

Initial studies using extracted primary nasal cells from surgically removed polyps cultured on collagen gels (Laoukili, Perret et al. 2001) showed that IL-13 increased the number of goblet cells through differentiation. A recent study (Turner, Roger et al. 2011) modulated the differentiation of these cultures by stimulating the cells with IL-13 during the differentiation period; they specifically cultured primary cells for 14 days in the presence or absence of IL-13 in the growth medium to see if this altered the proportions of each cell type once differentiated. Stimulation with IL-13 caused an increase in the number of mucus-secreting goblet cells, and a reduction in ciliated cells after which the cells reverted back to their 'normal' proportions of cell numbers upon removal of IL-13 (Turner, Roger et al. 2011). This has provided a model for studies of

diseased airways as exacerbations of asthma and CF are associated with an increase in the number of mucous cells, known as mucous cell metaplasia. Mucous cell metaplasia has been extensively studied in animals, specifically mice, in order to understand the underlying signalling mechanisms that cause increased differentiation of Clara and ciliated cells into goblet cells. It is thought that cytokines released by T helper 2 (Th2) cells are involved in the development of mucous cell metaplasia as an inflammatory response to allergic reactions and asthma, for example.

IL-13 and IL-4 share the receptor subunit IL-4R α 1. IL-13 induces STAT6 release through the IL-4R α 1, which in turn causes increased expression of goblet cell markers (e.g. SPDEF and GABA_AR) and decreased expression of ciliated cell markers (e.g. FoxA2) (Curran and Cohn 2010) to start transformation into a goblet cell. STAT6 is also thought to be associated with MUC5AC expression. Murine models ruled out IL-4 as a major cause of this, suggesting the involvement of IL-13 (Curran and Cohn 2010). However it is thought that IL-13 is the second signal required to induce mucous cell metaplasia. It is hypothesised that EGFR release prevents the cell from undergoing apoptosis, allowing IL-13 to have an effect as a second signal (Curran and Cohn 2010). A recent publication by Kettle et al (2010) found that neuregulin1 β 1, part of the EGFR signalling pathway, also induces increased goblet cell differentiation, further suggesting both are involved in the induction of mucous cell metaplasia.

1.6. The Immune Response

A pathogen is a bacterium, virus, or other microorganism that can cause disease in another organism. As the resultant evolutionary development of the defence mechanisms known as the immune system occurred, so did evolution of the pathogen in an attempt to evade the actions of the immune response.

Broadly, the immune response is made up of two components: the adaptive (specific) response and the innate (non-specific) response. The adaptive response takes time to generate a specific response to pathogens, inferring long-lasting protection to future challenges. The innate response generates an immediate inflammatory response to invading foreign material or endogenous signalling. This inflammatory response recruits and activates immune cells at the site of injury/pathogen invasion, resulting in oedema and erythema as well as causing the capillaries to dilate, and allowing movement of white blood cells. This inflammatory response is a crucial first line

response to infection and foreign material, attempting to prevent spread of infection while the adaptive immune system responds. Despite this essential role in host defence, aberrant immune responses, both innate and adaptive, are also in part the cause of diseases such as COPD and asthma.

1.6.1. Neutrophils

Neutrophils, also known as polymorphonuclear neutrophils, are one of the major leukocytes that contribute to the immune response. They are short lived, fast moving polymorphonuclear (PMN) granulocytes representing around 50-60% of the total number of leukocytes circulating in the blood (Smith 1994). The main function of neutrophils is to engulf and degrade bacteria either to prevent infection or control commensal populations. They achieve this function through use of one of three mechanisms of action upon contact with targets. (1) Upon recruitment to the appropriate site, extracellular bacteria and debris is engulfed into the phagosome. The phagosome fuses with the lysosome to form the phagolysosome, in which a process known as respiratory burst occurs (Segal 2005). During respiratory burst, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase produces the reactive oxygen species superoxide, which decays to form hydrogen peroxide and is then converted by myeloperoxidase to form hypochlorous acid which kills the bacteria (Segal 2005). (2) The neutrophil cytoplasm contains granules which contain a variety of potentially harmful proteases, including elastase, which are involved in the degradation of pathogens and extracellular matrix proteins. A process called degranulation releases the granules when the neutrophil is activated. In the absence of activation signals they go through programmed cell death and are then engulfed and cleared by macrophages (Savill, Wyllie et al. 1989). Apoptosis is a tightly controlled anti-inflammatory process as it promotes clearance of neutrophils by macrophages to prevent tissue damage (Akgul, Moulding et al. 2001). (3) The formation of NETs was first described by Brinkmann et al (2004). They trap and kill bacteria extracellularly through the formation of web like structures consisting of fibres of chromatin and serine proteases and were found to form as early as 10 minutes after activation, a process which is quicker than apoptosis (Brinkmann, Reichard et al. 2004). NETs may provide a localised collection of anti-microbial components that can be used to control infection by trapping and killing bacteria independently of phagocytosis, as well as prevention of further infection in neighbouring areas by formation of a physical barrier to limit bacterial spread.

1.6.2. Macrophages

Macrophages are traditionally thought to differentiate from PBMCs which are initially released from the bone marrow into the peripheral blood. As monocytes transmigrate to the tissues they differentiate into macrophages (Mosser and Edwards 2008). Macrophages are much longer-lived than neutrophils, often remaining in tissues for several months. As a result of this, macrophages have a key role in the adaptive immune response, interacting with T- and B-cells to function as antigen-presenting cells, as well as roles in the innate immune response. The major function of macrophages is to phagocytose dead/dying cells, cellular debris and pathogens, during tissue remodelling and especially in the early stages of chronic inflammation when neutrophils are present. After ingestion, the pathogen is trapped in a phagosome, which fuses with a lysosome to form a phagolysosome, in the same way as neutrophils. Phagolysosomes contain a variety of enzymes and toxic agents used to kill the bacteria contained within it (Aderem and Underhill 1999). Eventually the macrophage produces enough of these digestive compounds, as a result of repeated bacterial ingestion and killing, to kill itself. Most phagocytosis is performed by macrophages that are localised to the specific tissue; these are then responsible for production of a range of cytokines to recruit more macrophages and neutrophils to the site of inflammation (Mosser and Edwards 2008).

Macrophages are also key molecules in generating long lasting immunity via the adaptive immune system by acting as antigen presenting cells. After ingestion of a pathogen the macrophage 'presents' the antigen from the pathogen by incorporating parts of it into its surface, resulting in the production of antibodies to provide a memory of the pathogen. The next encounter of this pathogen results in recruitment and proliferation of the specific antibody to aid phagocytosis.

1.7. The Inflammatory Response

Predominantly the inflammatory response is initiated by pattern recognition receptors (PRRs) present on the surface of innate immune cells (Medzhitov and Janeway 2000; Akira, Uematsu et al. 2006). PRRs are activated upon exposure to/recognition of, one of two signals: the pathogen-associated molecular pattern (PAMP) triggers inflammation in response to infection, while the damage associated molecular pathogen (DAMP) triggers inflammation as a result of tissue injury (Medzhitov and Janeway 2000; Akira, Uematsu et al. 2006). This results in secretion of inflammatory cytokines and

chemokines. Key PRRs that recognise both PAMPs and DAMPs are Toll-like receptors (TLRs).

1.7.1. Toll-Like Receptors

TLRs are a family of trans-membrane proteins/receptors. The *toll* gene was initially identified in *Drosophila* where it is crucial for normal development (Anderson, Bokla et al. 1985). Initially there were five sequences identified with sequence homology and protein architecture to the *Drosophila* Toll that were designated as TLRs (Rock, Hardiman et al. 1998), however it is now known there are ten human TLRs distinctly recognising a variety of extracellular and endosomal PAMPs to induce nuclear factor (NF)- κ B and IFN signalling and the mitogen activated protein kinase (MAPK) pathway to secrete pro-inflammatory cytokines, chemokines and co-stimulatory molecules. Most cells express at least one TLR and cell surface expression of TLRs is up-regulated by proinflammatory cytokines (Muzio, Bosisio et al. 2000). The association of TLRs with the immune response results in a tightly controlled signalling system to prevent impaired responses that may lead to disease.

1.7.2. Interleukin-1 Receptors

There are two types of receptor to the potent pro-inflammatory cytokine IL-1: IL-1RI and IL-1RII. They bind to the ligand at three extracellular immunoglobulin-like domains at the N-terminal, a key feature of the immunoglobulin class they belong to.

IL-1RI (80kDa) is exclusively responsible for IL-1 signalling (Sims, Gayle et al. 1993). It binds IL-1 with high affinity in the presence of the IL-1 accessory protein (AcP). The AcP does not have any affinity for IL-1 itself, but is recruited as the ligand binds to form a heterotrimeric complex, resulting in signal transduction (Greenfeder, Nunes et al. 1995)

IL-1RII (67kDa) acts as a 'decoy' receptor, attenuating the potency of IL-1 by acting as a sink (Colotta, Re et al. 1993). With its high avidity for IL-1 β it is able to form the heterotrimeric signalling complex; however this does not result in signal transduction or cellular responses (Lang, Knop et al. 1998). Both types are structurally similar, but IL-1RI has a much longer cytoplasmic tail (215 amino acids for IL1-R1 and 19 amino acids for IL-1RII), and are predominantly found on different cell types (IL-1RI is

predominantly found on T cells and fibroblasts and IL-1RII on B cells and neutrophils) (Greenfeder, Nunes et al. 1995).

1.7.3. TIR Domain

TLR and IL-1 receptors function to identify and detect danger signals, resulting in initiation of the immune response. The connection between TLRs and IL-1R is shown through the use of common signalling components, more specifically TLR/IL-1R (TIR) cytoplasmic receptor domain. The TIR domain is a stretch of approximately 200 amino acids arranged in three conserved domains, known as boxes, in the intracellular portion of TLR/IL-1R. The signature sequence of the family is box1, however box2 and box3 are also important in signalling (Fitzgerald and O'Neill 2000). The most common inflammatory route involving this domain utilises the adaptor molecule myeloid differentiation factor 88 (MyD88), triggering the MyD88 dependent signalling pathway.

1.7.4. MyD88-Dependent Signalling

MyD88 was initially discovered as a myeloid differentiation response gene induced in response to IL-6 stimulation in M1 myeloblastic leukaemia cells (Lord, Hoffman-Liebermann et al. 1990). Sequence analysis of MyD88 identified a C-terminal TIR domain, that mediates interactions between receptor and adaptor TIR domains and is critical for signalling (Xu, Tao et al. 2000) and a 'death domain (DD)' containing N-terminus which is now known to mediate protein-protein interactions (Feinstein, Kimchi et al. 1995). These link MyD88 to functions as an adaptor molecule for downstream signalling actions.

Activation of the receptor causes MyD88 to recruit and interact with IL-1R-associated kinases (IRAKs) (Wesche, Henzel et al. 1997). The IRAK family consists of four members, IRAK-1, IRAK-2, IRAK-4 and IRAK-M (also known as IRAK-3), which are encoded from different areas of the genome, transcribing separate gene products with similar amino-acid sequences that are all associated with TLR/IL-1R signalling. IRAK-4 and IRAK-1 are recruited by binding with the death domain of MyD88, where IRAK-4 rapidly phosphorylates IRAK-1 to activate IRAK-1s kinase domain leading to auto-phosphorylation of IRAK-1 (Burns, Janssens et al. 2003). Phosphorylation of IRAK-1 causes a reduction in its affinity to MyD88 but increases affinity to TNF receptor-associated factor 6 (TRAF6) (Cao, Xiong et al. 1996; Wesche, Henzel et al. 1997).

TRAF6 belongs to a family of TNF receptor-associated adaptor proteins that are evolutionary conserved. It is recruited to the TIR domain by the TRAF-C domain, which mediates binding between the C-terminal TRAF6-binding motif (Pro-X-Glu-X-X-aromatic/acidic residue) in IRAK-1 and TRAF6 (Ye, Arron et al. 2002). Upon binding to IRAK-1, the zinc-finger and RING effector domains within the N-terminus (Rothe, Wong et al. 1994; Cao, Xiong et al. 1996) are activated, allowing the IRAK:TRAF6 complex to dissociate from the receptor complex and bind to the membrane bound transforming growth factor β (TGF β)-activated kinase 1 (TAK1) signalling complex (Ninomiya-Tsuji, Kishimoto et al. 1999).

TAK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK or MAP3K) family member directly linked to the activation of NF- κ B and its subsequent up-regulation of pro-inflammatory cytokines (Yamaguchi, Shirakabe et al. 1995; Ninomiya-Tsuji, Kishimoto et al. 1999). For its functions in signalling, TAK1 is associated with adaptor proteins: TAK1-binding protein-1 (TAB1) (Shibuya, Yamaguchi et al. 1996), TAB2 (Takaesu, Kishida et al. 2000) and TAB3 (Cheung, Nebreda et al. 2004). As the IRAK:TRAF6 complex binds to the TAK1 signalling complex, phosphorylation (by an as of yet unknown kinase) of TAK1 and TAB2 releases all components except IRAK-1 into the cytosol (Jiang, Ninomiya-Tsuji et al. 2002). TAB2 and TAB3 act as adaptor molecules, allowing TRAF6 to activate the kinase domain of TAK1 (Kanayama, Seth et al. 2004) TAB1 enhances activation of TAK1 (Shibuya, Yamaguchi et al. 1996), resulting in release of NF- κ B (a nuclear factor kappa enhancer binding protein) from inhibitory proteins.

1.7.5. Canonical NF- κ B Activation

NF- κ B is the primary transcription factor activated by TLR/IL-1R signalling. The NF- κ B family of transcription factors consists of five proteins, RelA (p65), RelB, c-Rel, p105 (p50/NF- κ B1) and p100 (NF- κ B2), characterised by the presence of an N-terminus region of around 300 amino acids known as the Rel-homology domain (RHD). When activated, NF- κ B exists as a dimer in the cytosol of most mammalian cell types, the most common consisting of RelA and p105 (Mercurio and Manning 1999). It also exists in the cytoplasm in resting cells, where it is held in its inactive form by a complex of inhibitory proteins termed I κ Bs (Baeuerle and Baltimore 1988). There are six mammalian I κ B family members, however only three of these, I κ B α , I κ B β and I κ B ϵ

appear to be important for controlling NF- κ B activity (Baeuerle and Baltimore 1996; Whiteside, Epinat et al. 1997).

IKKs are large (700-900kDa) cytoplasmic complexes that phosphorylate I κ Bs at the N-terminal sites to control turnover (Zandi, Rothwarf et al. 1997). They contain two serine kinases IKK α (IKK1) and IKK β (IKK2) and a regulatory component IKK γ (NEMO). The IKK complex is targeted by activated TAK1, where it phosphorylates serine residues in the activation loop of IKK α and IKK β , marking these molecules for proteasomal degradation by the 26S proteasome (Chen, Hagler et al. 1995). This allows NF- κ B to translocate to the nucleus and up-regulate target genes, including pro-inflammatory cytokines

1.7.5.1. MAPK Activation

There are also other targets activated by TLR/IL-1R signalling, such as mitogen-activated protein kinases (MAPKs), which are also involved in many essential processes, including cell growth, differentiation, stress and inflammatory responses. MAPKs are serine/threonine protein kinases that are activated by phosphorylation of both threonine and tyrosine residues (Anderson, Maller et al. 1990). MAPKs are divided into three sub-categories: the extracellular signal regulated kinase (ERK), p38 MAPK and the Jun N-terminal kinase (JNK), also known as the stress-activated protein kinase (SAPKs).

The first MAPK family member to be cloned were the ERKs (Boulton, Yancopoulos et al. 1990; Boulton, Nye et al. 1991) Further study showed the phosphorylation of ERKs led to activation of other transcription factors, for example Elk-1 (Gille, Sharrocks et al. 1992; Marais, Wynne et al. 1993). The MAPK activation system is a three-tiered phosphorylation cascade. MAPKKK phosphorylates MAP kinase kinases (MAPKKs) in order to activate MAPK. TAK1, as mentioned previously (Section 1.3.4.) is a key molecule in NF- κ B signalling and is a MAPKKK and is responsible for the branching of the signalling pathways.

The p38 MAPK was originally identified in mammalian cells as a 32kDa protein that underwent tyrosine phosphorylation in response to endotoxin stimulation and extracellular changes in osmolarity (Han, Lee et al. 1994). The p38 MAPKs consist of four isoforms, p38 α , which is the original form of p38 and shown to be activated in

response to IL-1 (Freshney, Rawlinson et al. 1994), p38 β (Jiang, Chen et al. 1996; Stein, Yang et al. 1997), p38 γ (Lechner, Zahalka et al. 1996; Li, Jiang et al. 1996) and p38 δ (Goedert, Cuenda et al. 1997; Jiang, Gram et al. 1997; Kumar, McDonnell et al. 1997).

The JNK family of proteins are encoded by three genes, JNK1, JNK2 and JNK3 (also known as SAPK γ , SAPK α and SAPK β respectively) which exist as ten spliced isoforms (Derijard, Hibi et al. 1994; Kyriakis, Banerjee et al. 1994; Gupta, Barrett et al. 1996). Activation by phosphorylation is initiated by a variety of cellular stresses, including inflammation.

Both the p38 MAPKs and JNKs are involved in activation of the transcription factor activator protein-1 (AP-1). AP-1 exists as a heterodimer and is activated by phosphorylation of the trans-activating domain of ATF2, resulting in up-regulation of transcription of AP-1 components (Karin, Liu et al. 1997). Activated AP-1 is translocated to the nucleus where it is involved in transcription of genes for the inflammatory response (IL-1, TNF), cell adhesion molecules (E-selectin) and proteases (Karin, Liu et al. 1997; Read, Whitley et al. 1997).

An overview of NF- κ B signalling can be found in Figure 1.3.

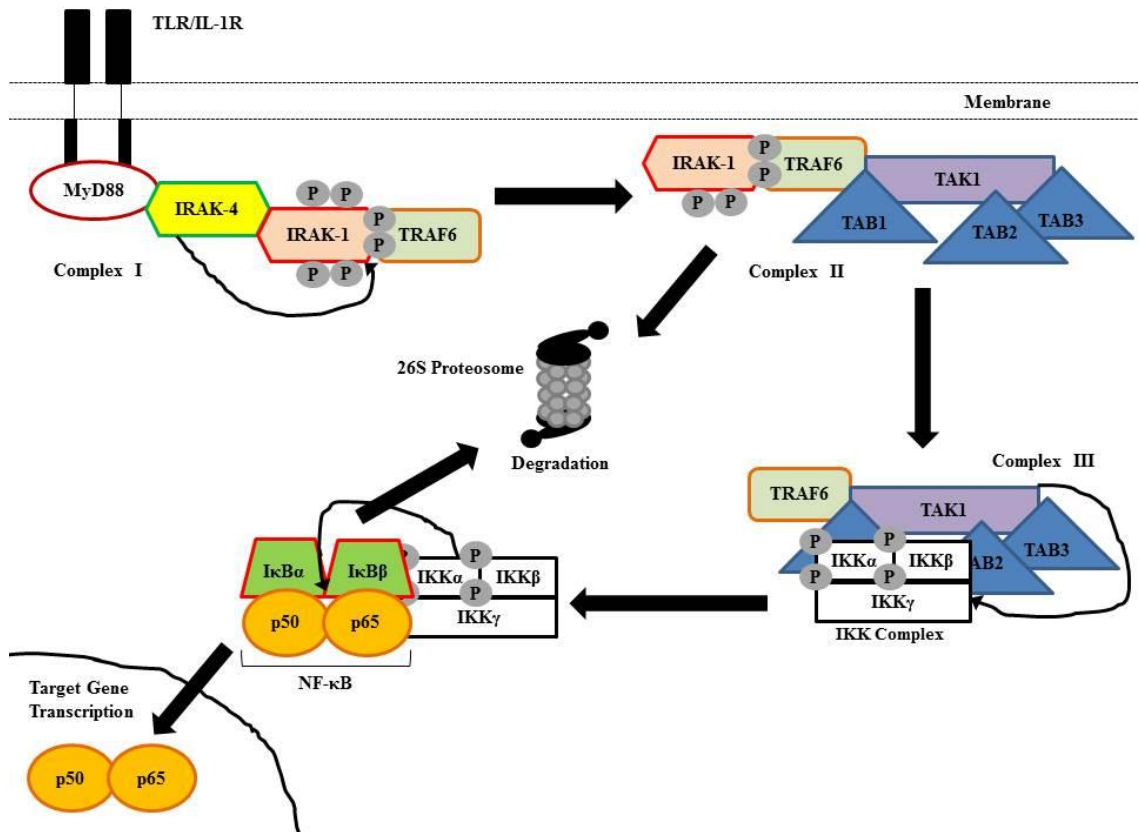


Figure 1.3: An overview of canonical NF-κB signalling

Activation of TLR/IL-1R at the membrane results in formation of complex I as MyD88 is recruited to the receptor complex. This leads to hyperphosphorylation (P) of IRAK-1 by IRAK-4 and subsequent binding of TRAF6. The IRAK-1:TRAF6 heterodimer leaves the receptor to bind to TAK1 and its associated binding proteins TAB1, TAB2 and TAB3 to form complex II. Formation of complex III occurs when IRAK-1 releases TRAF6:TAK1:TAB1/2/3 from the membrane, where it activates the IKK complex by phosphorylation. Phosphorylated IKK activates NF-κB by targeting the inhibitory proteins bound to it, the IκBs, for degradation. This allows NF-κB to translocate to the nucleus where it is responsible for up-regulating target genes.

1.7.6. MyD88-Independent Signalling Pathways

All TLR/IL-1R signalling occurs through MyD88, except TLR3, which signals through the TIR domain-containing adaptor inducing IFN- β (TRIF) (also known as TICAM-1), and TLR4, which signals through both MyD88 and TRIF (Yamamoto, Sato et al. 2002; Oshiumi, Matsumoto et al. 2003). The TRIF sequence contains a TIR domain for TIR-TIR interactions and a receptor interacting protein (RIP) homotypic interaction motif (RHIM) in the C-terminus to mediate interactions of the RIP family (Meylan, Burns et al. 2004), which leads to activation of TAK1 and subsequently the IKK complex as seen in canonical NF- κ B (Cusson-Hermance, Khurana et al. 2005).

TRIF was discovered using MyD88 deficient mice, which activated NF- κ B and MAPKs as a late phase response associated with activation of Type I IFNs, in response to LPS and viral stimuli (Kawai, Adachi et al. 1999; Kawai, Takeuchi et al. 2001). Mice deficient in functional TRIF show early phase activation of NF- κ B and MAPK signalling, but reduced proinflammatory cytokine and Type I IFN production, suggesting a requirement for TRIF for Type I IFN induction/responses and maximal production of cytokines in response to LPS (Hoebe, Du et al. 2003; Yamamoto, Sato et al. 2003).

TRIF and TLR4 do not bind directly; they require an adaptor molecule, TRIF-related adaptor molecule (TRAM), to activate TRIF-dependent signalling processes in response to LPS (Yamamoto, Sato et al. 2003). TLR3 binds to double stranded RNA (dsRNA), a product of replication by many viruses, and poly(I:C), a synthetic dsRNA TLR3 agonist (Alexopoulou, Holt et al. 2001). In response to poly(I:C), TRIF knockout mice do not elicit a response, whereas MyD88 and TRAM knockout mice respond normally, leading to the conclusion that TRIF is the only required adaptor for signalling through TLR3 (Yamamoto, Sato et al. 2003). A summary of MyD88 independent signalling can be found in Figure 1.4.

1.7.7. Non-Canonical NF- κ B Signalling

Non-canonical NF- κ B signalling was discovered during study of p100 processing (Xiao, Harhaj et al. 2001). Two NF- κ B family members, p100 and p105, are produced as precursor proteins that undergo proteolytic processing to become the active transcription factors, p52 and p50 respectively, and are involved in activating non-canonical NF- κ B signalling pathways. The precursor proteins function in the same manner as I κ B in

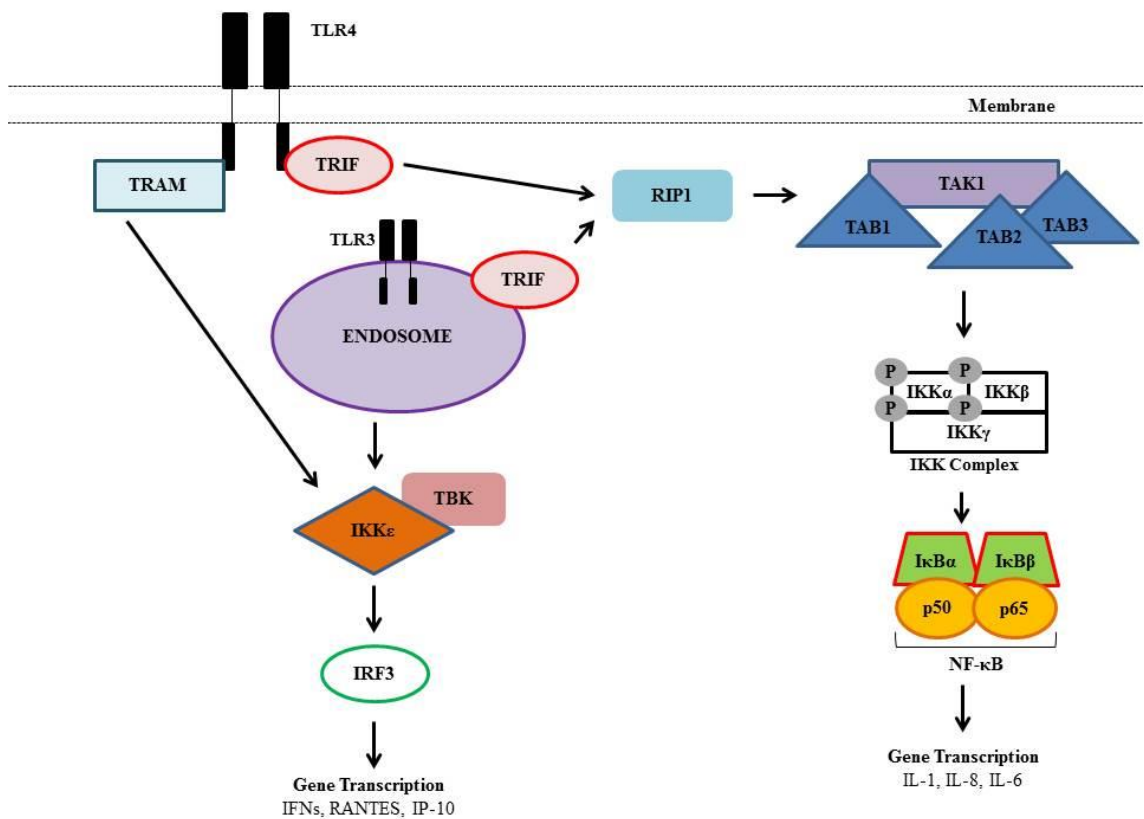


Figure 1.4: An overview of MyD88-independent signalling

TLR3 is activated in response to viral dsRNA, resulting in activation of TRIF. TRIF recruits RIP1 which leads to activation of NF-κB transcription through TAK1 and the IKK complex. Activated TLR3 can also activate the transcription factor IRF3 through IKKε/TBK kinases. TLR can also activate MyD88-independent pathways through TRIF, to activate NF-κB, and TRAM, to activate IRFs.

canonical signalling, by holding them in an inactive form in the cytoplasm. Activation of the p100/p105 signalling pathways causes partial degradation of the precursors C-terminal domains, removing the I κ B like ankyrin repeats to release active p52/p50 fragments. Although the unmasked RHD allows the proteins to bind to DNA, transactivation domain (TAD) required for activating gene transcription is not present (Zhong, May et al. 2002). To overcome this, p52/p50 fragments form heterodimers with RelB, c-rel or p65, whereas homodimers of p52 and p50 have been shown to inhibit gene transcription (Zhong, May et al. 2002).

The transcription factor p105 undergoes constitutive processing, by partial degradation by the proteasome, to generate p50 fragments. This is thought to be a co-translational mechanism to generate p50 fragments for formation of the p50/p65 heterodimers utilised in canonical NF- κ B signalling (Fan and Maniatis 1991; Palombella, Rando et al. 1994; Lin, DeMartino et al. 1998). This processing is inhibited by formation of homo- or heterodimers with other NF- κ B proteins (Harhaj, Maggirwar et al. 1996; Cohen, Orian et al. 2001), suggesting its role is dependent upon the cells availability of NF- κ B.

The processing of p100, however, is much more tightly regulated by both positive and negative domains, with only minimal amounts of processing in unstimulated cells (Heusch, Lin et al. 1999; Xiao, Harhaj et al. 2001). The functions of p100 are to prevent nuclear translocation of RelB and regulate its transcriptional activity (Dobrzanski, Ryseck et al. 1995; Solan, Miyoshi et al. 2002). The processing of p100 has been linked to receptor activation of immune response molecules, including BAFF-R (Kayagaki, Yan et al. 2002), CD40 (Coope, Atkinson et al. 2002) and LT β R (Dejardin, Droin et al. 2002). BAFF-R and CD40 activation is linked to B-cell maturation and survival and LT β R binds to TNF superfamily members. The pathway is also activated by some TNF family members, however, TNF α does not induce p100 processing (Coope, Atkinson et al. 2002), but does lead to up-regulation of p100 and RelB (Derudder, Dejardin et al. 2003).

Activation of the p100 non-canonical signalling pathway has been linked to expression of the NF- κ B inducing kinase (NIK); overexpression leads to p100 processing to p52 (Senftleben, Cao et al. 2001), whereas deficiencies in functional NIK result in significantly reduced levels of p52 but normal p100 levels (Xiao, Harhaj et al. 2001). NIK contain a kinase domain that has some conserved sequence homology to the kinase

domains of MAP3Ks; it has also been shown to interact with (Regnier, Song et al. 1997) and phosphorylate IKK α (Ling, Cao et al. 1998). This interaction of IKK α and NIK is independent of factors known to be required in canonical NF- κ B signalling such as IKK β and IKK γ (Senftleben, Cao et al. 2001; Xiao, Harhaj et al. 2001). Upon binding with NIK, IKK α has been shown to directly phosphorylate p100, which leads to its processing to p52 (Senftleben, Cao et al. 2001).

Non-canonical signalling has not been studied as extensively as canonical NF- κ B signalling, therefore it is likely there are many as of yet unidentified factors that act as mediators and activators, however, key molecules identified so far are summarised in Figure 1.5.

1.7.8. TLR3 Signalling

TLR3 could play a prominent role in detection of viruses as it has been shown to bind double-stranded RNA (dsRNA) (Alexopoulou, Holt et al. 2001), which is a product of viral replication or part of the viral genome. TLR3 has also been shown to bind to poly(I:C) the stable synthetic dsRNA, which it does preferentially over dsRNA derived from viral sources (Okahira, Nishikawa et al. 2005). Binding of dsRNA and TLR3 is dependent on an acidic pH (de Bouteiller, Merck et al. 2005). It was originally shown to not recognise single stranded RNA (ssRNA) (Alexopoulou, Holt et al. 2001), however, more recent work showed that mRNA, which is predominantly single stranded acts as a ligand to TLR3, showing that it is capable of binding to ssRNA (Marshall-Clarke, Downes et al. 2007). TLR3 activation leads to induction of type I IFN and NF- κ B activation through distinct pathways.

TLR3 is a type I transmembrane receptor so has a similar structure to the other TLR family members (Jin and Lee 2008). Structural studies show that in solution it exists as a monomer, however can form homomultimers in the membrane, which takes place upon ligand binding (Bell, Botos et al. 2005; Bell, Botos et al. 2006; Leonard, Ghirlando et al. 2008; Liu, Botos et al. 2008).

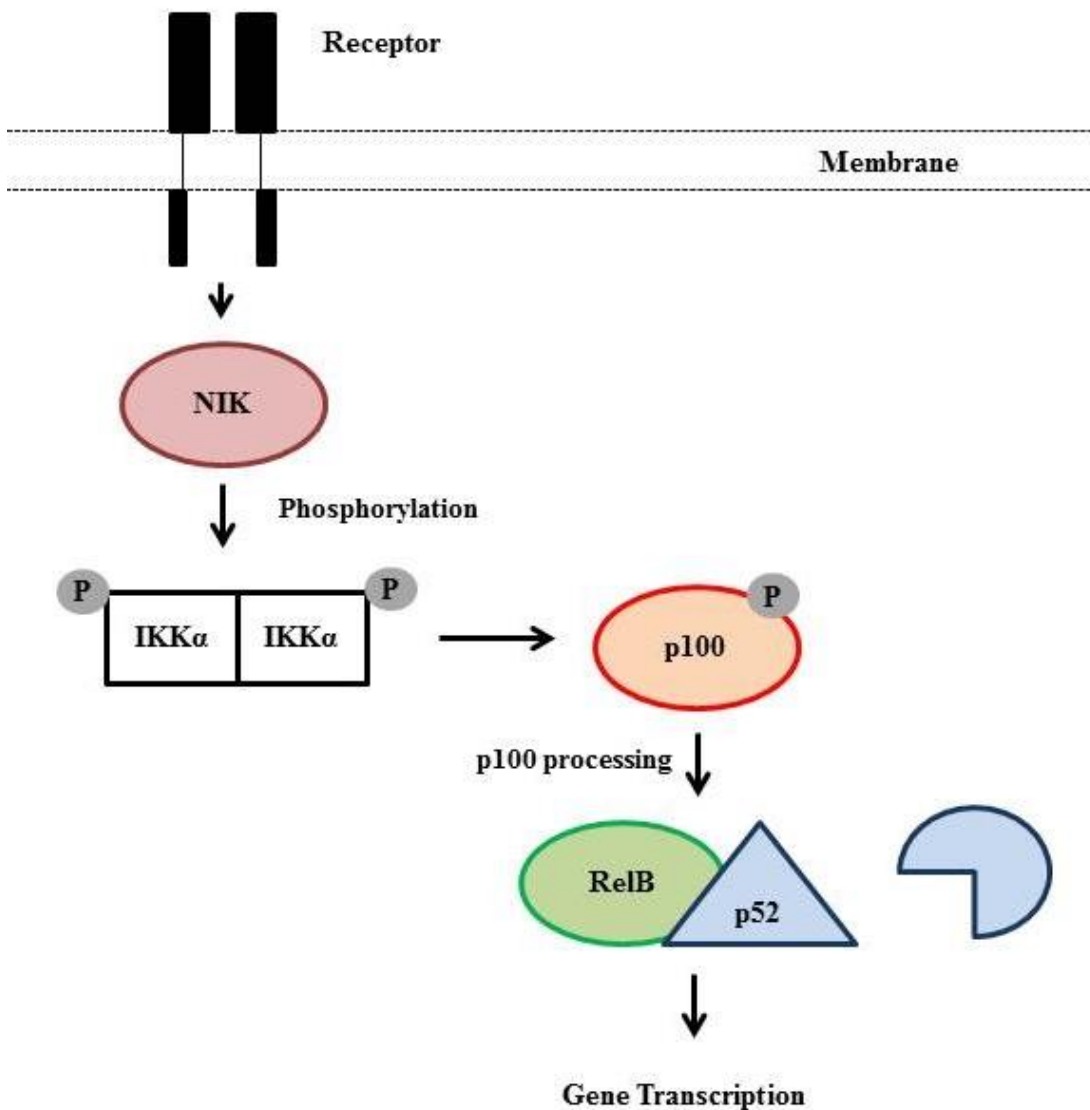


Figure 1.5: The key mediators of non-canonical NF- κ B signalling

Activation of the non-canonical signalling pathway is mediated through the kinase NIK. NIK is involved in phosphorylating an IKK complex that only consists of two IKK α units. The activated IKK complex phosphorylates p100, causing processing of p100 to its active form p52, which then dimerises with RelB, which contains the transactivation domain (TAD) required for nuclear translocation and subsequent up-regulation of target genes.

TLR3 activation results in phosphorylation at two C-terminal tyrosine residues (Sarkar, Peters et al. 2004), leading to recruitment of TRIF. Stimulation of TLR3 leads to activation of distinct pathways that result in activation of NF- κ B and the IRFs (See section 1.3.7.1). NF- κ B is activated by TLR3 through receptor interacting protein 1 (RIP1) protein but has been shown to be negatively regulated by RIP3, another RIP protein family member (Meylan, Burns et al. 2004). TRIF contains a RIP homotypic interaction motif in its C-terminus that mediates its binding to RIP proteins in order to activate signalling. This recruits RIP1 to the TLR3-TRIF signalling complex (Meylan, Burns et al. 2004). RIP1 recruitment activates TAK1 and subsequently the IKK complex which leads to the degradation of I κ B, as seen on canonical NF- κ B signalling (Cusson-Hermance, Khurana et al. 2005). There are likely to be other mediators involved in interactions between RIP1 and TAK1 that are yet to be identified. However, TRAF6 has been suggested as a likely candidate as TRIF contains three TRAF6 binding motifs and over expression of dominant negative TRAF6 has shown inhibition of TRIF-induced NF- κ B activation (Sato, Sugiyama et al. 2003; Jiang, Mak et al. 2004). IRF activation and subsequent activation of both type I and III IFNs occurs when TRIF binds to TRAF3. TRAF3 recruits kinases involved in phosphorylating and activating IKK ϵ and TANK binding kinase 1 (TBK1) IRF family members (Hacker, Redecke et al. 2006; Oganessian, Saha et al. 2006).

1.7.8.1. IRFs

The IRF family consists of nine different transcription factors (IRF1-9) that are all involved in induction of Type I and III IFNs. They all contain a DNA binding domain consisting of five conserved tryptophan-rich repeats in the N-terminus forming a helix-turn-helix that recognises the IFN regulatory element upstream of IRF-regulated genes (Mamane, Heylbroeck et al. 1999; Taniguchi, Ogasawara et al. 2001). The C-terminal domains, which are less conserved, mediate interactions with other signalling mediators and transcription factors.

The key IRFs involved in antiviral immunity are IRF3 and IRF7 (Marie, Durbin et al. 1998; Yoneyama, Suhara et al. 1998; Au, Yeow et al. 2001); both of which have been found to activate type I and III IFNs in response to viral stimuli, however IRF1 and IRF5 have also been associated with type I IFN reduction in response to viruses (Mamane, Heylbroeck et al. 1999; Taniguchi, Ogasawara et al. 2001; Takaoka, Yanai et al. 2005). Upon viral infection, phosphorylation at specific serines in IRF3s C-terminal

regulatory domain results in homodimerisation or heterodimerisation with IRF7 and subsequent assembly of the nuclear holocomplex. This holocomplex consists of IRF dimers associated with co-factors, either cyclic adenosine monophosphate (AMP) responsive element binding protein (CREB) binding protein (CREBBP) or p300, which are involved in binding to DNA and up-regulating target gene transcription (Lin, Heylbroeck et al. 1998; Sato, Tanaka et al. 1998; Weaver, Kumar et al. 1998; Yoneyama, Suhara et al. 1998).

IRF7 is strongly induced by type I IFN-mediated signalling but in contrast to IRF3 it is expressed in small amounts in most cell types. Binding of type I IFNs to the IFN receptor results in IRF7 up-regulation, suggesting that IRF7 is involved in late-phase IFN production as part of a positive feedback loop. Activation of IRF7 is similar to that of IRF3, with phosphorylation of C-terminal serine domains leading to production of homodimers or heterodimers with IRF3 which then translocate to the nucleus to up-regulate transcription of target genes. Different combinations of IRF3 and IRF7 homo- and heterodimers have different effects on target gene transcription; IRF7 activates both *IFNA* and *IFNB* genes, whereas IRF3 is a potent *IFNB* gene activator, but not *IFNA* (with the exception of *IFNA4*) (Marie, Durbin et al. 1998; Sato, Hata et al. 1998; Sato, Suemori et al. 2000).

Originally, it was thought that only IRF3 was involved in the early phase of *IFNB* gene induction and that this type I IFN production leads to production of IRF7 for the late phase induction of *IFNA* and *IFNB* genes. However, a study conducted using an *irf7* deficient (*irf7*^{-/-}) mouse model, specifically *irf7* deficient mouse embryonic fibroblasts, showed a severe impairment in type I IFN induction in response to an ssRNA viral stimulus, suggesting the constitutively produced small amount of IRF7 is vital for early phase IFN production (Honda, Yanai et al. 2005).

1.7.9. RIG-1/MDA5 Signalling

It was initially thought that TLR3 was the key mediator involved in viral detection in host cells until a study conducted using TLR3 knockout cells showed production of IFN in response to both the dsRNA viral mimic poly(I:C) and viral infection (Yoneyama, Kikuchi et al. 2004). This led to identification of the involvement of retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), members of the RIG-I-like receptor family of proteins, in sensing viral RNA in the

cytoplasm (Yoneyama, Kikuchi et al. 2004). RIG-I and MDA5 both contain two repeated CARDs in the N-terminal and a DExD/H-box RNA helicase domain in the C-terminal. The CARD domains have been implicated in IRF3, IRF7 and NF- κ B activation, and subsequent activation of IFN- β (Yoneyama, Kikuchi et al. 2004). The RNA helicase domain has been shown to bind in an ATP dependent manner to poly(I:C) (Saito, Hirai et al. 2007). The C-terminus of RIG-I has been shown to act as a repressor domain (RD) in order to keep it in the cytosol in its latent form (Saito, Hirai et al. 2007).

RIG-I deficient mouse embryonic fibroblasts were shown to exhibit impaired IFN release when infected with Sendai virus (SeV), Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), influenza virus or Japanese encephalitis virus (JEM) or stimulated with dsRNA transcribed *in vitro*. IFN release was unaffected in MDA5 deficient mouse embryonic fibroblasts showing that RIG-I and MDA5 recognise different viruses. MDA5 deficient mouse embryonic fibroblasts did show impaired IFN release in response to infection with Picornaviridae virus family members, which isn't seen in the RIG-I deficient cells (Loo, Fornek et al. 2008). Recent studies have identified the binding site for RIG-I as a 5'-triphosphate moiety in viral RNA. This 5'-triphosphate moiety is usually capped in host endogenous mRNA (Hornung, Ellegast et al. 2006; Pichlmair, Schulz et al. 2006). The structure recognised by MDA5 has yet to be elucidated.

1.7.10. Final Stage of Signalling: Cytokine and Chemokine Production

Cytokines are intracellular signalling peptides (8-30kDa) that act at any range (auto-, para- and endocrine) on a variety of cell types including leukocytes and epithelial cells. Their main function is to manage the immune response by activating, inhibiting or drawing leukocytes to a site of infection by chemotaxis. Cytokines can be produced by a variety of nucleated cell types (Cannon 2000).

Infections stimulate the TLR/IL-1R signalling pathway to activate various transcription factors, including NF- κ B, to produce cytokines, some of which have a pro- or anti-inflammatory effect. Local proinflammatory responses stimulate leukocyte activity, including proliferation, cytotoxicity and proteolytic enzyme release. They also synthesise prostaglandins and initiate a cascade of "secondary" anti-inflammatory cytokine synthesis and secretion. Systemic pro-inflammatory cytokine release induces

fever by raising the thermoregulatory set point and creates a hostile growth environment for growth of some bacterial species by redistributing iron from extracellular to intracellular sites. The central nervous system is targeted to cause a reduction in energy consumption and skeletal muscle is broken down to provide amino acids for acute phase plasma proteins which function to work with leukocytes to remove pathogens (Cannon 2000).

Chemokines are a subset of cytokines that act as chemoattractants to immune cells to recruit them to the site of infection/injury. They are small proteins (8-10kDa) sub-grouped based on their primary structure. The four sub-groups are labelled CC, CXC, CX3C and C, which are defined by the position of two N-terminal conserved cysteine residues (Murphy 1994; Choi and An 2011). Chemokines exert their effects by binding to G-protein receptors on the cell surface, triggering cellular responses including chemotaxis. The two major groups are the CC and CXC groups; CC chemokines are involved in monocyte, lymphocyte and basophil recruitment, whereas CXC chemokines are involved in neutrophil recruitment (Choi and An 2011).

The cytokines and chemokines that are utilised as a stimulus or measure of inflammatory responses throughout this thesis are discussed individually.

1.7.10.1. Interleukin-1

IL-1 is produced by cells in response to pathogens via the NF- κ B pathway. There are two agonist forms of IL-1, IL-1 α and IL-1 β , both are found in the same 430kb gene cluster (in humans chromosome 2q13) but are produced as independent gene products (Nicklin, Weith et al. 1994). Both forms bind the same receptors, however IL-1 β is the most common mediator of systemic inflammation.

Transcription of the IL-1 β gene produces an inactive proform protein that is activated by caspase-1 cleavage. It is largely produced by immune cells but can also be produced by epithelial and endothelial cells (Cannon 2000). It is a potent pro-inflammatory cytokine, capable of inflicting detrimental effects on host tissues; therefore evolution of a network of regulatory mechanisms was necessary to attenuate its potency. The IL-1 receptor antagonist (IL-1ra) (a 17kDa protein) acts as competitive inhibitor to regulate potency by binding with high affinity to IL-1RI, and with lower affinity to IL-1RII (Dripps, Verderber et al. 1991). All three proteins share similar amino acid sequences,

gene structure and chromosomal location (Arend 1993; Nicklin, Weith et al. 1994) IL-1ra restricts IL-1 β activity through competitive inhibition.

1.7.10.2. Interleukin-6

Interleukin-6 (IL-6) is a 212 amino-acid member of the 4 α -helix haematopoietic family of cytokines. IL-6 is another gene regulated by NF- κ B; is not expressed constitutively but is up-regulated in response to a wide range of PAMPs and DAMPs, including LPS, viral infection, IL-1 β , TNF- α and IFN- γ . It is produced by both immune and non-immune cells, including macrophages, lymphocytes, dendritic cells, fibroblasts and endothelial and epithelial cells (May, Ndubuisi et al. 1995; Jawa, Anillo et al. 2011).

IL-6 acts in both a pro- and anti-inflammatory capacity with knock-out murine studies demonstrating that it has a key role in mediating the immune and acute phase response (Kopf, Baumann et al. 1994). One of the many effects it has on the immune response is involvement in immune cell maturation, including T cell differentiation and antibody production in B-cells, but it doesn't appear to be involved in proliferation of activated B cells (Muraguchi, Hirano et al. 1988; Naka, Nishimoto et al. 2002). Its targeting of endothelial cells causes an induction of chemokines and adhesion molecules that attract and recruit leukocytes to the site of inflammation (Cronstein 2007). IL-6 also induces phospholipase A₂ (PLA₂) (Crowl, Stoller et al. 1991), leading to the production of key pro-inflammatory mediators including prostaglandins and PAF (Tilg, Dinarello et al. 1997), which is involved in neutrophil priming, a process potentiated by IL-6 (Biffi, Moore et al. 1994).

Inflammation is tightly controlled in order to avoid unnecessary damage to the host. The pleiotropic effects of IL-6 are important anti-inflammatory functions. It has been shown, *in vivo*, to down-regulate the potent effects of IL-1 and TNF α by inducing production of IL-1ra and TNFR (Tilg, Trehu et al. 1994). It has also been shown to induce the tissue inhibitor of matrix metalloproteinase(MMP)s (TIMP) which reduces damage to cells (Jawa, Anillo et al. 2011).

IL-6 is activated by binding to a receptor complex containing IL-6R and gp130 subunits. Activation of the IL-6 receptor results in activation of the JAK/STAT pathway and transcription of IL-6 stimulated genes.

1.7.10.3. Interleukin-8

Interleukin-8 (IL-8 or CXCL8) is a CXC chemokine that is a potent chemoattractant for neutrophils, but not monocytes, and is produced by a wide range of cell types, including monocytes, neutrophils, T lymphocytes, fibroblasts, endothelial cells and epithelial cells (Schroder, Mrowietz et al. 1987; Walz, Peveri et al. 1987).

There is a strong link between IL-8 and inflammatory diseases in the respiratory tract as increased levels of IL-8 have been identified in BAL fluid obtained from asthma (Fahy, Kim et al. 1995) and COPD patients (Keatings, Collins et al. 1996). This is a key factor in the establishment of a neutrophilic inflammatory environment within the airways of these patients (Fahy, Kim et al. 1995).

IL-8 has the ability to bind to two different receptors, CXCR1 and CXCR2. These receptors are expressed on a variety of cell types including a variety of leukocytes and non-haematopoietic cells such as endothelial and epithelial cells. However, they are much more commonly associated with cells of a myeloid lineage, especially chemoattraction of neutrophils (Stillie, Farooq et al. 2009). Transduction of signals through both receptors plays a role in antimicrobial activity of neutrophils (Baggiolini and Clark-Lewis 1992), but CXCR1 plays a more dominant role in neutrophil chemotaxis than CXCR2 (Hammond, Lapointe et al. 1995; Quan, Martin et al. 1996).

1.7.10.4. RANTES

Also known as CCL5, RANTES is a CC- type chemokine discovered in a differential screen between T- and B- leukocytes. It is an IRF-stimulated gene that exerts its effects by binding to four different chemokine receptors, CCR1 (Gao, Kuhns et al. 1993; Neote, DiGregorio et al. 1993), CCR3 (Daugherty, Siciliano et al. 1996), CCR4 (Power, Meyer et al. 1995) and CCR5 (Combadiere, Ahuja et al. 1996; Raport, Gosling et al. 1996). Production is inducible upon mitogen or antigen stimulation of T-cell lines and circulating lymphocytes and viral stimulation of epithelial cells (Schall, Bacon et al. 1990). It functions as a potent chemoattractant for monocytes, but is less effective than monocyte chemotactic protein (MCP)-1 (Schall, Bacon et al. 1990; Uguccioni, D'Apuzzo et al. 1995) and has no effect on neutrophils. It is an important chemotactic agent for many T-lymphocyte subsets including CD4⁺ and CD45R0⁺ in endothelial free assays (Schall, Bacon et al. 1990) and CD4⁺ and CD8⁺ in trans-endothelial systems, where it has been shown to be the most potent CC chemokine for CD8⁺ T-cells (Roth, Carr et al. 1995). As well as attracting and activating natural killer (NK) cells (Taub,

Sayers et al. 1995; Maghazachi, Al-Aoukaty et al. 1996), it also attracts and is secreted by eosinophils (Rot, Krieger et al. 1992; Lim, Wan et al. 1996) and it targets basophils, causing them to release histamine (Kuna, Reddigari et al. 1992).

1.7.10.5. IP-10

Produced in response to viral stimulus, the IRF-stimulated gene IP-10, also known as CXCL10, is a CXC chemokine that binds to the G-protein coupled receptor CXCR3 (Loetscher, Gerber et al. 1996). IP-10 is produced from a variety of cell types including monocytes, keratinocytes, T-lymphocytes, fibroblasts and endothelial and epithelial cells in response to IFN γ stimulation (Luster, Unkeless et al. 1985). It does not attract or activate neutrophils (Dewald, Moser et al. 1992), but has been linked to chemotaxis of T-lymphocytes (Murphy, Tian et al. 1996; Taub, Longo et al. 1996) and tumour-infiltrating leukocytes (Liao, Rabin et al. 1995).

1.7.10.6. MCP-1

MCP-1, also known as CCL2, exists in two forms, a precursor and mature form, of 23 and 76 amino acids in length (Furutani, Nomura et al. 1989; Yoshimura, Yuhki et al. 1989). Dominant negative inhibition studies showed that it functions as a dimer (Zhang and Rollins 1995). It is primarily secreted by monocytes, macrophages and dendritic cells; however it requires cleavage by the metalloproteinase MMP-12 to become activated. It is linked to chemotaxis and activation of monocytes (Ugucioni, D'Apuzzo et al. 1995), CD4⁺ and CD8⁺ T-lymphocytes (Loetscher, Seitz et al. 1994) and basophils (Bischoff, Krieger et al. 1992), however it does not attract neutrophils or eosinophils. Activation of basophils results in histamine release (Alam, Lett-Brown et al. 1992; Bischoff, Krieger et al. 1992; Kuna, Reddigari et al. 1992). MCP-1 exerts its effects by binding to CCR2 (Charo, Myers et al. 1994; Yamagami, Tokuda et al. 1994) and CCR4 (Power, Meyer et al. 1995).

1.7.10.7. Interferons (IFNs)

The IRF stimulated family of cytokines, the IFNs, share significant amino acid homology and exhibit similar cellular effects on target cells including modulation of the immune response, anti-viral immunity and anti-proliferative effects. There are three major IFN subgroups: Type I (INF α , IFN β , IFN ω , IFN κ and IFN ϵ), Type II (IFN γ) and Type III (IFN λ 1, IFN λ 2 and IFN λ 3).

The genes encoding all Type I IFNs share significant sequence homology and are located in a cluster on chromosome 9 (Diaz, Pomykala et al. 1994; Stark, Kerr et al. 1998). Type I IFN have been shown to possess potent anti-viral activity which includes up-regulation of proteins that bind to dsRNA (Williams 1999), inhibition of protein translation (Kerr and Brown 1978; Jacobs and Langland 1996), inhibition of viral ribonucleoprotein complex trafficking (Weber, Haller et al. 2000) and induction of apoptosis in virally infected cells (Takizawa, Ohashi et al. 1996; Der, Yang et al. 1997; Balachandran, Kim et al. 1998; Gil and Esteban 2000), showing them to possess potent anti-viral activity. The essential role of these cytokines is confirmed in the Type I IFN receptor knockout mouse, which is highly susceptible to viral infections (Hwang, Hertzog et al. 1995; Steinhoff, Muller et al. 1995).

IFN γ , the only Type II IFN, shares little amino acid sequence homology with the Type I IFNs (Stark, Kerr et al. 1998). Studies using an IFN γ receptor knockout mouse (Huang, Hendriks et al. 1993; Lu, Ebensperger et al. 1998) and humans with IFN- γ receptor mutations (Dorman, Picard et al. 2004) show that it does not play a significant role in anti-viral immunity. It has been associated with both cell-mediated immune responses to intracellular pathogens and anti-tumour immune responses (Donnelly and Kotenko 2010).

The more recently discovered Type III IFNs have also been shown to be involved in anti-viral responses (Kotenko, Gallagher et al. 2003; Sheppard, Kindsvogel et al. 2003). The genes encoding all three IFN λ s are clustered on chromosome 19, with proteins sharing between 5-18% sequence homology to the Type I IFNs (Donnelly and Kotenko 2010) and induction occurs in response to the same stimuli as Type I IFNs (Coccia, Severa et al. 2004; Ank, West et al. 2006). They are often co-expressed in viral infected cells, confirming their joint roles in anti-viral immunity (Kotenko, Gallagher et al. 2003; Sheppard, Kindsvogel et al. 2003). Type III IFNs have been strongly associated with responses of human primary epithelial cells to RSV infection (Spann, Tran et al. 2004).

Each IFN type binds a specific receptor; Type I bind to the IFNR1 α and IFNR α heterodimeric complex, Type II to the IFN γ R1/IFN γ R2 tetrameric receptor complex that consists of two of each of the receptor domains, and Type III IFNs bind to the IFN λ R1/IL-10R2 heterodimeric receptor complex. Activation of any of these receptors activates the JAK/STAT pathway and leads to transcription of IFN stimulated genes

(ISG) through STAT binding to IFN stimulated response elements (IRSE) for Type I and III IFNs or IFN γ associated sequence (GAS) for Type II IFNs (Donnelly and Kotenko 2010).

1.7.11. TNF α -Signalling

Tumour necrosis factor- α (TNF- α), a member of the TNF-related superfamily of proteins is a pro-inflammatory cytokine up-regulated in response to acute inflammation. First identified as a lymphocyte and macrophage product that caused lysis of certain cell types, including tumour cells (Granger, Shacks et al. 1969; Carswell, Old et al. 1975), it has since been shown to be produced by a wide variety of cells, including macrophages, CD4⁺ and CD8⁺ T-lymphocytes, B lymphocytes, NK cells, neutrophils, smooth muscle cells and endothelial cells (Vilcek and Lee 1991). It plays important roles in the immune system include activation of immune cells, including monocytes and platelets, and enhancement of macrophages and NK cells abilities to kill viral, bacterial and parasitic pathogens. TNF- α has also been associated with causing cell death via apoptosis and necrosis (Fiers 1991; Beyaert and Fiers 1994). Membrane bound TNF- α is proteolytically cleaved by TNF- α converting enzyme (TACE) to produce a 17kDa soluble form which forms a homotrimer with two other soluble TNF- α proteins to activate the TNF receptor (TNFR) (Vilcek and Lee 1991).

There are two different TNF receptors that bind TNF- α : TNFR1 and TNFR2 and both bind with high affinity to the TNF- α homotrimer. TNFR1 and TNFR2 are type I transmembrane glycoprotein receptors, however they have very little sequence homology in their intracellular domains, suggesting activation of separate signalling pathways. Most of the biological effects of TNF- α occur through TNFR1; the few specific actions of TNFR2 include proliferation of thymocytes and possible sink activity for TNF- α as a way to reduce inflammation-mediated host damage (Peschon, Torrance et al. 1998). TNFR1 activation leads to NF- κ B activation.

TNF- α signalling through TNFR1 is essential for a successful defence response to a variety of invading pathogens (Flynn, Goldstein et al. 1995; Marino, Dunn et al. 1997). Overproduction of TNF- α or membrane TNFR1 accumulation can result in damage to the host. This damage has been associated with the pathogenesis of some diseases, including multiple sclerosis and rheumatoid arthritis (Korner, Lemckert et al. 1997; McDermott, Aksentjevich et al. 1999).

TNF-receptor-associated protein with a death domain (TRADD) and the 74kDa serine-threonine kinase receptor interacting protein (RIP1) are recruited to the receptor complex upon activation of TNFR1 by TNF- α (Chan 2007). When this receptor complex has been assembled, the N-terminal TRAF binding domain of TRADD facilitates binding to the homotrimer TRAF2.

The role of RIP1 in this signalling pathway remains somewhat controversial with conflicting data arising from a number of studies. One group found normal NF- κ B activation in RIP1 knockout mouse embryonic fibroblasts (Wong, Gentle et al. 2010); whereas another group, using the same model, showed RIP1 is an essential part of NF- κ B activation (Devin, Cook et al. 2000). A third group showed abolishment of NF- κ B signalling in RIP1 deficient Jurkat cells (Ting, Pimentel-Muinos et al. 1996). Binding studies show that recruitment of the IKK complex is unaffected by RIP1 deficiency (Devin, Cook et al. 2000). Later reconstitution studies showed the kinase activity of RIP1 is not necessary for activation of NF- κ B through the TNFR1 signalling pathway (Lee, Shank et al. 2004) suggesting its activation may mediate signalling activity in other pathways. It has also been implicated in TRIF-dependent TLR3 antiviral responses (Meylan, Burns et al. 2004).

To activate NF- κ B, the TNFR1-TRADD-RIP1 complex assembles and is subsequently ubiquitinated with Lys-63 polyubiquitin chains (Ea, Deng et al. 2006). The IKK complex and its activating complex (TAK1-TAB2-TAB3) are recruited to the activated TNFR1 receptor through the ubiquitin binding domains. This then undergoes phosphorylation and lysine 63 ubiquitination (Fan, Yu et al. 2010), activating TAKs kinase domain resulting in IKK α and IKK β phosphorylation. The activated IKKs phosphorylate the I κ B α (the NF- κ B inhibitory protein) resulting in its proteasomal degradation, finally leading to activation to NF- κ B.

1.8. What was known

Prior to the start of this project, NTHi had been shown to elicit a response through TLR2 and TLR3, however very little work had been performed to characterise the cytokine response to NTHi, with only one study identifying early expression IL-1 α , IL-1 β , TNF- α , IL-6, IL-8 and MCP-1, resulting in secretion of TNF- α , IL-6, IL-8 and MCP-1 by the cells. RSV had been well characterised in cell line models, identifying

up-regulation of type III IFNs, IL-6, IL-8 IP-10, ICAM-1, RANTES and TNF- α from the airway epithelium, however studies in more complex airway models were more limited. The virus was known to have selective preference for ciliated cells in differentiated epithelial cell cultures but had been shown to infect undifferentiated (non-ciliated) cells as well. Both these pathogens had been shown to co-infect ‘diseased’ airways, with studies showing that an initial RSV infection aids the establishment of an NTHi infection.

1.9. Aims and Objectives

The airway epithelium functions as a respiratory surface in the lower airways and as a defensive tool in the upper airways. The thickness and cellular structure of the airway relates to the function. In the upper airways the epithelium consists of basal, secretory and ciliated cell types, producing mucus to trap particles that are moved towards the oropharynx by mucociliary clearance. ‘Diseased’ airways undergo cellular remodelling, altering the composition of the mucociliary phenotype and increasing the susceptibility of the airway to structural damage and infection with a variety of respiratory pathogens.

We hypothesise that airway epithelial cells can be used as models for the study of host pathogen interactions *in vitro*. We hypothesise that primary airway epithelial cells cultured at the ALI may represent a better model than established cell lines. Furthermore growth of these cultures can be modulated by altering growth factors to simulate the diseased airway. Exposing these cultures to pathogens could aid understanding of poorly understood disease processes and innate defence mechanisms.

The main aim of this study was to model the airway epithelium *in vitro* in order to use it as a tool to understand pulmonary innate defence mechanisms. Regardless of the disease state/phenotype of the airway, RSV is a major causative pathogen of respiratory infections in children, the immune-compromised and the elderly, specifically targeting ciliated cell types within the airway epithelium. Exhibiting a seasonal infection pattern, RSV manages to evade host defences and recur periodically throughout life. NTHi has also been found to be a leading bacterial cause of respiratory infection, particularly in the ‘disease’ phenotype, where it is frequently found colonising the airways, often in biofilm form. My initial objective was to establish the effectiveness of cell lines for use as an airway model, and optimise bacterial and viral doses.

As a result of the potential limitations of cell line studies my second objective was to reproducibly establish the ALI culture model with primary cells to allow for further studies; and characterise the differences between the ‘normal’ cultures and those differentiated in the presence of IL-13 to give a ‘diseased’ phenotype.

Upon establishment of the ALI culture model with primary cells, my third objective was to expose the cultures to the pathogens RSV and NTHi in order to establish infection to investigate the immune responses. These two pathogens have also been implicated in co-infections, particularly in ‘diseased’ airways. Thus a key objective through use of the ALI culture model was to establish RSV and NTHi infections, a biofilm-type infection/colonisation of the NTHi, and establish co-infections with both RSV and NTHi.

These models were subsequently used to understand early progression of the infections and the roles of specific host factors involved in the establishment and prevention of the infection process in both the normal and diseased airway.

2. Materials and Methods

2.1. Materials

A full list of materials can be found in Appendix 1

2.2. Cell Biology

2.2.1. Initial culture from frozen stock

2.2.1.1. Cell Lines

The appropriate medium (Appendix 2) was made, and a T75 flask containing 20mls was placed in an incubator to pre-warm. Cells were removed from liquid nitrogen storage and placed immediately on ice. The vial was then placed in water at 37°C and agitated for 90 seconds until cells were thawed. The cells were then added to the pre-warmed media and replaced into the incubator to grow.

2.2.1.2. Primary uHBE Cells

100mls of bronchial epithelial growth medium (BEGM) was made (Appendix 2) and 2 T25 flasks containing 10mls placed in the incubator and pre-warmed. Primary human bronchial epithelial (HBE) cells were removed from liquid nitrogen storage and placed immediately on ice. The vial was then placed in water at 37°C and agitated for 90 seconds until the cells were thawed. They were then opened in the laminar flow-hood and resuspended by pipetting up and down gently. Half the cells were placed in each pre-warmed T25 and placed back in the incubator. The BEGM was replaced after 24 hours and then every 2-3days until the flasks were approximately 80-90% confluent. Upon achieving 80-90% confluence, one T25 flask was sub-cultured to passage 3 and the other was frozen down for later use. Lower cell numbers in the vials required use of T25 flask as opposed to the T75 flask used for cell lines in section 2.2.1.

2.2.2. Sub-culture of cells

2.2.2.1. Cell Lines

After removing the media from the flask, the cells were gently washed with 5mls of Phosphate Buffered Saline (PBS) without Calcium/Magnesium. The PBS was then removed and 2mls of Trypsin-Ethylenediaminetetraacetic acid (EDTA) was added to the flask and placed back in the incubator until the cells began to detach. The side of the flask was tapped gently to detach any remaining loose cells and the trypsin was quenched with complete medium. This was pipetted up and down to re-suspend cells

and break up any clumps. Cells were counted using a haemocytometer, centrifuged, and seeded into T75 flasks at 10,000cells/cm². The flasks were then incubated at 37°C and passaged upon reaching 70-80% confluence.

2.2.2.2. Primary uHBE Cells

Cells were only sub-cultured to a maximum of Passage 4 as cells could not be used for ALI culture beyond passage 5. After removing the BEGM from the flask, the cells were gently washed with 5mls of PBS. The PBS was removed and 1ml of trypsin-EDTA was added to the flask which was then placed back in the incubator for 5-10minutes to allow the cells to begin to detach. The side of the flask was tapped gently to detach any remaining loose cells and the trypsin was quenched with 5ml serum-containing media. This was pipetted up and down to re-suspend cells and break up any clumps. Cells were counted using a haemocytometer, centrifuged, and seeded into T75 flasks at 10,000cells/cm². The flasks were then incubated at 37°C replacing the BEGM after 24 hours and then every 2-3 days until 80-90% confluent. Upon reaching confluence, the flasks were used to seed further flasks to Passage 4 or seeded onto transwells for ALI Culture.

2.2.3. Freezing of cells

The cells were detached using trypsin-EDTA as in 2.2.2 and counted. Cell lines were centrifuged and re-suspended in freezing medium (Appendix 2) at a cell density of 1x10⁶cells/ml. Primary cells were centrifuged and re-suspended in Cryo-SFM at a cell density of 0.5x10⁶cells/ml. Cells were placed in cryovials in 1ml aliquots and placed in a Mr Frosty in the -80°C freezer overnight so they could freeze gradually and stored in liquid nitrogen. Primary cells were frozen at a lower density and in different medium to cell lines.

2.2.4. Isolation of Human Neutrophils

Density gradient centrifugation coupled with negative magnetic selection, using a custom antibody cocktail (Stem Cell Technologies, Vancouver, Canada), was used to isolate human neutrophils from the venous blood of healthy human volunteers (Sabroe, Prince et al. 2003; Basran, Jabeen et al. 2013) using a protocol approved by the Sheffield Research Ethics Committee (UK).

2.3. Protein Analyses

2.3.1. Western Blot Analysis

2.3.1.1. Generation and characterisation of human-specific SPLUNC1 and LPLUNC1 antibodies

Two affinity purified anti-rabbit, peptide-specific, polyclonal antibodies against human SPLUNC1 and LPLUNC1 were generated by Eurogentec (Seraing, Belgium) using their DoubleX strategy. This involved the generation of two peptides from the same antigen which are immunized into the host animal together to improve success rate.

For SPLUNC1, the peptide sequences used for antibody A corresponded to amino acids 169-179 (AVRDKQERIHL) and to amino acids 192-207 (DGLGPLPIQGLLDSL) for antibody B. For LPLUNC1, the peptide sequences used for antibody A corresponded to amino acids 139-154 (TIRMDTSASGPTRLV) and to amino acids 472-484 (ASLWKPSSPVSQ) for antibody B; which is located at the extreme C-terminus of the protein in the second BPI domain. All peptides were chosen to have minimal sequence conservation between man and mouse (Appendix 3) and no similarity with other members of the PLUNC family. Bioinformatic analysis also showed that these epitopes had no significant sequence identity with any other human proteins.

2.3.1.2. Validation of antibodies by Western blotting of ALI cell culture secretions and BAL

Antibodies were validated using apical secretions from ALI cells provided by Dr Michael Campos (University of Miami) or by Dr Phillip Monk (Synairgen, Southampton). Secretions were collected, every 2-3 days, from differentiated TBE cells cultured at the ALI by washing each well of a 12-well plate with 100µl of PBS. Samples were centrifuged at 600xG to remove cellular debris and 5µl aliquots used for Western blotting as outlined below. BAL samples (provided by Dr Michael Campos, University of Miami) were obtained by instilling 60ml of normal saline in the non-diseased middle lobe or lingula of subjects with lung cancer undergoing clinically indicated bronchoscopy as part of their diagnostic work-up, under a protocol approved by the University of Miami's Institutional Review Board. They were collected and processed under standard conditions (Meyer, Raghu et al. 2012). 15µl of BALs were subjected to Western blotting. Aliquots of ALI cell culture secretions and BAL were digested overnight with PNGaseF according to the manufacturers' instructions and Western blotted.

2.3.1.3. Separation by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein separation was performed using the Bio-Rad Mini-PROTEAN 3 Gel Electrophoresis System. Glass plates for casting a 1.5mm thick gel were cleaned with 70% Industrial Methylated Spirits (IMS) and assembled in the casting stand. A resolving gel (Appendix 2) was prepared and transferred to the casting equipment using a Pasteur pipette to approximately 1.5cm below the top of the short plate. This was then overlaid with a thin layer of propan-2-ol to remove oxygen and allowed the gel to set as well as bursting any bubbles that may have formed; this was then washed off with distilled water. A stacking gel (Appendix 2) was prepared and transferred to the top of the resolving gel using a Pasteur pipette to the top of the glass plates. This helped to eliminate air bubbles upon insertion of a 1.5mm thick, 10-well comb which was carefully removed upon setting of the gel. The complete gel was then removed from the casting equipment and assembled in the running tank, which was filled with SDS-PAGE running buffer.

Samples for separation were prepared by addition of equal volumes of 2x SDS Lysis Buffer (Appendix 2) and heated to 100°C for 5 minutes. The samples were loaded alongside ColorPlus Prestained Protein Ladder (New England BioLabs). The gel was run at 120V until the blue dye front of the lysis buffer had passed through the stacking gel; it was then turned up to 150V-200V until the dye front had reached the bottom of the gel.

2.3.1.4. Transfer of Proteins to a membrane

Proteins were separated using SDS-PAGE as previously described. When electrophoresis was complete, the apparatus was disassembled and the stacking gel separated from the resolving gel. The resolving gel was then placed in transfer buffer (Appendix 2). Six pieces of Whatman blotting paper and polyvinylidene fluoride (PVDF) membrane were cut to the approximate size of the resolving gel. The PVDF was activated by soaking in methanol for 2 minutes and placed in transfer buffer. The Whatman paper was also soaked in transfer buffer. These components were placed on the surface of a Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell in the following order: (1) three pieces of Whatman paper (2) PVDF (3) the resolving gel and (4) three pieces of Whatman paper. Air bubbles were squeezed out to ensure efficient

transfer. For two gels transfer was conducted at 15V for 50 minutes, this was reduced to 40 minutes at 15V for one gel.

2.3.1.4.1. Dot-blotting

A grid of 1cm x 1cm boxes was drawn in pencil on a 0.2 μ M nitrocellulose membrane. 2 μ l of samples were spotted into the centre of the box and allowed to dry before being immuno-blotted as below.

2.3.1.5. Immunoblotting and Detection

Upon completion of transfer, the membrane was placed in blocking buffer (Appendix 2) on an orbital shaker for 1 hour in order to reduce non-specific binding of antibodies to the membrane. The membrane was then washed for 5 minutes in TBS (Appendix 2). The primary antibody was diluted in 5mls of blocking buffer and incubated overnight on a rolling platform at +4 $^{\circ}$ C (a full list of antibodies, including where they were purchased from and dilution factors, can be found in Appendix 1). Membranes were washed 3 times for 5 minutes in TBS-Tween (Appendix 2) on an orbital shaker, and then incubated with the appropriate horse-radish peroxidase (HRP)-conjugated secondary antibody, diluted in 5mls blocking buffer, (Table 2.1) on a rolling platform for 1 hour at room temperature.

After a further 3 washes in TBS-Tween HRP activity was detected by chemiluminescence using the EZ-ECL Chemiluminescence Detection Kit according to the manufacturers' instructions. The treated membranes were then placed between two pieces of acetate and any excess ECL and air bubbles eliminated. In the dark room the membrane was positioned in a Hypercassette and exposed to X-ray film (Hyperfilm) for varying periods of time to attain an optimum exposure with discrete bands and developed.

2.3.2. Human Cytokine Array

The human cytokine array was performed using a Human Cytokine Array Panel A Kit (R&D Systems). The cytokines and chemokines included in this kit are all involved in pro-inflammatory signalling in the innate and adaptive immune systems. Signalling pathways include both canonical and non-canonical NF- κ B signalling, JAK/STAT signalling, the complement system and IFN/IRF signalling.

Reagents from the kit were diluted or reconstituted according to the manufacturers' instructions. Hybridisation of the array was performed in accordance with the manufacturers' instructions. The Sample-Antibody-HRP bound cytokines were detected using the EZ-ECL Chemiluminescence Detection Kit for HRP as stated in section 2.4.3 and exposed to X-ray film in the dark room for 1, 5 and 10 minutes, before developing (section 2.4.3).

2.4. Histological Staining and Imaging

2.4.1. Fixation of membranes

Media was removed from the basal compartment of ALI cultures and replaced with 1ml of PBS, with a further 500µl added to the apical surface. Cultures were then placed on a plate shaker at 150rpm for 10 minutes to wash the cells. This wash was repeated twice to ensure all media was removed. Cells were fixed by the addition of 1ml 10% phosphate buffered formalin to the basolateral compartment and 500µl to the apical surface (Villenave, Touzelet et al. 2010). Plates were sealed with parafilm to prevent dehydration and stored at +4°C. Wells containing fluorescent-labelled pathogens were fixed for 15 minutes with 10% phosphate buffered formalin as mentioned above; this was then removed and replaced with PBS before storage at +4°C.

2.4.2. Haematoxylin and Eosin Staining

Transwell membranes supporting ALI cultures were formalin fixed and paraffin embedded. Processing, embedding and serial sectioning was performed by the Core Histology Facility in the Medical School at the University of Sheffield. After processing, the membranes were cut in half prior to embedding in paraffin blocks, with the straight (cut) edge towards the cutting surface, to provide lateral sections of the membrane. Sections were de-waxed by immersion in xylene for 5 minutes, and rehydrated to tap water by immersion for 5 minutes in 100%, 70% and 50% ethanol and finally tap water. Slides were immersed in Gills Haematoxylin for 2 minutes, washed in tap water, immersed in Scott's tap water for 10 seconds to 'blue' and washed in tap water. Sections were then stained with 1% aqueous Eosin for 5 minutes, quickly rinsed with tap water and dehydrated back through the alcohols to xylene before mounting under coverslips with DPX mountant.

2.4.3. Immunohistochemistry

The methods outlined in this section are the standard conditions used by and outlined in Bingle, Cross et al. (2005) and Bingle, Barnes et al. (2007).

2.4.3.1. Preparation

Serial sections were cut from formalin fixed and paraffin embedded membranes and mounted onto slides by the Core Histology Facility in the Medical School at the University of Sheffield. The sections were then drawn round using a solvent resistant pen and placed in a slide rack. These were de-waxed twice in xylene for 5 minutes. Slides were then immersed twice in 100% ethanol for 5 minutes to rehydrate the sections. The slides were finally immersed in 3% hydrogen peroxide diluted in methanol for 20 minutes to quench any endogenous peroxidases present in the samples. Some sections were subjected to antigen retrieval by boiling the slides in a microwave for 8 minutes in 0.01M tri-sodium citrate (Bingle, Barnes et al. 2007). Sections were then incubated in 100% normal serum for 30 minutes in a humidified chamber at room temperature (Bingle, Cross et al. 2005; Bingle, Barnes et al. 2007). The species of serum chosen was that from the animal used to raise secondary antibody. Blocking with normal serum is important as it blocks non-specific activity.

2.4.3.2. Primary Antibody

During the blocking period, the primary antibody was diluted with 100% normal serum (again from the animal used to raise the secondary antibody) (a full list of antibodies, including where they were purchased from, dilution factors and normal serum used, can be found in Appendix 1). After blocking, sections were incubated with primary antibody overnight at +4°C in a humidified chamber. Control slides were incubated with 100% normal serum overnight at +4°C (Bingle, Cross et al. 2005; Bingle, Barnes et al. 2007). Slides were then placed in a slide rack and washed in PBS twice for 5 minutes each. The slide rack was raised inside the wash container to allow for the use of a magnetic stirrer to mix the PBS during the washing step.

2.4.3.3. Secondary Antibody

A biotinylated secondary antibody was provided in a Vectastain Elite ABC kit for the appropriate species. The Elite ABC Reagent is a complex avidin and HRP conjugated biotin to enhance signal. Slides were then placed in a slide rack and washed twice for 5 minutes in PBS as before.

2.4.3.4. Colour Development

The Vector NovaRed Substrate kit was used for colour development. The reagent was mixed according to the manufacturers' instructions and applied to the sections for no more than 5 minutes before submersion in distilled water to stop the reaction. Sections were then counterstained with haematoxylin, dehydrated to xylene, mounted using DPX mountant and allowed to dry (Bingle, Cross et al. 2005; Bingle, Barnes et al. 2007).

2.4.4. Immunofluorescent staining of ALI cultures

2.4.4.1. Immunostaining

In the presence of fluorescently labelled pathogens, the plate was covered in foil throughout the following steps to preserve fluorescence. The formalin/PBS was aspirated from the apical and basolateral compartments of fixed membranes, washed by the addition of 1ml PBS to the basolateral compartment and 500µl PBS to the apical surface and placed on a plate shaker at 150rpm for 10 minutes. This was repeated twice to ensure thorough removal of formalin. The cells were then permeabilised by addition of 200µl of permeabilisation buffer to the apical surface. The plate was then placed on a plate shaker at 150rpm for 1 hour at room temperature. The permeabilisation buffer was removed and replaced with 200µl of primary antibody diluted in permeabilisation buffer and placed on a plate shaker at +4°C overnight.

Antibody was removed from the apical surface, and the cells washed 3 times with PBS, on a plate shaker for 20 minutes at 150rpm at room temperature. The secondary antibody was diluted in permeabilisation buffer, and 200µl added to the apical surface. The plate was covered in foil (to prevent photo-bleaching of the secondary antibody) and placed on a plate shaker at 150rpm at +4°C for 1 hour. The secondary antibody was removed and the cells washed 3 times in PBS for 30 minutes at room temperature on a plate shaker at 150rpm, (the plate remained covered in tin foil to shield the secondary antibody). The membrane was then cut out using a scalpel and placed on a microscope slide with the cell side facing up. The filter was mounted by addition of 1 drop of Vectashield mounting medium containing DAPI (4',6-diamidino-2-phenylindole), a coverslip added and sealed with nail polish. Slides were covered with tin foil until the nail varnish had set. Slides were stored in the refrigerator until they could be viewed on an Olympus FV-1000 Confocal Microscope and imaged in the Light Microscope Facility in the Department of Biomedical Science at the University of Sheffield. This protocol was adapted and optimised from Villenave *et al* (2010).

2.5. Molecular Biology

2.5.1. RNA isolation and purification

Samples lysed in TRI reagent first underwent phase separation by the addition of 200µl of chloroform per ml of TRI reagent used. The sample was mixed vigorously for 15 seconds and allowed to stand for 2- 15 minutes at room temperature before centrifuging at 13,000 rpm for 15 minutes at 4°C, this resulted in separation of the mixture into three phases; a protein containing red organic phase, a DNA containing interphase and a clear RNA containing upper aqueous phase. The upper aqueous phase was transferred to a fresh tube and 500µl of isopropanol added per ml of TRI reagent used in the initial lysis. This was mixed, allowed to stand at room temperature for 5-10 minutes and then centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was carefully removed without touching the pellet. A minimum of 1ml 75% ethanol per ml of TRI reagent was added to wash the RNA pellet and centrifuged at 13,000 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet air-dried for 5-10 minutes. The RNA pellet was not allowed to dry completely as this reduces solubility. The pellet was resuspended in up to 50µl of sterile water by repeated pipetting and stored at -20°C for the short-term and -80°C for long-term storage. RNA quality and yield was measured using a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK) and a small amount of sample visualised (using RNA gel electrophoresis) to further examine quality.

2.5.2. RNA Gel Electrophoresis

A gel tank was cleaned and set up for casting. A gel was prepared by mixing an appropriate amount of RNase-free agarose in water and heating in a microwave until the agarose had fully dissolved. This was allowed to cool before the addition of 10x MOPS and formaldehyde (Appendix 2). The gel was poured in the fume hood and allowed to set. The pouring apparatus was removed and the gel tank filled with 1xMOPS as running buffer. 1µl of sample was mixed with 2µl of RNA loading buffer and loaded into the wells. The gel was run at 70V until the dye front had travelled approximately 1.5cm. The gel was then visualised using a Bio-Rad Chemi-Doc™ XRS+ system and an image was captured.

2.5.3. cDNA synthesis by Reverse Transcription

All work involving RNA was performed using RNase-free filter pipette tips. RNA was reverse transcribed into complementary DNA (cDNA). RNA samples were quantified

using a Nanodrop-1000 spectrophotometer and an aliquot diluted to 200ng/ μ l-1000ng/ μ l depending on amount when quantified. 2 μ l of RNA was added to 0.75 μ l of oligo(dT) and 8 μ l of RNase free water . This was then heated at 70°C for 5 minutes to anneal the oligo(dT) and then placed in ice for 5 minutes. A master mix; containing 5 μ l of AMV-RT buffer, 0.75 μ l of AMV-RT enzyme, 0.75 μ l of RNasin, 10 μ l 10mM deoxyribonucleotide triphosphate (dNTP)s and 5.75 μ l RNase-free water per reaction was made and 15 μ l was added to the tubes containing RNA, oligo(dT) and water. The reactions were placed in a GRI PTC-200 DNA Engine PCR machine in 'block' mode at 42°C for 1 hour, followed by 95°C for 5 minutes to inactivate enzyme activity, and finally at 4°C until the reactions could be removed and stored at -20°C. The cDNA was not quantified after reverse transcription.

2.5.4. Polymerase Chain Reaction (PCR)

Primers (Appendix 2) were reconstituted by adding an equal volume of water to weight (μ g) to give a final concentration of 1 μ g/ μ l. These were then diluted 1:10 to provide a 0.1 μ g/ml working dilution. A master mix; containing 5 μ l of 5X Green GoTaq@Flexi Buffer, 2 μ l MgCl₂, 0.25 μ l of GoTaq® DNA Polymerase, 2 μ l 10mM dNTPs and 12.75 μ l of water per reaction, was prepared. 1 μ l of cDNA, 1 μ l of forward primer and 1 μ l of reverse primer were added together in individual reaction tubes, water was used in place of cDNA in the negative control. 22 μ l of master mix was added to each tube and mixed. The tubes were then placed in the PCR machine for 35 cycles (as a default program); consisting of a 94°C denaturation step for 1 minute, a 60°C annealing step for 1 minute and a 72°C extension step for 1 minute; reactions were then subjected to a prolonged extension step of 7 minutes at 72°C and cooled to 12°C until removed. Exon-spanning RT-PCR primers were designed using Primer3 (<http://simgene.com/Primer3>) and were chosen to have a default annealing temperature of 60°C.

2.5.5. Agarose Gel Electrophoresis

Successful amplification of DNA by PCR was assessed using agarose gel electrophoresis. A gel tank was set up for casting of a gel and a 1-2% gel was prepared by mixing an appropriate amount of agarose in 1x Tris-acetate-EDTA (TAE) (Appendix 2) and heating in a microwave until the agarose had fully dissolved. This was allowed to cool before the addition of ethidium bromide (1 μ l per 100ml of gel mixture). The gel was poured and allowed to set, the pouring apparatus removed and the gel tank filled with 1xTAE buffer. Samples were loaded alongside size markers, either Hyperladder I

or Hyperladder IV, and run at 70-90V until fully resolved. The gel was visualised using a Bio-Rad Chemi-Doc™ XRS+ system and an image was captured.

2.5.6. qPCR

Primers were reconstituted by the addition of an equal volume of sterile water per weight (μg) to give a final concentration of $1\mu\text{g}/\mu\text{l}$ and diluted 1:10 to provide a $0.1\mu\text{g}/\text{ml}$ working dilution. A master mix; containing $10\mu\text{l}$ of Luminaris Color HiGreen High ROX qPCR Master Mix (SYBR green), $7\mu\text{l}$ of sterile water, $1\mu\text{l}$ of forward primer and $1\mu\text{l}$ of reverse primer per reaction were mixed by vortex. $19\mu\text{l}$ of master mix was added to the appropriate well in a 96-well plastic qPCR plate. $1\mu\text{l}$ of sample was added in triplicate per primer pair. The plate was sealed and centrifuged at 13,000 rpm for 1 minute to ensure the sample was at the bottom of the well. The plate was placed in an Applied Biosystems 7900HT Fast Real-Time PCR System for amplification. The data generated was analysed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

2.6. Respiratory Syncytial Virus (RSV)

Red-tagged RSV (rr-RSV) stock was gifted to us by Mark Peeples from the Nationwide Children's Hospital in Columbus, Ohio (Guerrero-Plata, Casola et al. 2006).

2.6.1. Propagation of RSV

HeLa cells were grown to 60-80% confluence in a minimum of 4 T75 flasks in 5% Serum HeLa medium (Appendix 2). Media was removed from the cells and replaced with 5mls of a 1:2500 dilution of virus ($8\mu\text{l}$ in $19992\mu\text{l}$ 2% Serum HeLa medium). This was incubated at 37°C for 2 hours, gently shaking the flasks every 15 minutes. 8mls of 2% Serum HeLa medium was then added and cells were incubated for 3 days at 5% CO_2 and 37°C . On day 3, the approximate percentage of fluorescent cells was calculated, and if less than 80% then 10mls of media were added to prevent cell death, and infection of cells was monitored by fluorescent microscopy. If more than 80% then virus was harvested from cell as on day 5. On day 5, the media was removed from the flask into a 50ml Falcon tube. 5mls of the original media was put back into the flask and cells were then removed with a cell scraper and pipette and then passed through a 21 and 19 gauge needle 2-3 times in order to shear the cells. Cells were then 'snap' frozen in liquid nitrogen, thawed at 39°C to prevent killing the virus with large hot/cold rapid temperature changes and centrifuged at 300rpm for 10 minutes at 4°C to pellet the

cell debris. The supernatant was removed (as this contains the virus), aliquoted into 8ml samples and snap frozen in liquid nitrogen. These aliquots were then stored at -80°C .

2.6.2. Purification of RSV

To ensure complete dissolution, I-block (0.1% Casein Solution) was prepared at least three days before virus purification. Vivaspin tubes were washed in ethanol and air dried in a tissue culture hood. 5mls of I-block were added to each tube, centrifuged at 1000g for 5 minutes at 4°C , and then removed from the collecting tube of the vivaspin. 20mls of a 1:10 dilution of RSV stock in cold serum free Dulbecco's Modified Eagle Medium (DMEM) was added to the vivaspin tube and centrifuged at 2500g for 40 minutes at 4°C . The filtrate in the bottom contained the HeLa cells and cytokines, and the retentate in the top chamber contained the RSV. The RSV was removed from the top chamber and any remaining virus washed off with 1ml of serum free DMEM. The purified virus was then diluted 1:2 with serum free media, aliquoted into 1ml or 500 μl samples, snap frozen in liquid nitrogen and stored at -80°C . 12mls of the filtrate (3mls from each vivaspin tube) were also stored to use as controls to ensure successful virus purification.

2.6.3. RSV Plaque Assay

2.6.3.1. HeLa cell Infection

HeLa cells were seeded at a density of 2.5×10^5 cells/ml (100 μl well of a 96-well plate in 5% serum HeLa medium) and incubated overnight at 37°C . The plate was inverted to remove the media and 90 μl of 2% Serum HeLa medium added to each well. The stock of RSV was thawed quickly as previously described (section 2.10.1). 10 μl diluted virus was added in triplicate to row A to give a concentration of 1×10^{-1} and pipetted up and down gently to mix. 10 μl from each of these wells was removed and placed in row B, mixed gently and the process repeated to row H to give final concentrations between 1×10^{-1} to 1×10^{-8} . Filtrate from the purification was also added to the plate in triplicate of neat filtrate and dilutions of 1×10^{-1} and 1×10^{-2} . The plate was then incubated for 48 hours at 37°C .

2.6.3.2. Immunostaining

The plate was inverted to remove the media and fixed in 200 μl of ice cold 50:50 PBS: acetone solution for 5 minutes at room temperature. The plate was washed 3 times with 200 μl of wash buffer (0.1% BSA solution diluted in PBS), 5 minutes per wash. 50 μl of

RSV antibody (AbD Serotec, 7950-0004) diluted 1:500 in wash buffer was added to the wells and incubated for 1 hour at room temperature on a rotating platform. The wells were washed a further 3 times for 5 minutes. 50µl rabbit anti-goat peroxidase conjugated antibody (diluted 1:500) was added to the wells and incubated for 1 hour at room temperature on a rotating platform. The plate was then washed 3 times for 5 minutes with wash buffer.

A 30mg 4-chloronaphthol substrate tablet was dissolved in 10ml of methanol and 1 ml was added to 10mls of 1x TAE buffer along with 12µl of 30% hydrogen peroxide. The plate was incubated for 10-20 minutes with 50µl of substrate until black colonies appeared. Cells were washed with distilled water and allowed to air dry. To determine the plaque forming units (pfu)/ml, the number of black colonies was counted at each dilution in triplicate, the mean number calculated, and multiplied by 10 to calculate the pfu/ml.

2.7. Non-Typeable Haemophilus influenzae (NTHi)

2.7.1. Culture of NTHi

Strains of Non-typeable Haemophilus influenzae were obtained from the Medical Microbiology Department in the Royal Hallamshire Hospital on chocolate agar plates. For broth culture, the bacteria were grown in brain heart infusion (BHI) broth supplemented with yeast extract, hemin and β-NAD. Strains were maintained on chocolate agar plates (Appendix 2). A 5% CO₂ level was required in the incubator (37°C) for growth.

The NTHi strain, 86-028NP-GFP, was a gift from Dr Glen McGillivray and Dr Lauren Bakaletz at the Nationwide Children's Hospital, Columbus, Ohio (Mason, Munson et al. 2003). Provided in frozen glycerol stock the bacteria was streaked onto chocolate agar plates (Appendix 2) containing 20µg/ml of kanamycin (required to preserve the green fluorescent protein (GFP) tag), and added to BHI broth supplemented with yeast extract, hemin, β-NAD and 20µg/ml of kanamycin (Appendix 2). Both plate and broth were incubated at 37°C with a 5% CO₂ level overnight. Growth was maintained on kanamycin supplemented chocolate agar at regular intervals, never longer than two weeks.

In order to make glycerol stocks for long term storage, 86-028NP-GFP was grown overnight in two broth cultures (as before). These were centrifuged at 13000 rpm for 2 minutes. The pellets were resuspended in 1ml of BHI broth and 1ml of 60% glycerol added to give a final glycerol percentage of 30%. 1ml aliquots in cryovials were stored at -20°C in the short term and in liquid nitrogen for long term storage.

2.8. Statistics

All statistics are listed in the figure legends. We assumed normal distribution of data therefore despite the small replicate numbers parametric statistical analyses were performed as they test group means as opposed to medians. They are a more ‘powerful’ statistical test, making them more likely to detect a significant effect when one exists.

2.8.1. Paired *t* test

There was only one viral dose to compare against the control in gene expression studies. As the samples obtained were ‘matched pairs’ a paired *t* test was used to analyse the differences between control and treated/ RSV infected samples.

2.8.2. ANOVA

Repeated measures one-way ANOVA with Dunnetts multiple comparisons test was performed on expression data from NTHi infections. ANOVA was used as there are two different doses being compared to a control (3 independent groups); it is possible to have performed multiple *t* tests, however this increases the chance of committing a type 1 error.

ANOVA was used to analyse the variation in bacterial count data. One-way ANOVA was used to analyse variance in bacterial count data from independent 1 hour, 3 day and 7 day infections. Two-way ANOVA was used to analyse the variation in bacterial count data from two different doses of bacteria over independent short term infections.

3. Establishment of an infection model using cell lines and undifferentiated primary human bronchial epithelial cells (uHBE)

3.1. Introduction

In vitro studies provide a means of examining biological effects using components of an organism taken from their biological niche. In lung research, *in vitro* studies have traditionally employed established cell lines which are generally derived from lung tumour tissue and as such can be considered immortal. Cell lines no longer possess the usual senescence apparatus, allowing them to undergo infinite replication, making them a useful tool for optimisation studies, and generating replicate data as there are no issues of inter-donor variability. This obvious advantage has to be balanced against the concern that such immortal cells will, by definition, have abnormal growth characteristics that may result in them generating inappropriate responses to stimuli. In addition cell lines have undergone genetic mutation to become tumorigenic, the impact upon the cell type specific characteristics found in the tissue of origin is unclear. This is particularly true with respect to airway epithelial gene expression, for example, study performed by Stewart, Torr et al. (2012) showed the BEAS-2B cell line did not form the tight junctions between cells in the airway, and in Calu-3 cells evidence of secretory cell types was clear with the identification of MUC5AC, however β -tubulin staining suggested cilia had not formed.

Two established cells lines that have been used extensively to study the airway epithelium are A549 and NCI-H292. A549 cells are a human adenocarcinoma, alveolar basal cell line (Giard, Aaronson et al. 1973) and NCI-H292 cells are mucoepidermoid carcinoma cells (Yoakum, Korba et al. 1983; Banks-Schlegel, Gazdar et al. 1985; Carney, Gazdar et al. 1985).

Primary HBE cells purchased from commercial companies or when freshly isolated from tissue, undergo a period of de-differentiation and can be considered to provide cultures of cells with an undifferentiated/basal cell phenotype. These cells do however have a finite lifespan, and exhibit significant donor variability.

The A549 and NCI-H292 cell lines and undifferentiated primary human bronchial epithelial (uHBE) cells can be used to model aspects of the airway epithelium, eliciting responses upon stimulation or infection.

RSV is a common respiratory pathogen, frequently infecting children, the elderly and immuno-compromised. RSV exhibits a seasonal infection pattern, whilst evading host defence mechanisms between periods of exacerbations. The mechanism by which the virus infects the lung and is able to evade the immune system remains only partially characterised. Previous work from Sheffield has focused on the role that dendritic cells may play in this process, finding high numbers of dendritic cells during and after an RSV infection in hospitalised infants. We have found that RSV is able to infect dendritic cells with viral replication re-activated by stimulation with nitric oxide (Jones, Morton et al. 2006; Hobson and Everard 2008; Everard, Ugonna et al. 2009; Ugonna, Bingle et al. 2014). These studies focussed on the possibility that dendritic cells may be where the virus is able to lie dormant between infections but latterly our lab has become interested in the role that the epithelium plays.

Haemophilus influenzae species, both encapsulate serotypes (a-f) and non-encapsulate (non-typeable, NT) strains are readily identified in the flora of normal airways (Hilty, Burke et al. 2010). Routine use of the Hib vaccine against type b strains, which was the most common cause of respiratory infection by encapsulate strains, reduced the number of invasive Hib infections. NTHi strains are more frequently identified as the causative agent in localised respiratory tract infections (Gilsdorf, Chang et al. 1992). They are frequently the cause of COPD exacerbations and are involved in colonisation of the CF airway. They are thought to be the first pathogen to infect the CF airway, allowing further pathogens to colonise. The large amount of heterogeneity between strains results in different combinations of surface antigens/features, therefore no single feature has been identified as essential for establishment of infection (Clemans, Bauer et al. 2000).

With specific relevance to the studies contained in my thesis, these three cell lines have a history of use in infection studies. A549 cells have frequently been used to study RSV infections (Fiedler, Wernke-Dollries et al. 1996; Choudhary, Boldogh et al. 2005; Spann, Tran et al. 2005; Martinez, Lombardia et al. 2007; Eckardt-Michel, Lorek et al. 2008; Gibbs, Ornoff et al. 2009; Munday, Emmott et al. 2010), and have also been used to introduce NTHi infections to RSV-infected cells to study adherence (Jiang, Nagata et al. 1999; Fukasawa, Ishiwada et al. 2009). NCI-H292 cells have also been subject to viral sensitivity testing, including for RSV (Hierholzer, Castells et al. 1993) and have also been used in NTHi adherence assays (Buscher, Burmeister et al. 2004).

Undifferentiated primary cells have been used to study NTHi infections (Jono, Xu et al. 2003; Chen, Lim et al. 2004; Teng, Slavik et al. 2010; Morey, Cano et al. 2011) and have also been used to study RSV, to identify immune responses and signalling mechanisms activated in response to infection, and to investigate which RSV proteins cytokine responses were elicited against (Becker, Reed et al. 1997; Kong, San Juan et al. 2003; Oshansky, Barber et al. 2010).

3.1.1. Aims

The aims of the data presented in this chapter were to:

- (1) Establish NTHi and RSV infections in A549 and NCI-H292 cell lines and uHBE cells and to potentially establish biofilms
- (2) Characterise NTHi and RSV infections in A549 and NCI-H292 cell lines and uHBE cells to determine the representativeness of cell lines for use as an airway epithelial model.

3.2. Methods

3.2.1. Infection with RSV

Prior to infection with RSV, A549 and H292 cells and undifferentiated primary human bronchial epithelial (uHBE) cells underwent a media change, RSV was diluted to concentrations of 5×10^5 , 1×10^6 and 5×10^6 pfu in 50 μ l of serum free media prior to addition to the surface of the cells.

3.2.1.1. Short term infections

A549 and H292 cells and uHBE cells were infected with 1×10^6 pfu of virus as described above. At time points of 1, 2, 4, 8, 24 and 72 hours post infection, media was collected and cells were lysed in TRI reagent for later RNA extraction.

3.2.1.2. Long term infections

A549 and H292 cells and uHBE cells were infected with RSV in 4-well chamber slides (and transwells for the undifferentiated primary cells only). The media was changed 48 hours after infection. At 5 days post infection, the experiment was terminated by collecting the media and fixing the cells with 10% phosphate-buffered formalin for 10 minutes. The fixative was then replaced with PBS for longer-term storage. For the transwells (uHBE infections) membranes were cut from the transwell using a scalpel and mounted with Vectashield containing DAPI, sealed with nail polish and imaged

using the Olympus FV-1000 Confocal Microscope (see section 2.4). Infections performed in chamber slides were also stored in PBS long-term. The chambers were removed and counter-stained and mounted in the same way as the transwell membranes.

3.2.2. Infection of cells with NTHi

A549 and H292 cells and uHBE cells underwent an antibiotic free media change for at least 3 days prior to infection. An appropriate amount of overnight broth growth was taken, diluted to an OD600 of 0.38 and centrifuged at 13000 rpm for 2 minutes to pellet the bacteria. This was resuspended in PBS and diluted to provide approximate MOIs of 5, 25 and 50 per 50 μ l.

3.2.2.1. Short term infections

A549 and H292 cells and uHBE cells were infected with bacterial doses of approximate MOI of 5 and 25 for periods of 1, 2, 4, 8, 24 and 72 hours. After which the media was removed and frozen. Prior to freezing some samples were used to perform serial dilutions and obtain viable bacterial counts. Two wells were lysed in 200 μ l of 2xSDS lysis buffer and two were lysed in TRI reagent for later RNA extraction.

3.2.2.2. Long term infections

A549 and H292 cells and uHBE cells were seeded onto transwells to allow for basolateral media changes during the long-term infection. Infections were conducted on cells that had been cultured in antibiotic free media for at least three days. Cells were infected with approximate MOIs of 5, 25 and 50 for 1 hour, 3 days and 7 days. After which the media was removed from the surface of the transwell and frozen. Wells were washed basolaterally in PBS to remove excess media and fixed for 10 minutes in 10% phosphate-buffered formalin (basolateral and apical compartments). After 10 minutes the formalin was removed and replaced with PBS for longer term storage. Membranes were cut from the transwell using a scalpel and mounted with Vectashield containing DAPI, sealed with nail polish and imaged using the Olympus FV-1000 Confocal Microscope (see section 2.4).

3.3. Results

3.3.1. Establishing infections in cell lines and uHBE cells

3.3.1.1. NTHi infections

The first series of studies were designed to develop protocols for reproducibly infecting lung derived cell lines with NTHi. As we were ultimately interested in establishing mid-term cultures with a view to generating biofilms, we initially studied infections up to 7 days post infection without any post infection washing of the cultures.

A549 (Figure 3.1), NCI-H292 (Figure 3.2) and uHBE (Figure 3.3) cells were infected for 1 hour (A and B), 3 days (C and D) and 7 days (E and F) with NTHi at an MOI of 25 and imaged using confocal imaging, to examine the effects of long term exposure on cell numbers/confluence and the development of biofilms on these cultures.

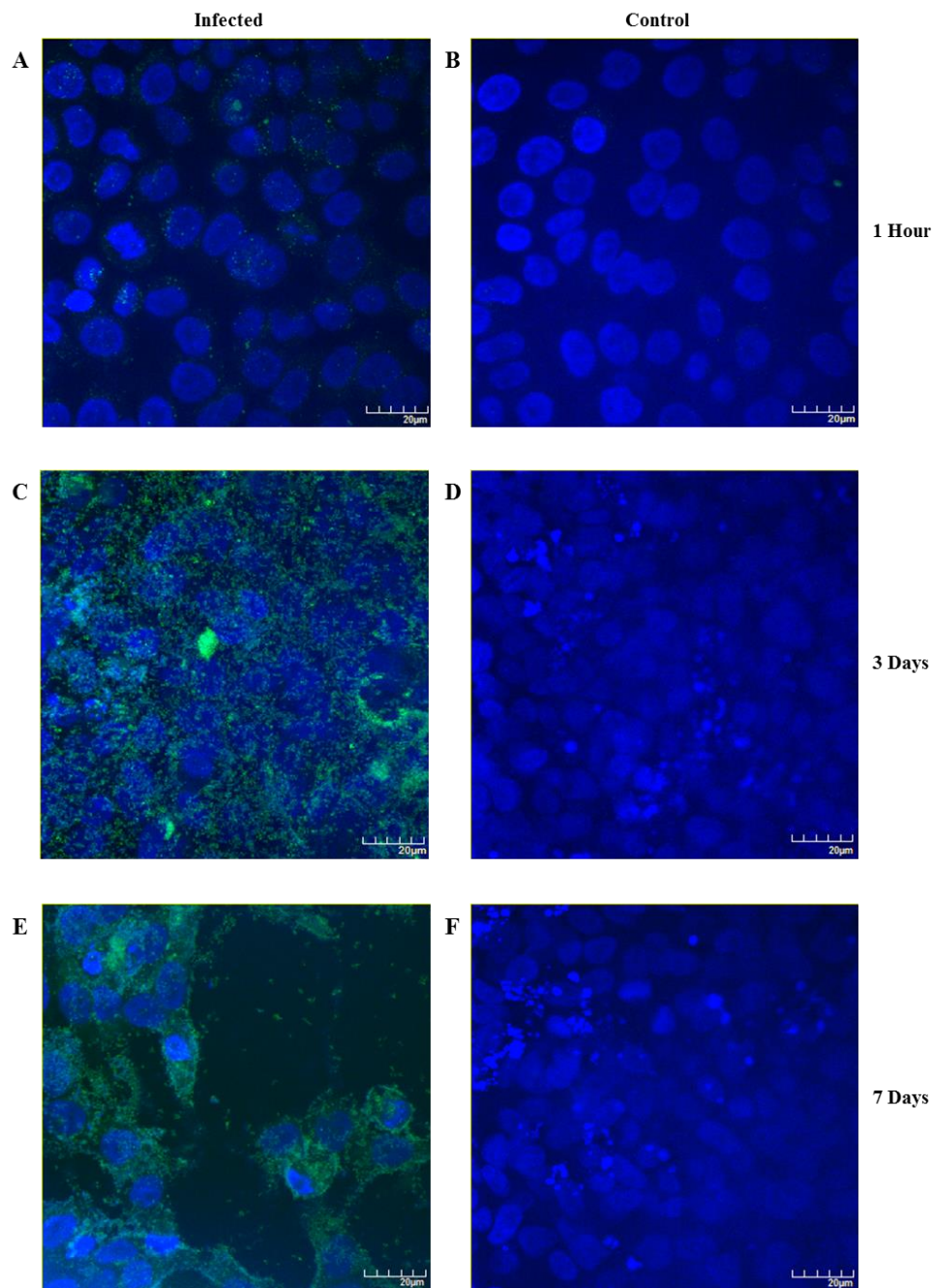


Figure 3.1: Infection A549 cells with NTHi for 1h, 3d and 7d

A549 cells were infected with NTHi at an MOI of 25 for 1 hour, 3 days and 7 days. Green fluorescence shows the presence of bacteria, blue fluorescence shows DAPI staining of nuclei. Control wells showed no green fluorescence. (Images are representative of n=3 infections).

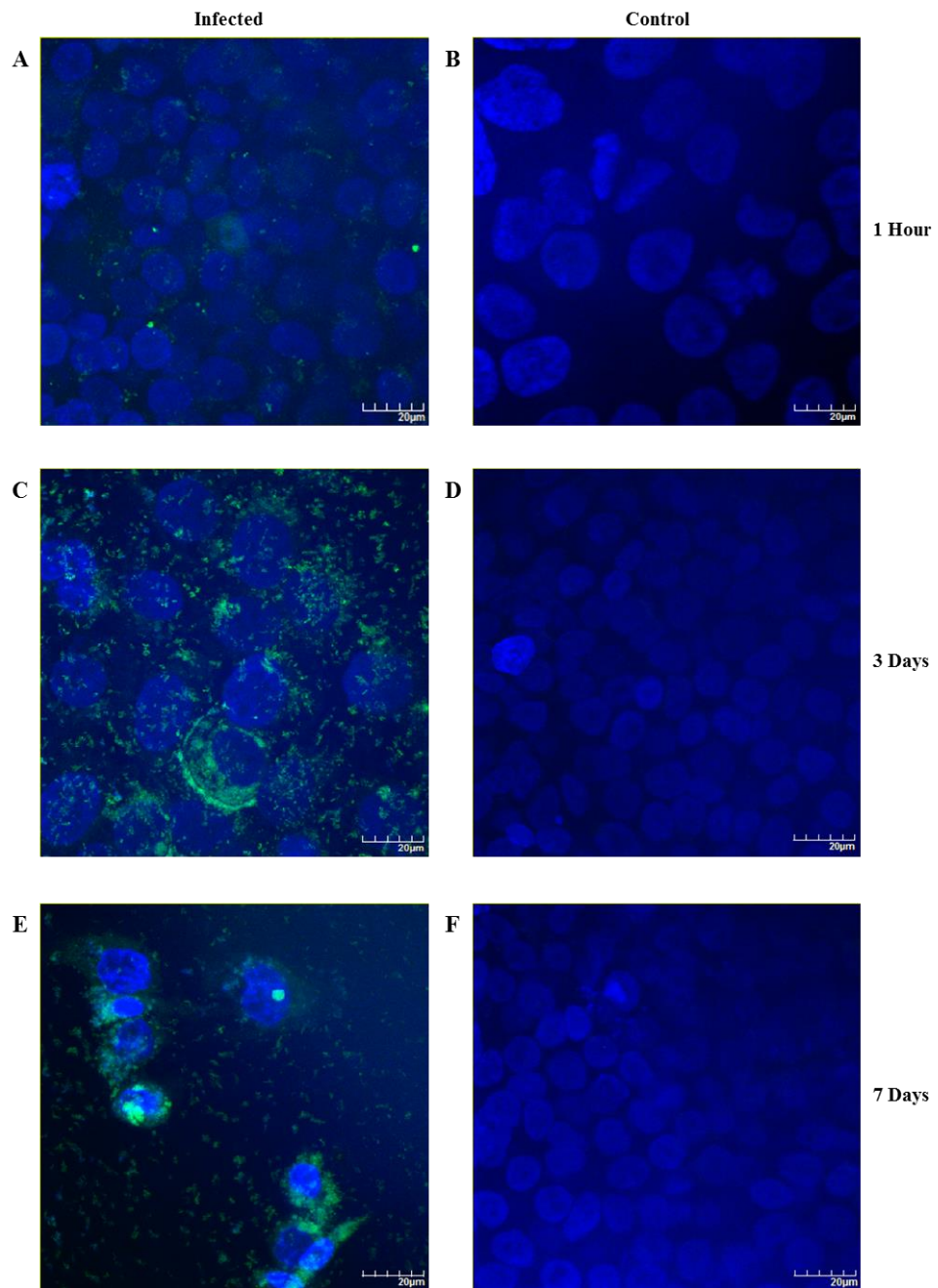


Figure 3.2: Infection of NCI-H292 cells with NTHi for 1h, 3d and 7d

NCI-H292 cells were infected with NTHi at an MOI of 25 for 1 hour, 3 days and 7 days. Green fluorescence shows presence of bacteria, blue fluorescence shows DAPI staining of nuclei. Control wells showed no green fluorescence. (Images are representative of n=3 infections).

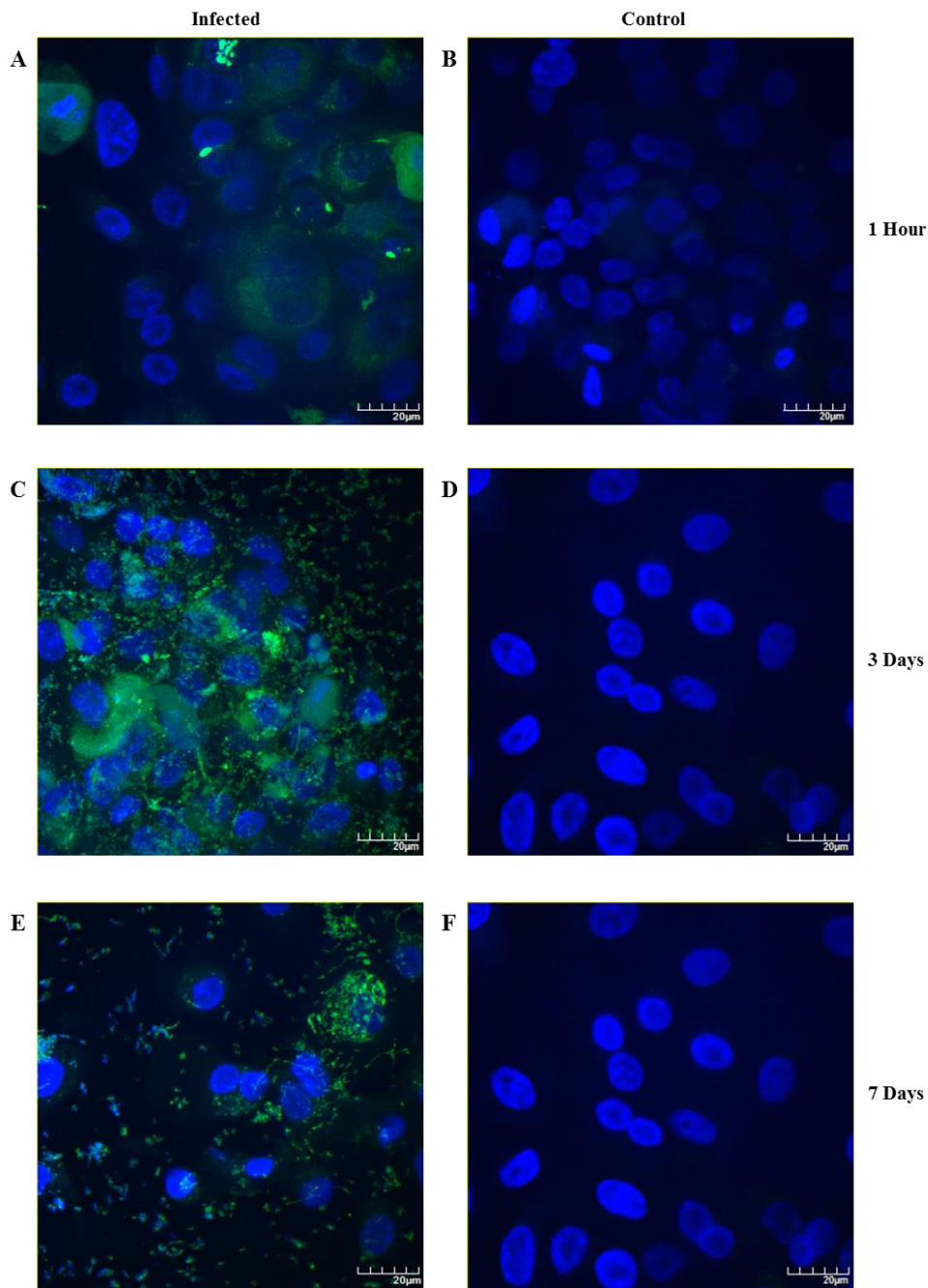


Figure 3.3: Infection uHBE cells with NTHi for 1h, 3d and 7d

uHBE cells were infected with NTHi at an MOI of 25 for 1 hour, 3 days and 7 days. Green fluorescence shows the presence of bacteria, blue fluorescence shows DAPI staining of nuclei. Control wells showed no green fluorescence. (Images show infection of cells from Donor 4, and are representative of =3 infections)

In all three cell types, cells at the 1 hour time point (A and B) were confluent, with small amounts of green fluorescence in the infected wells (A) showing that NTHi were beginning to establish an infection. By three days there appeared to be more green fluorescence in the wells. The A549 cell mono-layer was less tightly packed in the infected wells (C) compared to controls (D). The nuclei of NCI-H292 cells appeared enlarged, with some spaces in the mono-layer beginning to appear. There was also a higher level of NTHi (as shown by green fluorescence) in the uHBE cells at 3 days post infection. The uHBE cell layer did not appear to be adversely affected with spaces appearing in both the infected and control wells. By 7 days, all three cell types showed dramatically reduced cell numbers, although the presence of green fluorescence showed that there were still bacteria present. The bacteria appeared to have clustered around the remaining cells.

As the uHBE cells did not appear to be adversely affected by infection and showed a higher level of green fluorescence, imaging of the surface of uHBE cells infected for 3 days with NTHi at an MOI of 25 was performed. This showed clustering of bacteria within what appeared to be a fluorescent mesh-like structure that could perhaps represent a biofilm (Figure 3.4).

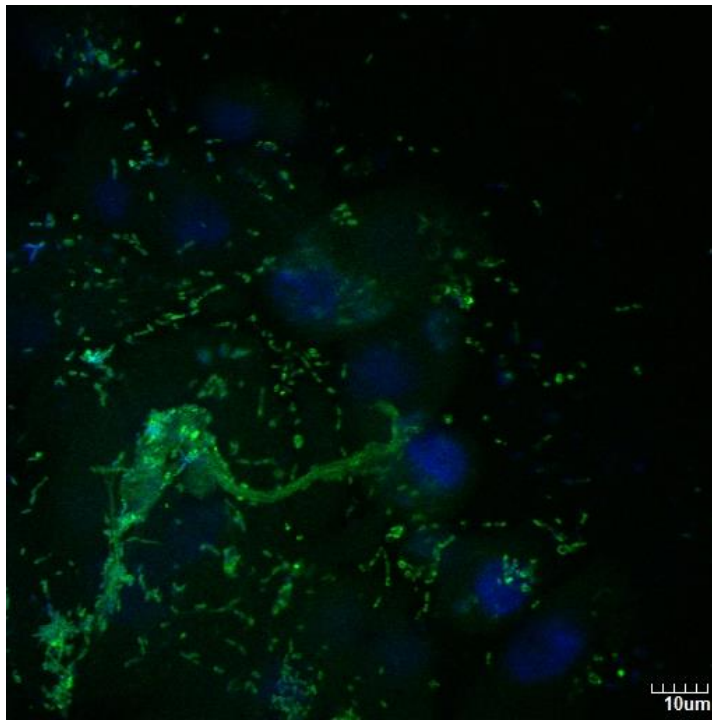


Figure 3.4: Surface of uHBE cells infected for 3 days

uHBE cells were infected with NTHi at an MOI of 25 for 3 days. Green fluorescence shows presence of bacteria and blue fluorescence shows DAPI staining of nuclei.(Donor 4)

3.3.1.2. RSV infections

To study whether we were able to infect A549, NCI-H292 and uHBE cells with RSV and to establish an effective dose for further studies, cells were seeded into chamber slides at an initial density of 10,000 cells per cm² for the cell lines and 20,000 cells per cm² for uHBE cells. This was necessary to achieve confluence at the same time as the primary cells grow much more slowly than cell lines. At approximately 80% confluence cells were infected with RSV at doses of 5×10^5 , 1×10^6 and 5×10^6 pfu for 5 days. Cells were fixed and imaged using a confocal microscope. An example of images captured with the 1×10^6 pfu dose show red fluorescence in all three cell lines (Figure 3.5A, C and E), and no fluorescence in the controls (Figure 3.5B, D and F).

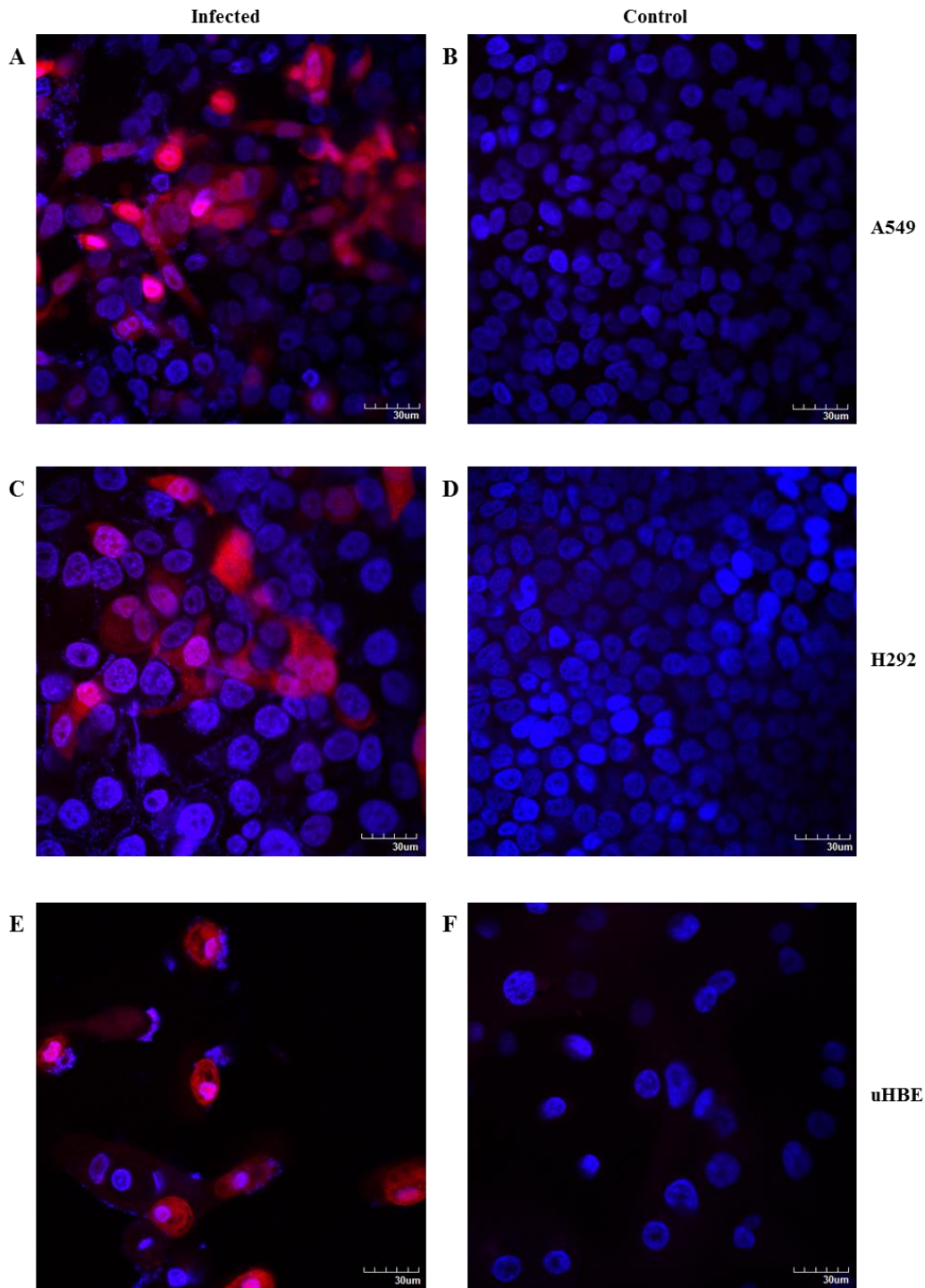


Figure 3.5: A549, NCI-H292 and uHBE Cells are susceptible to RSV infection

A549 (A), NCI-H292 (C) and uHBE (E) cells were infected with 1×10^6 pfu of red recombinant RSV for 5 days alongside controls (B, D and F). Red fluorescence shows infection with RSV and blue fluorescence shows DAPI staining of nuclei. (Images show infections from uHBE Donor 5, and are representative of $n=3$ infections).

3.3.2. Characterisation of NTHi and RSV infections in cell lines and uHBE cells

To obtain some quantitative measurement of the levels of bacteria present in cultures during the development of infection, viable bacterial counts were performed, and although none of the changes seen were significant, in A549 cells, the viable bacterial numbers had increased slightly by 3 days, but decreased to their lowest levels by 7 days but not lower than the initial inoculum (Figure 3.6A). Counts for NCI-H292 cells showed a small increase from 1 hour to 3 days and a sharp increase between 3-7 days (Figure 3.6B) and uHBE cells showed a decrease in viable bacteria by 3 days but this had increased to more than the 1 hour time point by 7 days (Figure 3.6C), suggesting that numbers of NTHi reach and maintain a threshold level.

Due to the loss of viable cells in the longer term cultures, shorter term infections with MOI's of 5 and 25 for periods up to 72 hours were also conducted on A549, NCI-H292 and uHBE cells with viable bacterial counts performed at 0, 1, 2, 4, 8 and 24 hours. All cell lines showed a slight decrease (approx. 1×10^6 cfu/ml in MOI 5 and 1×10^7 cfu/ml MOI 25) in viable bacteria during the initial stages of infection which increased through log phase and began to plateau by 8 hours as a result of bacteria utilising all available nutrients. By 24 hours the levels began to drop, as the media was not changed and the bacteria began to die. However, there were more viable bacteria remaining in the washes from wells initially infected with NTHi at an MOI of 5 (Figure 3.7).

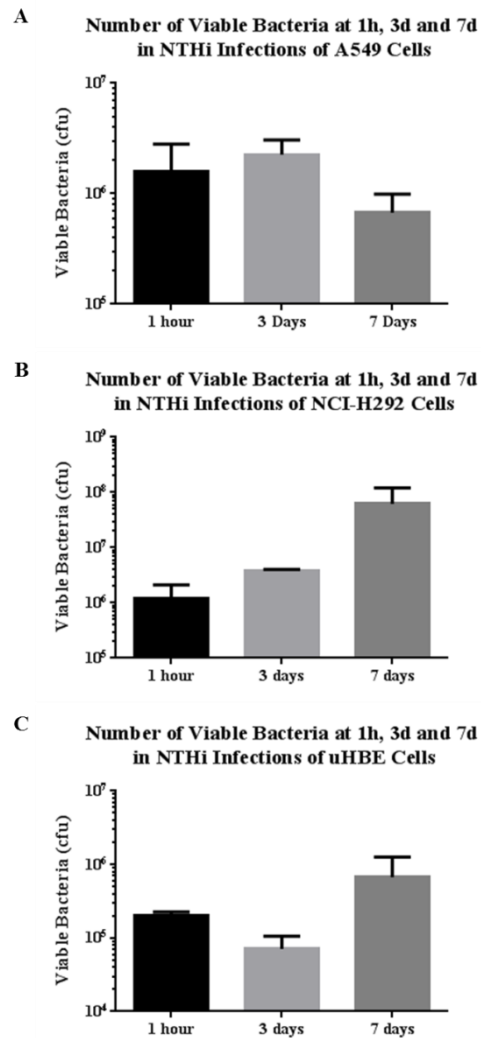


Figure 3.6: Viable Counts of 7day NTHi infections in A549, NCI-H292 and uHBE cell lines

Viable counts were taken at 1h, 3d and 7d in A549 (A), NCI-H292 (B) and uHBE (C) cells. A one-way ANOVA using T=1h as the control showed no significant difference between samples (n=3). Error bars show SEM.

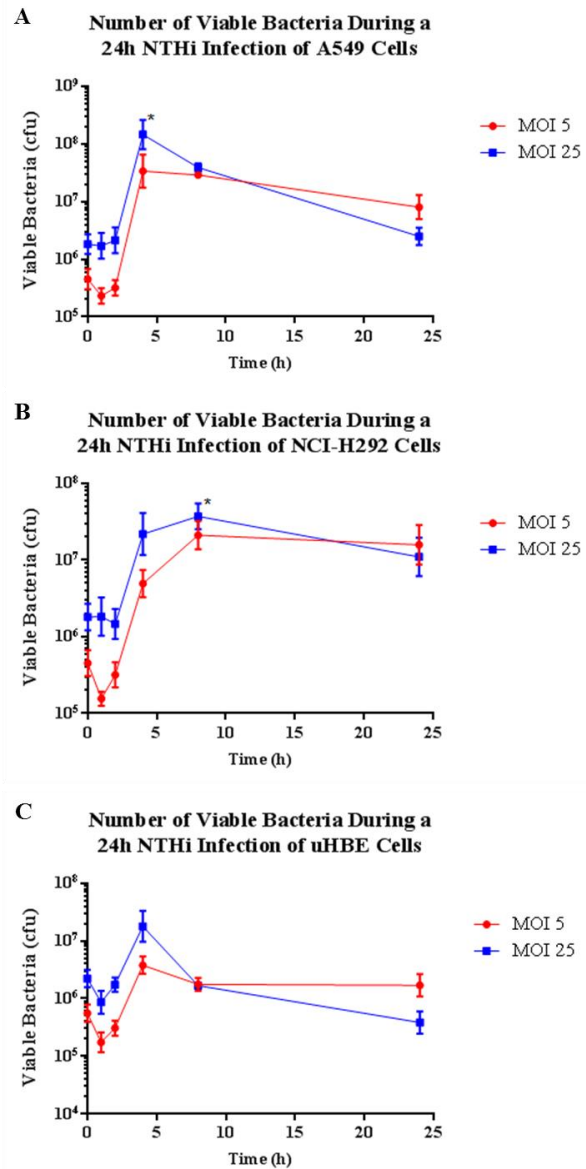


Figure 3.7: Viable bacterial counts during a 24h infection in A549, NCI-H292 and uHBE cells

Viable counts were taken at the start, 1h, 2h, 4h, 8h and 24h of short term infections performed using A549 (A), NCI-H292 (B) and uHBE (C) cells. Two way ANOVA with Dunnetts multiple comparisons test performed using T=0h as the control showed significance in the MOI of 25 at 4 hours in A549 cells and 8 hours in NCI-H292 cells ($p < 0.05$ for all) ($n=3$). Error bars show SEM.

Cells were cultured on transwells in these experiments to allow changing of media during the infection period. In order to gain information about the responses that the cells made to the infections, conditioned medium taken from the apical surface of A549 and NCI-H292 cells 7 days after infection, alongside controls, was used to perform a cytokine array. These studies were performed at the same time for all samples and thus all films were developed for the same length of time allowing accurate analysis of any differences.

Control A549 conditioned medium showed the presence of strong signals for GRO- α , MIF and SerpinE1 with less intense signals for CD40 ligand IFN- γ , IL-8 and IL-23 (Figure 3.8B). Infected A549 cells had similar levels of GRO- α , MIF and SerpinE1 and reduced signals for CD40 ligand, IFN- γ and IL-23. There was however, a 4-fold induction of IL-8 (Figure 3.8A), which is reflected in densitometry analysis (Figure 3.9A) Densitometry showed a small increase in GRO- α protein expression but a small reduction in MIF and SerpinE1 protein expression. Conditioned medium from control NCI-H292 cells showed the presence of GRO- α , MIF and SerpinE1 (Figure 3.8D). In the infected NCI-H292 cells SerpinE1 was increased and there was an induction of IL-8 release into the medium (Figure 3.8C) as shown by densitometry analysis, which also showed small increases in GRO- α and SerpinE1 but a reduction in MIF protein expression (Figure 3.9B). It is important to understand that as these results are from a single experiment they cannot be assessed for significance).

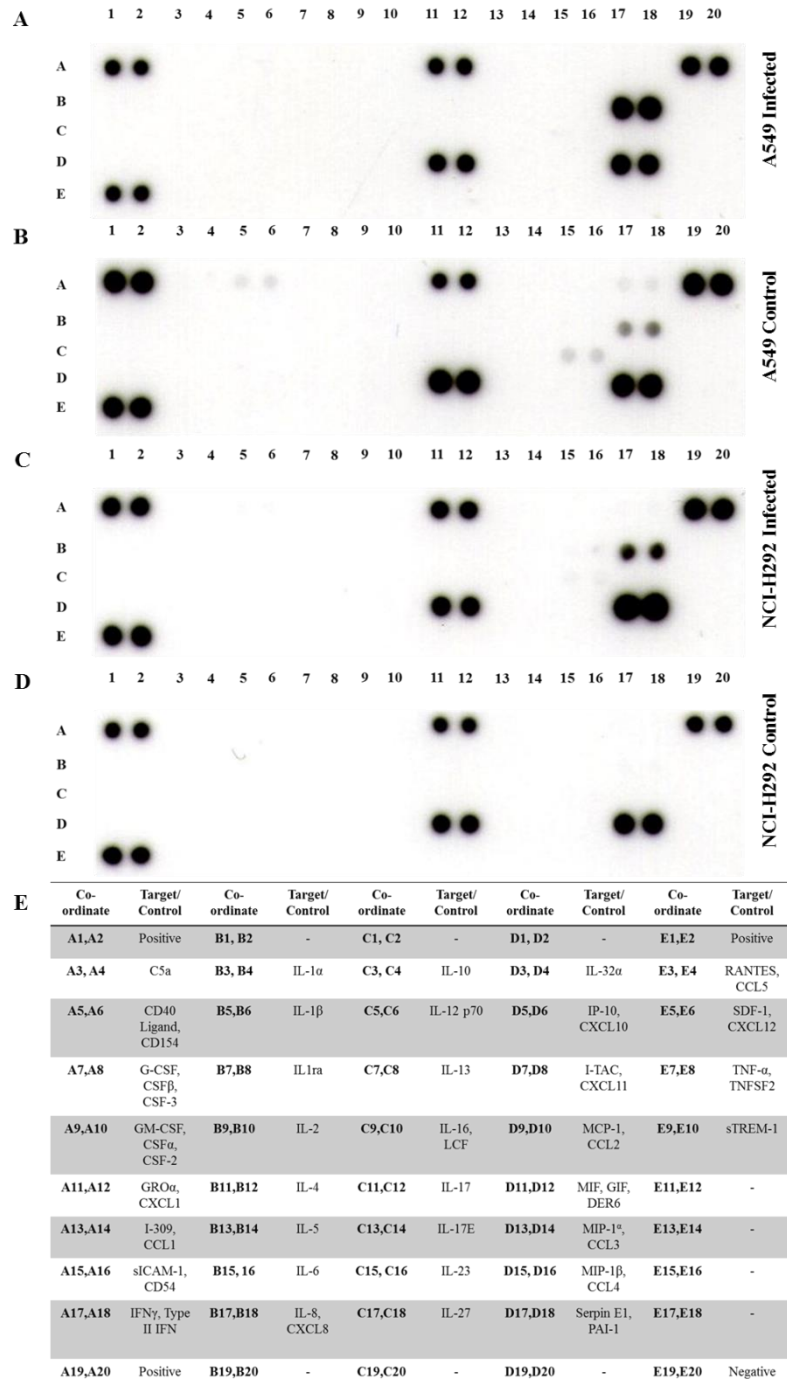


Figure 3.8: Cytokine Array from A549 and NCI-H292 cells with NTHi for 7d
A549 (A and B) and NCI-H292 (C and D) were infected with NTHi (A and C) alongside controls (B and D) for 7 days. The cytokine array data table is also shown (E) (n=1).

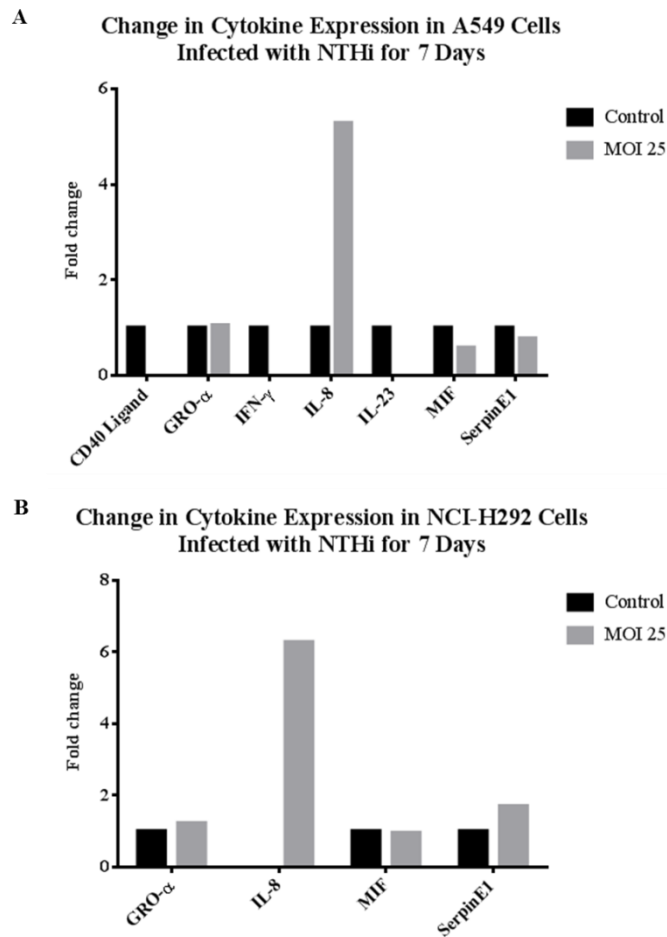


Figure 3.9: Densitometry analysis performed on Cytokine Array from A549 and NCI-H292 cells infected with NTHi for 7d

A549 and NCI-H292 were infected with NTHi alongside controls for 7 days (Figure 3.7). Densitometry was used to show the differences in levels of cytokines relative to the control in A549 cells (A) and NCI-H292 cells (B) (n=1).

Having examined cytokine secretion from the infected immortalised cell lines we went on to determine at the response generated by the primary airway cells (uHBE cells). Conditioned medium from the 24 hour infections in uHBE cells infected with NTHi at an MOI of 5 and 25 and RSV, were used in cytokine arrays alongside an uninfected control. Again arrays were performed at the same time so that differences between samples would be analysed from the same exposure of films. Uninfected uHBE cells showed protein expression of GRO- α , IL-8, MIF and SerpinE1, with less robust signals from G-CSF, GM-CSF, IL-1 α , IL-1ra and IL-6 (Figure 3.10D). As such these cells appeared to produce more cytokines than either A549 or NCI-H292 cells. Cells infected with both doses of NTHi appeared to generate a very limited cytokine response at this time point (Figure 3.10 A and B). NTHi infected wells expressed the same cytokines as the controls but in differing amounts, with NTHi showing changes in IL-6, IL-1 α , GM-CSF and SerpinE1 (these results are n=1 so cannot confirm significance) (Figure 3.11) Conditioned medium from the RSV infected cells (Figure 3.10C) showed induction of IP-10, decreased production of IL-1 α (1.75 fold) and increased GM-CSF production (1.75 fold) than the control cells (Figure 3.11).

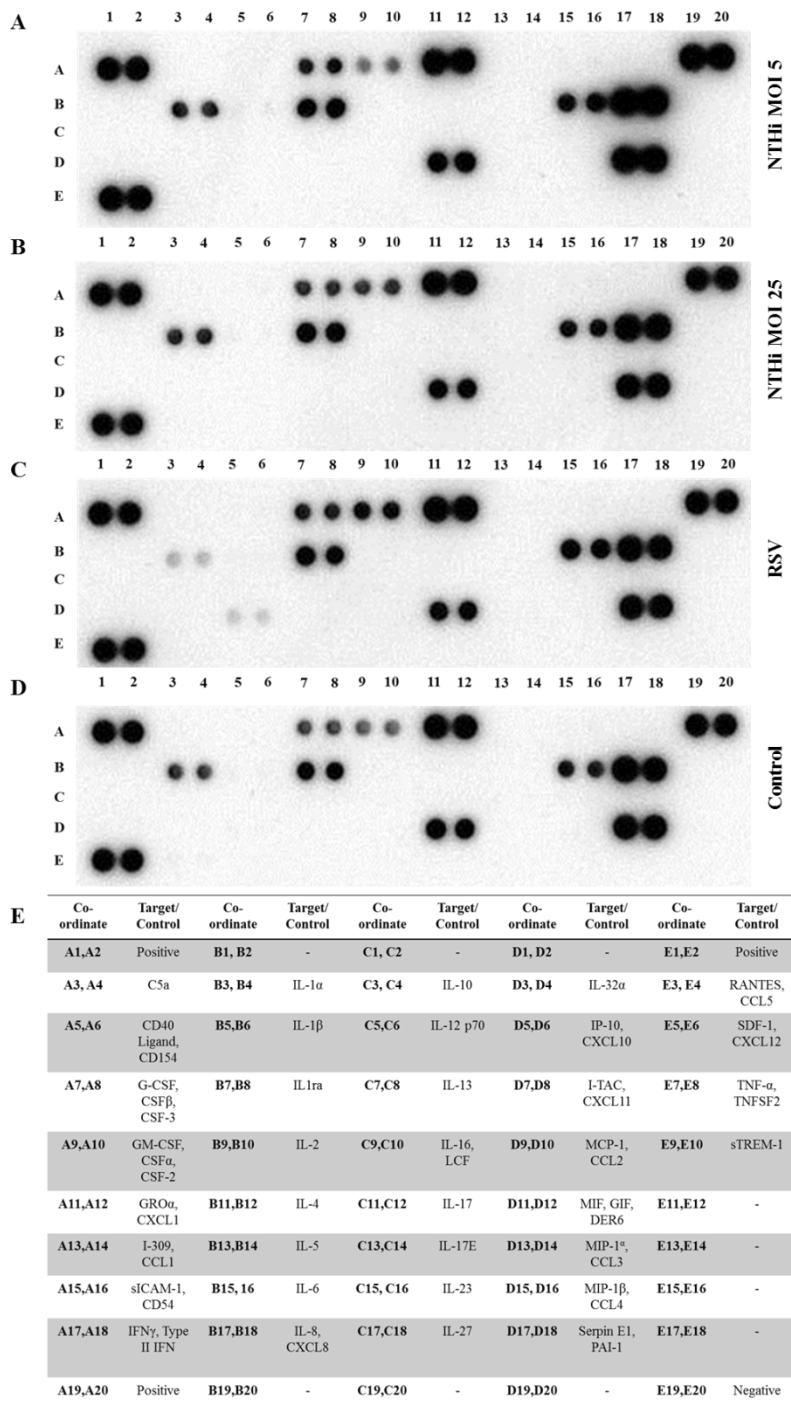


Figure 3.10: Cytokine Array of uHBE cells infected with NTHi and RSV for 24h

uHBE cells were infected with two different doses of NTHi (A and B) and RSV (C) for 24h alongside an uninfected control (D). The cytokine array data table is also shown (E). (Washes taken from an infection of Donor 3 uHBE cells) (n=1).

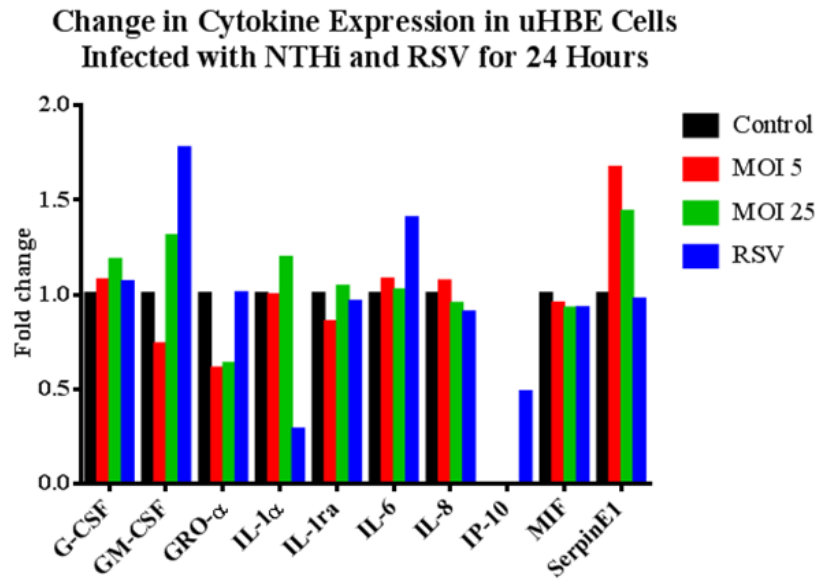


Figure 3.11: Densitometry analysis performed on the Cytokine Array of uHBE cells infected with NTHi and RSV for 24h

uHBE cells were infected with two different doses of NTHi and RSV for 24h alongside an uninfected control (Figure 3.10). Densitometry shows the differences in cytokines relative to the control (E) (n=1).

To complement the protein secretions studies, RNA extracted from A549, NCI-H292 and uHBE cells 24 hours after infection was used to quantify gene expression of IP-10, IL-6 and MUC5AC by qPCR. IL-6 was chosen as it had previously been identified as being up-regulated in response to NTHi LOS stimulation of human tracheal epithelial cells (Clemans, Bauer et al. 2000) and was also identified in our cytokine array data from 24h uHBE infections. IP-10 mRNA expression was not identified in our cytokine array of 24h uHBE infection, but it has previously been shown to be induced when cigarette smoke-exposed mice were challenged with NTHi, not in the lungs of control mice airways (Gaschler, Skrtic et al. 2009). MUC5AC has previously been shown to be induced by NTHi in multiple studies (Wang, Lim et al. 2002; Jono, Xu et al. 2003; Chen, Lim et al. 2004; Shen, Yoshida et al. 2008; Jono, Lim et al. 2012).

IP-10 and IL-6 mRNA expression was increased in A549 cells infected with NTHi at an MOI of 5 and 25 (Figure 3.12A and B), with IL-6 mRNA expression being lower in the MOI of 25 than in the MOI of 5. MUC5AC was decreased in the MOI of 5 and slightly increased in the MOI of 25 in comparison to the control (Figure 3.12C).

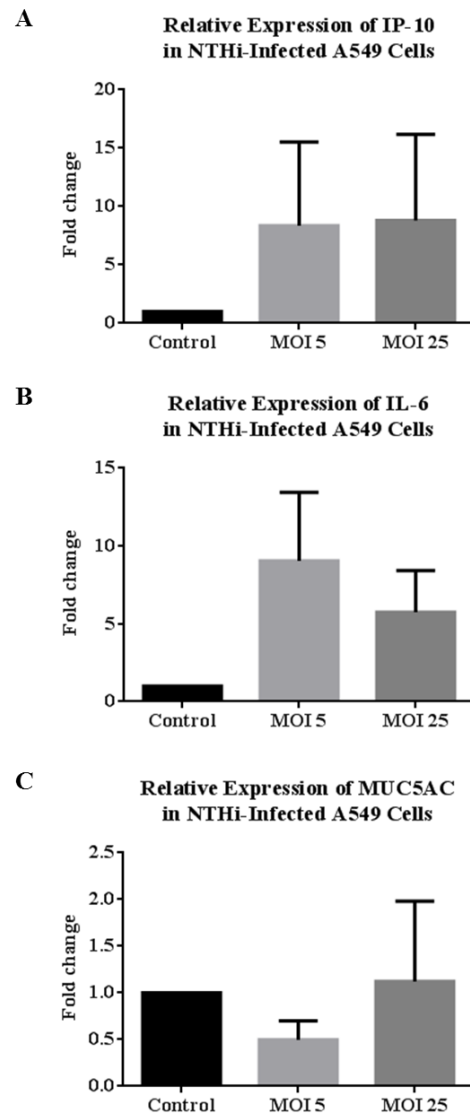


Figure 3.12: Comparison of IP-10, IL-6 and MUC5AC in A549 cells infected with two doses of NTHi

Quantitative data was generated using qPCR performed with primers against IP-10 (A), IL-6 (B) and MUC5AC (C) in samples from A549 cells infected with NTHi at two different doses for 24h. Repeated measures one way ANOVA with Dunnetts multiple comparisons test showed no significance between any samples (n=3). Error bars show SEM.

Expression of IP-10 (Figure 3.13A), IL-6 (Figure 3.13B) and MUC5AC (Figure 3.13C) all increased in NCI-H292 cells after infection with MOI 5 (4-fold increase in IP-10, 6-fold in IL-6 and 2-fold in MUC5AC) but infection with MOI 25 led to lower expression of MUC5AC than the control and expression of IP-10 and IL-6 was less marked with this higher dose of NTHi (Figure 3.13).

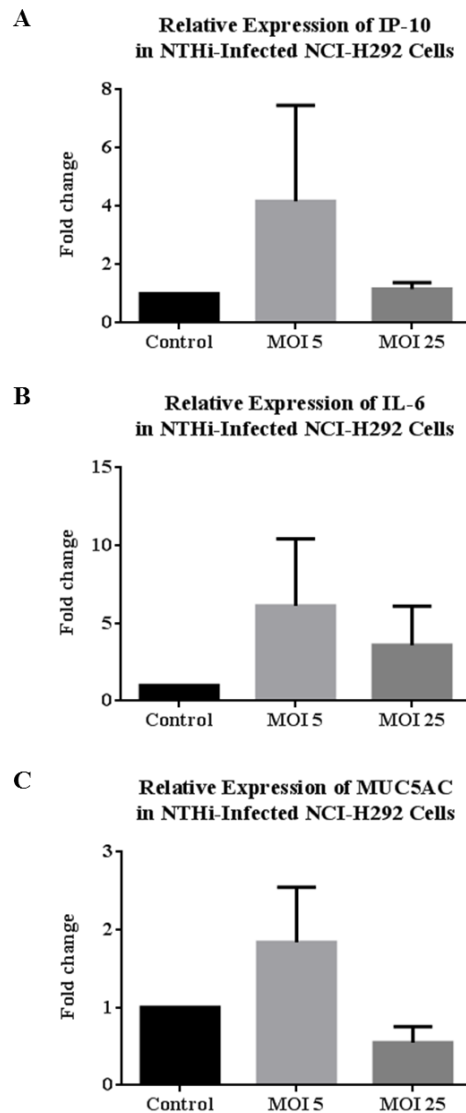


Figure 3.13: Comparison of IP-10, IL-6 and MUC5AC in NCI-H292 cells infected with two doses of NTHi

Quantitative data was generated using qPCR performed with primers against IP-10 (A), IL-6 (B) and MUC5AC (C) in samples from NCI-H292 cells infected with NTHi at two different doses for 24h. Repeated measures one way ANOVA with Dunnetts multiple comparisons test showed no significance between any samples

IP-10 (Figure 3.14A) and IL-6 (Figure 3.14B) mRNA expression was increased in uHBE, while MUC5AC mRNA expression is decreased (Figure 3.14C). IP-10 mRNA expression was higher in the lower NTHi dose (MOI 5) and lower in the higher dose (MOI 25), but both levels were increased in comparison to the control, similar to NCI-H292 cells. IL-6 increased with increasing MOI, and MUC5AC mRNA expression decreased more with the higher dose like IP-10 (Figure 3.14).

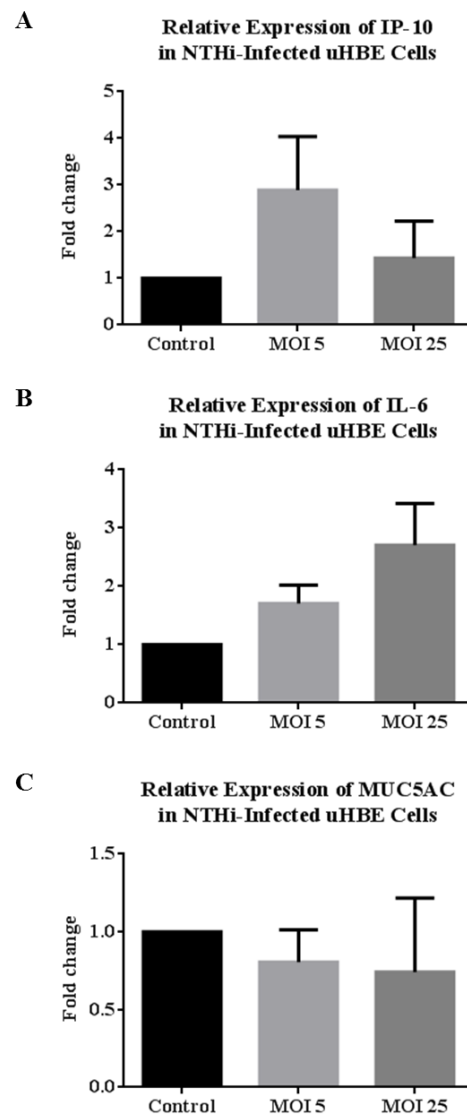


Figure 3.14: Comparison of IP-10, IL-6 and MUC5AC in uHBE cells infected with two doses of NTHi

Quantitative data was generated using qPCR performed with primers against IP-10 (A), IL-6 (B) and MUC5AC (C) in samples from uHBE cells infected with NTHi at two different doses for 24h. Repeated measures one way ANOVA with Dunnetts multiple comparisons test showed no significance between any samples (n=3). Error bars show SEM. (Data from Donor 1 and Donor 3 infections).

Statistical analysis showed that none of these differences were significant as a result of the high variability seen between replicates, the fold change data from the qPCR reactions for each individual replicate, for all cell lines, can be found in Appendix 4 alongside dissociation curves and DNA gels for some of the primer pairs.

To focus on the early cell responses, RNA extracted from uHBE cells exposed to 1×10^6 pfu of RSV at 1, 2, 4, 8 and 24 hour time points was reverse-transcribed and used PCR. Based on our cytokine array data (Figure 3.10) and because it had previously been shown to be up-regulated during RSV infection (Oshansky, Barber et al. 2010), we used primers against IP-10. As the changes in IP-10 in the cytokine array showed marked induction of IP-10, we chose to use standard RT-PCR to identify differences between time points. The results suggested that more relative induction was seen in latter time points (4, 8 and 24 hours post infection) but induction was present from 1 hour post infection (Figure 3.15). We decided to quantify this difference more definitively using qPCR. Based on this data and a study published by Seibold, Smith et al. (2013) qPCR was performed with primers for IP-10 and additionally IL-6 (Figure 3.16). There was an increase in IP-10 and IL-6 in all cell types at 24 hours. A paired *t* test showed a significant increase in IP-10 in uHBE cells ($p < 0.05$) (Figure 3.16E).

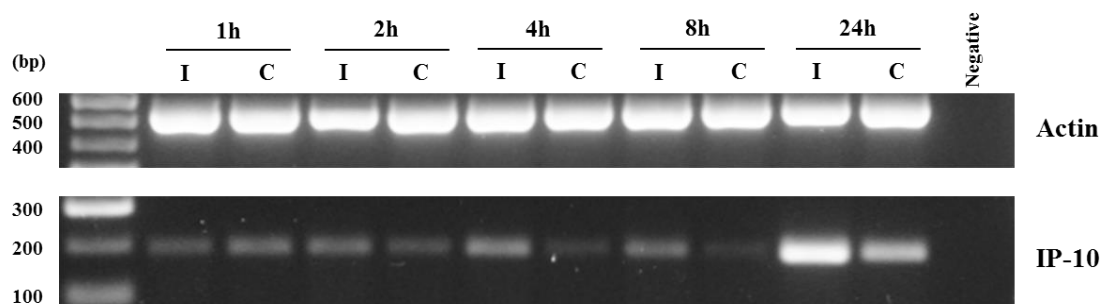


Figure 3.15: Comparison of gene expression in early time points using IP-10

PCR was performed on RNA extracted from RSV infected and control uHBE cells at 1, 2, 4, 8 and 24 hours using primers against IP-10 and actin to compare mRNA expression across the time points. Bands appear at the expected size for both primer pairs in all samples. (n=1)

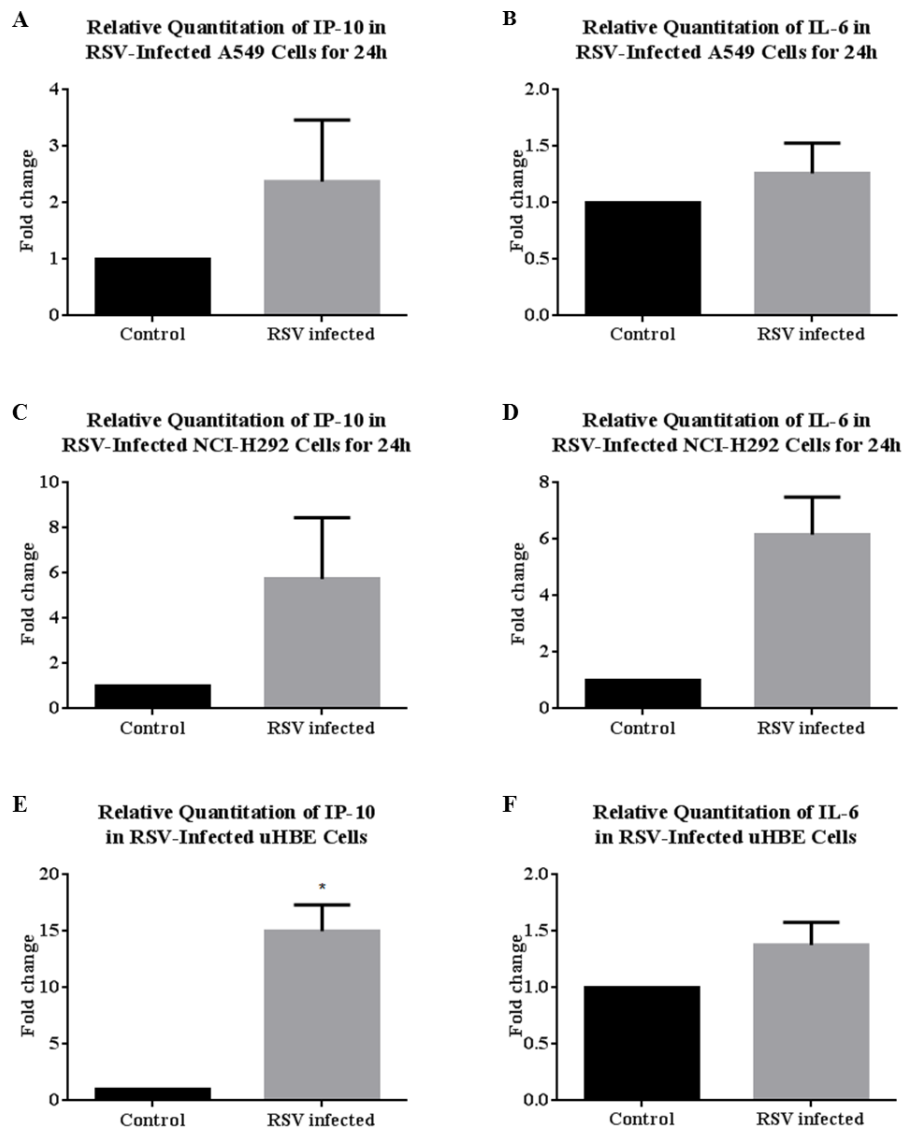


Figure 3.16: Comparison of expression of IP-10 and IL-6 in A549, NCI-H292 and uHBE cells infected with RSV

Quantitative data was generated using qPCR performed with primers against IP-10 (A, C and E) and IL-6 (B, D and F) in samples from A549 (A and B), NCI-H292 (C and D) and uHBE (E and F) cells infected with RSV for 24 hours. A paired *t* test showed a significant increase in IP-10 in uHBE cells ($p < 0.05$) ($n=3$). Error bars show SEM. (For uHBE cells data is from Donor 1 and Donor 3 infections).

3.4. Discussion

The aim of this chapter was to establish and characterise NTHi and RSV infection in cell lines, specifically the adenocarcinoma human alveolar basal cell line A549 (Giard, Aaronson et al. 1973), the mucoepidermoid carcinoma cell line NCI-H292 (Yoakum, Korba et al. 1983; Banks-Schlegel, Gazdar et al. 1985; Carney, Gazdar et al. 1985) and primary uHBE cells. These cells all showed poor survival when challenged with pathogens, and showed limited cytokine responses.

Lung derived cell lines can be used to study the biological effects of infectious agents and potential therapies *in vitro*. Cell lines essentially undergo infinite replication, making them useful tools for optimisation studies, and studying processes without the confounding problems of donor variability. Both of these lines have previously been utilised to study the effects of RSV and NTHi infection. Primary HBE cells have not been immortalised so do have a finite lifespan, and the use of these cells may introduce an element of inter-donor variability, however such cells are not transformed and are thought by many people to more closely represent the normal cells of the lung. Using these as an airway model, alongside immortalised cell lines, provides a direct comparison between cell types when establishing an infection model.

One of the aims of this project was to establish NTHi biofilms in an appropriate airway model; therefore infections with NTHi were performed on cell lines for periods of up to 7 days as it was felt that longer periods of infection would allow the best chance for biofilm formation. Infection studies performed on A549, NCI-H292 and uHBE showed that NTHi was able to establish an infection on these cells, but the prolonged infection period resulted in reduced cell numbers, affecting the confluence of the cell mono-layer, suggesting that the infection was leading to loss of cell numbers with a parallel reduction of viable bacteria. We could have assessed cell death/loss of cell numbers using a number of techniques, including live/dead staining such as Hoescht pI, flow cytometry, analysis of cell death markers such as lactate dehydrogenase and counting the number of DAPI stained nuclei in confocal imaging. However, we did not quantify cell death as they would not have allowed the use of the well for imaging and counting nuclei would require more images than were able to be taken.

As noted in the introduction, biofilms are a complex of aggregated cells, enclosed in EPS matrix. *In vivo* biofilms consist of multiple bacterial species and micro-organisms

that perform different condition related metabolic functions, however some bacterial species will form single-species biofilms. Not all bacterial species are able to form biofilms and initial doubts about the ability of NTHi to form biofilms were disproved in work conducted using a chinchilla model of otitis media (Ehrlich, Veeh et al. 2002). NTHi is thought to switch gene expression during the attachment phase to form a glue-like structured matrix, composed of multiple molecules and macromolecules (Moxon, Sweetman et al. 2008). No specific gene or factor has yet been identified as necessary for NTHi biofilm formation as heterogeneity of strains suggests that multiple combinations of factors are involved (Plaut, Qiu et al. 1992; St Geme, Falkow et al. 1993; Noel, Love et al. 1994; Gilsdorf, Tucci et al. 1996; Clemans, Bauer et al. 2000; Murphy and Kirkham 2002; Fink, Buscher et al. 2003; Webster, Wu et al. 2006). Our confocal imaging of a 3 day (and later) uHBE infections showed clustering of bacteria together within a structure that had a mesh-like appearance. The mesh-like structure could represent the glue-like matrix observed by Moxon, Sweetman et al. (2008). Comparing this to biofilm studies performed on NTHi and other bacterial species, this appears to be consistent with initiation of the formation of a biofilm structure (Starner, Zhang et al. 2006; Hall-Stoodley, Nistico et al. 2008; Ma, Conover et al. 2009; Juneau, Pang et al. 2011).

It was also important to ensure that all three cell types used in this study were susceptible to RSV infection. To do this we used a virus that expresses a red fluorescent protein tag when it is actively replicating. Confocal imaging showed that infecting both these cell lines and uHBE cells results in expression of the red fluorescent protein showing that they were successfully infected and could be used to study RSV infections in our hands. This is consistent with previous data as A549 lung epithelial cells have been used extensively to study RSV infections (Fiedler, Wernke-Dollries et al. 1996; Choudhary, Boldogh et al. 2005; Spann, Tran et al. 2005; Martinez, Lombardia et al. 2007; Eckardt-Michel, Lorek et al. 2008; Gibbs, Ornoff et al. 2009; Munday, Emmott et al. 2010; Bian, Gibbs et al. 2012; Chirkova, Boyoglu-Barnum et al. 2013; Krzyzaniak, Zumstein et al. 2013), NCI-H292s have also been shown to be sensitive to RSV infection (Hierholzer, Castells et al. 1993) and primary airway epithelial cells obtained using bronchial brushings from children have also been shown to be highly susceptible to RSV infection (Fonceca, Flanagan et al. 2012).

During the initial work with NTHi, bacteria were sub-cultured into the growth media preferred by the cells (Complete culture medium-Appendix 2) but this did not result in significant growth of the bacterium. *Haemophilus influenzae* has been shown to require a chemically defined media for growth (Coleman, Daines et al. 2003) which may have explained the absence of growth in the cultures. The growth of the bacteria in the presence of cells suggests that they need some factor produced by the cells in order to replicate in the airway model cultures.

Confocal images of all three cell types showed the presence of bacteria which appeared to increase (as judged by microscopy) by 3 days and were still present at 7 days. Viable bacterial counts showed that there were still live bacteria present in 7 day cultures; however there did not appear to be large differences between 3 and 7 day washes. Cell death in the 7 day cultures could have caused lifting of the adhered bacteria when washes were performed that may have given a falsely low level of viable bacterial counts. The viable bacterial counts did not take into account the bacteria clustered and associated with the epithelium in the form of biofilms, so in making these counts we were just taking viable counts of the planktonic bacteria present in the infected cultures. This may well have led to an underestimation of bacterial numbers in the cultures. The long term nature of these infections showed that some cells were able to survive the longer infection period required to form biofilms, however there were not enough cells to maintain a confluent mono-layer. Different times and infective doses are important in establishing infection models, to ensure infection is achieved without losing large numbers of cells. MOIs of 25 and 50 were investigated for 1 hour, 3 days and 7 days. As there was better cell survival in the MOI of 25 experiments we decided to show the results from these infections. No additional infection studies were performed in this work, but in order to effectively utilise cell lines as airway model, further studies using different doses and times might allow minimum dose and time required for successful infection/biofilm formation to be determined.

To investigate the cellular responses in long term infection of A549 and NCI-H292 cells, conditioned medium from day 7 infections was analysed using cytokine arrays. Strikingly, these cell lines were not influenced by the infection, as only IL-8 protein expression altered. As cytokines are released early in the response to pathogens it seems likely that the major cytokine responses elicited during these infections had been missed

by analysing the 7 days post infection samples. It is also possible that the infection had overcome the cells at this late time point.

To begin to address the cell survival issues, all three cell types were infected with NTHi and RSV for shorter periods of time. In the NTHi infections, viable bacterial counts were made up to the 24 hour time point and showed growth of bacteria in the first 8 hours, which plateaued by 24 hours. The number of bacteria present at 24 hours in the short term infection was similar to those in the previous studies of longer term infections. The data also showed that infections with a lower dose of NTHi (MOI 5) have higher viable counts by 24 hours (on average twice as many viable bacteria). Washes from the 24 hour infection of uHBE cells with the two doses of NTHi and a single dose of RSV as well as a control wash were used to perform a cytokine array. Cells infected with NTHi secreted the same cytokines as the control cells, however densitometry analysis showed varying levels of cytokine secretion. In the RSV infected cells, the most notable change was the induction of IP-10, an observation that has been shown previously (Oshansky, Barber et al. 2010; Smith, Kulkarni et al. 2013). IL-6 and GM-CSF were also increased the most. IL-6 and GM-CSF were frequently identified in immune studies against RSV infection using cell lines or undifferentiated primary cells (Cromwell, Hamid et al. 1992; Noah and Becker 1993; Arnold, Humbert et al. 1994). IL-6 acts in both a pro- and anti- inflammatory manner and is important in regulating the inflammatory response, preventing tissue damage. Bukreyev, Belyakov et al. (2001) generated a recombinant strain of RSV that expressed GM-CSF and found that viral replication was attenuated by GM-CSF, and they also saw increased levels of antigen presenting cells.

As a result of the cytokine array data (predominantly the uHBE cell data) showing alterations on secreted proteins we also studied the effect on gene expression, qPCR was performed with primers against IP-10 and IL-6 as both have previously been shown to be changes in mRNA expression compared to non-infected cells in NTHi and RSV infections (Clemans, Bauer et al. 2000; Gaschler, Skrtic et al. 2009; Oshansky, Barber et al. 2010; Smith, Kulkarni et al. 2013). MUC5AC expression was also studied as this has previously been shown to up-regulate MUC5AC mRNA expression (Wang, Lim et al. 2002; Jono, Xu et al. 2003; Chen, Lim et al. 2004; Shen, Yoshida et al. 2008; Jono, Lim et al. 2012). In contrast to the cytokine array for A549 cells, which did not identify either IP-10 or IL-6, the qPCR data showed increases in IP-10 and IL-6 mRNA

expression suggesting that they are expressed but not secreted at levels sufficient for the array membrane to detect them. MUC5AC mRNA expression was reduced in the lower dose infections but significantly changed in the higher dose infection. This does not agree with the study by Wang, Lim et al. (2002), which shows increased MUC5AC transcription in NTHi infected A549 cells by qPCR, although this study only infected cells for 5 hours. Further studies in A549 cells in response to NTHi reported induction of ICAM-1 (Avadhanula, Rodriguez et al. 2006) and activation of p38 MAPK and NF- κ B resulting in induction of cyclooxygenase (COX)-2 and prostaglandin E2 (PGE2) (Xu, Xu et al. 2008).

The NCI-H292 cell data was inconsistent. Cells infected with NTHi at an MOI of 5 showed increases in IP-10, IL-6 and MUC5AC whereas with an MOI of 25, IP-10 was increased slightly in comparison to the control; IL-6 increased to a lower level than that with an MOI of 5 and MUC5AC mRNA expression was decreased in comparison to the control. The cytokine array data did not identify either IP-10 or IL-6 as being secreted in detectable amounts by these cells. Only one study has used NCI-H292 cells with NTHi and this was to identify a putative toxin-antitoxin locus of NTHi (Daines, Jarisch et al. 2004) thus there is no confirmation of cytokine profiling data.

NTHi infected uHBE cells show increased mRNA expression of IP-10 and IL-6, but decreased mRNA expression of MUC5AC with both doses. IP-10 was not identified in the cytokine array data. IL-6 shows a different pattern. Densitometry showed more IL-6 secretion in the MOI of 5 whereas the highest expression is in the MOI of 25 sample, suggesting increased expression but reduced secretion. The decrease in MUC5AC is in contrast to the study of Jono, Xu et al. (2003) which noted an increase in MUC5AC in normal primary human airway epithelial cells infected with NTHi. As in the study by Wang, Lim et al. (2002), cells were infected for 5 hours which could explain the discrepancy with our data. Other studies in uHBE cells identified pathways involved in regulating NTHi induced MUC5AC transcription and the involvement of TLR3 in response to NTHi (Wang, Lim et al. 2002; Jono, Xu et al. 2003; Teng, Slavik et al. 2010).

RSV infected A549 cells showed increased expression of both IP-10 and IL-6, as did NCI-H292 cells and uHBE cells. A549 cells have frequently been used for RSV studies, which have characterised cytokine and chemokine responses over time (Arnold,

Humbert et al. 1994; Jiang, Kunimoto et al. 1998; Zhang, Luxon et al. 2001; Martinez, Lombardia et al. 2007). Increases have been noted in IP-10 (Rudd, Burstein et al. 2005) and IL-6 (Arnold, Humbert et al. 1994; Jiang, Kunimoto et al. 1998) and more recently the use of clinical isolates has identified strain specific responses including RANTES induction (Levitz, Wattier et al. 2012). Few studies have utilised the NCI-H292 cell line for RSV infection. One such study showed that the cells were sensitive to RSV infection (Hierholzer, Castells et al. 1993) and the other that type 1 and 2 cytokines regulate expression of programmed death-1 ligand (PD-L) 1, PD-L2, B7-H3 and inducible co-stimulator ligand (ICOS-L) (Stanciu, Bellettato et al. 2006). uHBE cells have also been used to study RSV infections; IP-10 expression increased upon RSV infection (Oshansky, Barber et al. 2010) and in a more recent study changes were noted in a range of chemokines including increases in IP-10 and IL-6 (Smith, Kulkarni et al. 2013). uHBE cells have not been extensively used to study the effects of RSV infections. A preference has been shown towards the use of primary cells in an ALI culture model, of differentiated airway epithelium as this is felt to generate a more accurate model of the airways for study (Villenave, Thavagnanam et al. 2012; Smith, Kulkarni et al. 2013).

Analysis of the control cytokine array data show the limited cytokine profiles of the cell lines used prior to infection. As cytokine secretion did not change much upon infection, under the conditions we have investigated this suggests that the cell lines exhibit a limited response to infection.

The aims of this chapter were to establish and characterise NTHi and RSV infections in cell lines and uHBE cells. In doing this we had to establish how representative/effective the cells were as an airway model, as well as investigate their ability to withstand infection. Infections were performed multiple times, encompassing time periods ranging from 1 hour to 7 days. Confocal imaging and characterisation of the responses of the cells indicated that the cell lines and uHBE cells could not tolerate high infection MOIs for prolonged periods of time. This coupled with exhibition of limited inflammatory responses suggested that cell lines and uHBE cells were not a representative model of the human airway epithelium. This led to the conclusion that the model needs to be able to withstand the infection process and elicit the complex responses seen in the human airway. This further suggests that a better model of the airway epithelium is required to successfully establish an infection model.

4. Establishment of the Air-Liquid Interface (ALI) Culture Model

4.1. Introduction

Culture of differentiated primary cells at an ALI was first performed using human tracheobronchial cells in 1990 (Wu, Martin et al. 1990). The key feature of this technique was the development of a mucociliary phenotype, such that the epithelial layer developed a pseudostratified appearance mimicking the human airway. As a result of this phenotypic organisation, the model is becoming an increasingly popular experimental tool. Since its initial description a number of varying protocols have been established (Koo, Yoon et al. 1999; Nlend, Bookman et al. 2002; Campos, Abreu et al. 2004; Ross, Dailey et al. 2007; Kettle, Simmons et al. 2010; Parker, Sarlang et al. 2010; Dvorak, Tilley et al. 2011; Pezzulo, Starner et al. 2011; Turner, Roger et al. 2011; Horani, Nath et al. 2013; Villenave, Shields et al. 2013). In general, these subtly different methods allow the cells to achieve similar levels of differentiation as judged by morphology and through the use of gene expression analysis. Whole genome transcriptional analysis, through the use of microarrays, has identified a number of genes specifically induced during the process of differentiation, including SPLUNC1, LPLUNC1, MUC5B, MUC5AC and tubulin (Ross, Dailey et al. 2007). These are ideal candidates to monitor and confirm differentiation of specific cell types within the cultures.

The ALI culture method can be performed using primary cells from a variety of sources, including commercially available cells as well as those directly obtained from patients via bronchoscopic brushing or from resected tissue. A major drawback in the use of primary cells, particularly those from human volunteers, is inability to obtain sufficient cells for replicates of experiments to be performed, particularly when the inter-donor variations of cell phenotype and responses are considered. However, the responses that ALI cells make to stimuli are considered to be representative of the airway epithelial lining than those from cell lines making their use invaluable.

Multiple growth factors mediate proliferation and differentiation of airway epithelial cells, and identification of the pathways involved has provided an understanding of the pathophysiology of a number of airway diseases. The initial cell culture studies identified key roles for retinoic acid, T3 and EGF (Koo, Yoon et al. 1999; Takeyama, Dabbagh et al. 1999; Gray, Koo et al. 2001) and modification of levels of these in the

culture medium allows phenotypic alteration of the cultures at will. Other exogenous mediators can modulate cell growth and differentiation for example the rho-associated protein kinase (ROCK) inhibitor Y27632. ROCKs are downstream effectors of Rho-A GTPase, which directly affects the cells cytoskeletal proteins. ROCK inhibitors also enhance basal cell proliferation in human airway epithelial cells at ALI without any subsequent effects on the differentiation of the cells (Horani, Nath et al. 2013).

Multiple immune molecules are able to act on the cultures to alter cell growth and differentiation. Two key molecules shown to regulate differentiation and gene expression in these cultures are IL-13 and IFN- γ . Culturing primary epithelial cells in the presence of IL-13, during the differentiation period, has been shown to result in increased numbers of goblet cells with a corresponding increase in the expression of goblet cell markers (Laoukili, Perret et al. 2001; Turner, Roger et al. 2011). IL-13 is a Th2 cytokine that has been implicated in the development of mucous cell metaplasia in murine models and is frequently detected in airway diseases such as CF and during exacerbations of asthma when there is an inflammatory response (Whittaker, Niu et al. 2002; Berry, Parker et al. 2004; Tyner, Kim et al. 2006; Curran and Cohn 2010). Therefore, treatment of the cultures with IL-13 can potentially provide an effective model for studying diseased airways. Recent work investigating the role of SPLUNC1 in the respiratory tract showed inhibition of SPLUNC1 expression in differentiated mouse tracheal epithelial cells treated with IFN- γ . This finding was partially corroborated in humans, through use of the NCI-H292 cell line treated in the same manner (Britto, Liu et al. 2013) as these cells also showed a reduced expression of SPLUNC1 following treatment with IFN- γ .

4.1.1. Aims

The aims of this chapter were to:

- (1) Reproducibly culture primary HBE cells at an ALI, to modulate the phenotype of these cultures through the addition of IL-13 and to validate these cultures using a variety of markers.
- (2) Investigate the effect of IFN- γ on the expression of SPLUNC1, LPLUNC1 and IP-10 (a molecule that is dependent on IFN- γ for induction) in differentiated primary HBE cells.

4.2. Methods

4.2.1. Culture of Primary Human Bronchial Epithelial Cells at ALI

Primary HBE cells were purchased from Promocell, who provided 500,000 cells at passage 2. The cells are from the healthy lungs of deceased subjects.

4.2.1.1. Seeding cells onto transwells

Cells were detached from the flasks using trypsin-EDTA as detailed in section 2.2.3, counted and centrifuged. They were re-suspended in ALI media (Appendix 2) to a cell density of 0.5×10^6 cells/ml. Transwell inserts (transparent PET membrane with $0.4 \mu\text{m}$ pores (BD Falcon)) were placed into the central wells of a 24-well-plate. $100 \mu\text{l}$ of 0.5×10^6 cells/ml were added to the apical surface of the transwell with additional media to make a final volume of $250 \mu\text{l}$, and $750 \mu\text{l}$ of ALI media was added to the basolateral compartment of each well. The media was changed in both compartments after 24 hours and every 2 days and cultured in sub-merged conditions until confluence was reached; typically 7 days. At confluence, the media was removed from the apical surface to expose the cells to an ALI. Apical medium was frozen for further studies.. Non-confluent cells do not undergo differentiation.

4.2.1.2. Differentiation at the ALI

Some cells were stimulated with 20ng/ml of IL-13 in the basolateral compartment to modulate differentiation to generate a more mucous phenotype which consists of a more 'goblet' cell-rich culture (Turner, Roger et al. 2011). IL-13 was added to an appropriate amount of media immediately prior to each media change; any remainder was disposed of and made fresh each time. After exposure to the air apical washes of all cells were performed daily or at most every 48 hours, for the duration of the differentiation period. Washes were frozen from three wells at each time point. ALI media was changed in the basolateral compartment every 48 hours during the differentiation period of a minimum of 21 days. This was either ALI media, or ALI media supplemented with IL-13 (Wu, Martin et al. 1990; Koo, Yoon et al. 1999; Kettle, Simmons et al. 2010; Dvorak, Tilley et al. 2011; Pezzulo, Starner et al. 2011).

Overall the culture of cells at ALI was performed eighteen times and each complete differentiation experiment took 5-6 weeks to perform (Figure 4.1). Primary cells obtained from five individual donors were used throughout these studies (details of which can be found in Appendix 1). The progress of mucociliary differentiation was

initially studied through Western blotting for SPLUNC1 and LPLUNC1 as they have been shown to be tightly associated with the process of differentiation (Campos, Abreu et al. 2004; Bingle, Barnes et al. 2007; Candiano, Bruschi et al. 2007; Ross, Dailey et al. 2007; Kesimer, Kirkham et al. 2009; Bingle, Wilson et al. 2010).

4.2.2. Stimulation of ALI cells with IFN- γ

ALI cells were media changed prior to stimulation. The apical surface of the cells was washed with 180 μ l of PBS and the washes frozen. IFN- γ was diluted to a concentration of 50ng/ml with sterile PBS and 30 μ l added to the apical surface of the cells in duplicate for 18 hours (based on the study by Britto, Liu et al. (2013)), sterile PBS was used as controls. After 18 hours, the apical surface of the cells was washed with 180 μ l of PBS. The wells were then lysed in TRI reagent, which was pooled to give greater volume for RNA extraction.

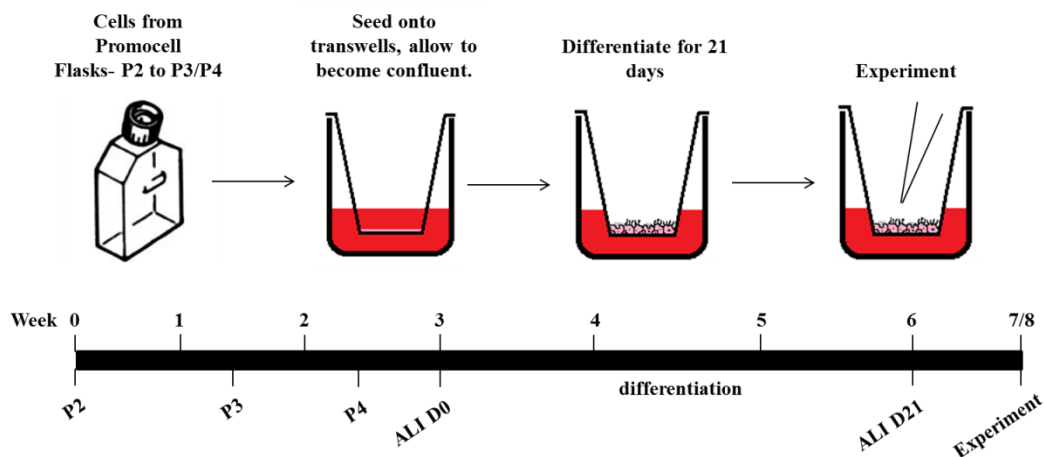


Figure 4.1: Schematic to show timeline of ALI culture experiments

uHBE cells were purchased from Promocell and cultured in flasks to increase numbers through to Passage 4. At a maximum passage of 5, cells were seeded onto transwells (100 μ l of cells at 0.5×10^6 cells/ml) and cultured in sub-merged conditions until confluence was reached. After removal of apical medium, cultures were differentiated for 21 days before being used experimentally.

4.3. Results

4.3.1. Validation of PLUNC antibodies

At the beginning of this study there were limited commercial antibodies available to PLUNC proteins. As we wished to use these markers to follow the process of differentiation we therefore generated antibodies against SPLUNC1 and LPUNC1 for use in the validation of our ALI cultures. We chose to generate two affinity-purified antibodies using unique peptide epitopes present in each of the two proteins. The antibodies were generated by Eurogentec and the epitopes used are shown in Appendix Figure 2.

4.3.1.1. SPLUNC1 antibodies

The SPLUNC1 antibodies were initially validated by Western blotting with samples of ALI washes taken from fully differentiated cultures using a range of antibody dilutions. Similar immuno-reactive products of the appropriate size were clearly seen with both antibodies, although the SPLUNC1B antibody also generated a smaller product (Figure 4.2A). We directly compared these products with those generated in a blot probed with a commercial SPLUNC1 monoclonal antibody which gave very similar reactive products (Figure 4.2B), suggesting that our antibodies were indeed reacting with SPLUNC1.

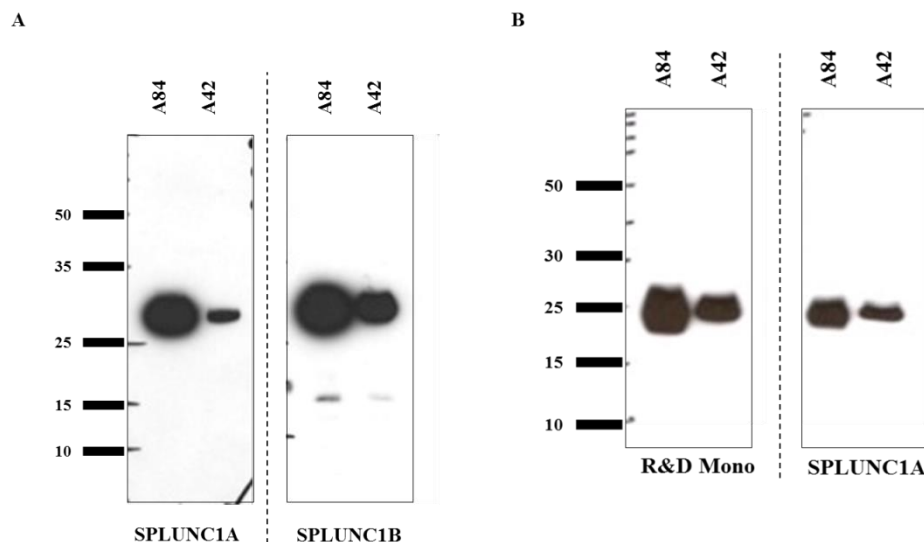


Figure 4.2: Validation of SPLUNC1 antibodies

The two SPLUNC1 antibodies were used to probe 2 ALI wash samples. SPLUNC1A was used at 1:1000 and SPLUNC1B at 1:200 (A). The R&D monoclonal SPLUNC1 antibody and SPLUNC1A was used to probe 2 ALI wash samples (B). Both blots show presence of bands at the appropriate size for all samples.

4.3.1.2. LPLUNC1 antibodies

The LPLUNC1 antibodies were initially validated by Western blotting with recombinant proteins (LPLUNC1, SPLUNC1 and SPLUNC2) produced both by *in vitro* transcription/translation and by transfected mammalian cells. These samples were provided by Dr Frances Barnes. An example of this validation is presented in Appendix Figure 1 (Appendix 3). We chose to do this for LPLUNC1 as at the time this was the first antibody generated and used against LPLUNC1.

Western blots of ALI washes from cultures as they underwent the process of differentiation were performed to validate the LPLUNC1 antibodies with ALI secretions and biological samples (BAL fluid) provided by Michael Campos at the University of Miami. ALI secretions were collected every 2-3 days by washing each well of a 12-well plate with 100µl of PBS, pooled and centrifuged to remove cellular debris. BAL fluid was obtained by instilling 60ml of saline in the non-diseased lingula or middle lobe from subjects undergoing clinically indicated bronchoscopy using a protocol approved by the University of Miami Institutional Review Board. LPLUNC1 protein was readily detectable as two distinct immuno-reactive products (Figure 4.3A) and levels increased as the cells underwent differentiation. Both protein bands were larger than the predicted size of LPLUNC1 (52.4kDa) suggesting that the protein might be subjected to post-translational modification. To investigate this PNGaseF (which hydrolyses most N-glycan chains) was added to the LPLUNC1, after which both immuno-reactive bands reduced in size (Figure 4.3B). BAL fluid (obtained from the non-diseased middle lobe (or lingula) of patients undergoing clinically indicated bronchoscopies and provided by Michael Campos at the University of Miami) treated with PNGaseF demonstrated similar immuno-reactive LPLUNC1 bands, suggesting that the protein product of the differentiated ALI cells is glycosylated in the same way as that secreted into the airways *in vivo* (Figure 4.3B).

Having validated the PLUNC antibodies we were then able to use them to study the differentiation of the ALI cell cultures.

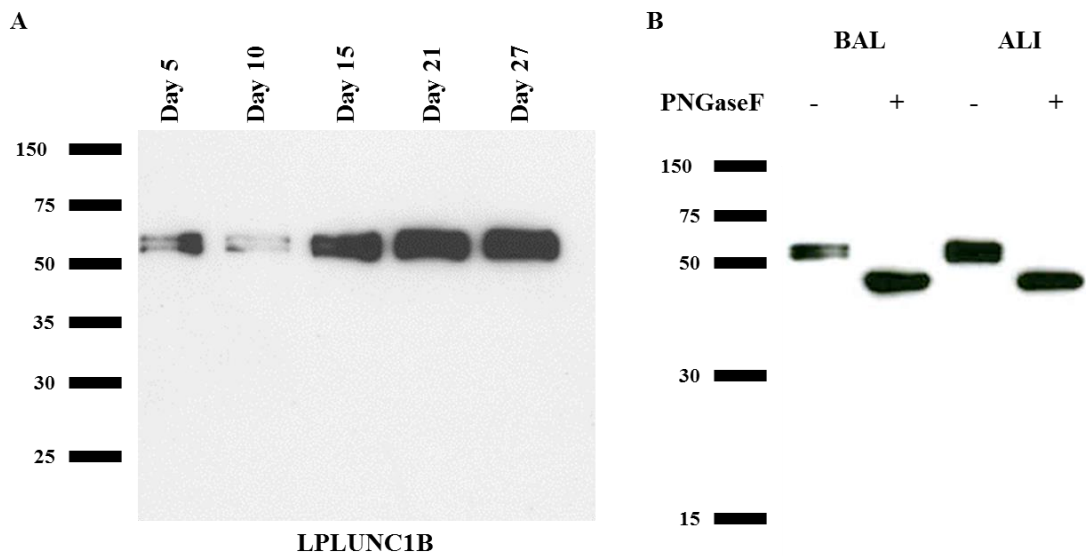


Figure 4.3: Validation of LPLUNC1 antibodies

Secretion of LPLUNC1 into apical secretions harvested from ALI cell cultures was shown by Western blotting. Samples were harvested from the cells every 2-3 days (A). Deglycosylation studies were performed using BAL fluid and ALI secretions as described in the materials and methods section. The resultant reaction products were subjected to Western blotting and detected with the LPLUNC1B antibody (B). Both blots show presence of bands at the appropriate size for all samples with a reduction in size for those samples used in deglycosylation reactions as expected.

4.3.2. ALI Cell Model

To follow differentiation in cell cultures, ALI washes from Day 0, 7, 14 and 21 were separated using SDS-PAGE, Western blotted and probed for SPLUNC1 and LPLUNC1. The results showed bands at the expected size in the day 14 and day 21 washes for LPLUNC1 and in the day 21 wash for SPLUNC1 (Figure 4.4A). To identify when transcription of LPLUNC1 and SPLUNC1 genes was induced during the differentiation process, PCR data was generated using RNA samples isolated from day 0, 2, 7 and 14 of ALI differentiation experiments. This data showed transcriptional activation of both LPLUNC1 and SPLUNC1 genes from day 2 of the differentiation process (Figure 4.4B) which is consistent with studies previously performed in our lab using cell samples provided by the Campos Lab in Miami, USA (Bingle, Barnes et al. 2007).

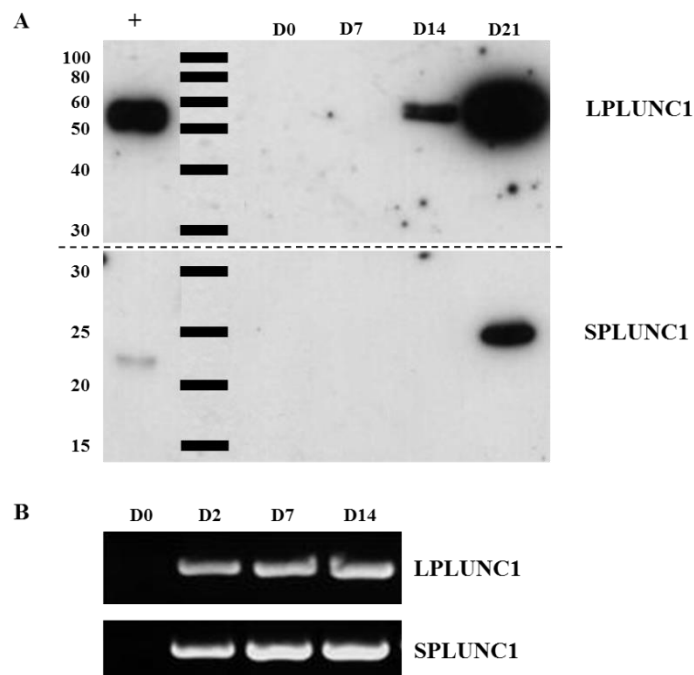


Figure 4.4: Western blot and PCR of ALI samples to track differentiation status

(A) 10 μ l of day 0, 7, 14 and 21 daily apical washes taken from throughout the differentiation period show bands at the appropriate size for LPLUNC1 and SPLUNC1, as well as bands at the correct size in the positive control (an apical secretion from ALI cells cultured by Synairgen and provided to us for a previous study). (B) RNA samples from Day 0, 2 7 and 14 were reverse transcribed and PCR performed with primers against LPLUNC1 and SPLUNC1 show presence of bands from day 2 (data generated by and taken from the Master’s dissertation of Miss Varsha Ganesan) (Images representative of all Donors (18 replicates), as all were successfully differentiated).

To compare differences between the relative amounts of SPLUNC1, LPLUNC1, MUC5AC and MUC5B secreted from cells undergoing differentiation with and without IL-13 treatment, 2 μ l samples of apical secretions (from three experiments) were spotted onto nitrocellulose membranes and probed. A clear variation in SPLUNC1 and LPLUNC1 secretion between individual cultures can be seen in Figure 4.5 A and C. Densitometry analysis showed increased SPLUNC1 (Figure 4.5 A and B), LPLUNC1 (Figure 4.5 C and D) and MUC5AC (Figure 4.5 E and F) in 14 day cultures following the addition of IL-13. There was no difference in the secretion of MUC5B (Figure 4.5 G and H). While the fold change data is portrayed, a ratio paired *t* test performed on the

raw data values showed differences in LPLUNC1 ($p < 0.01$) and MUC5AC ($p < 0.05$) are significant but not the SPLUNC1 levels.

To further monitor the differentiation status and examine the morphology of the epithelium during the differentiation 0, 7, 14 and 21 days ALI cultures were fixed in 10% phosphate-buffered formalin and paraffin embedded. Serial sections were cut from day 0, 7 and 14 samples, but not day 21 as initial differentiations became infected at day 16, and stained using haematoxylin and eosin to examine the morphology of differentiated cultures (Figure 4.6). This clearly shows different morphologies and cell compositions between day 0 (Figure 4.6A), day 7 (Figure 4.6B) and day 14 cultures (Figure 4.6C and D). The simple single celled epithelial layer seen initially undergoes significant thickening by day 7 and gains a pseudostratified appearance with ciliated cells (indicated by red arrow) clearly visible by day 14. Increased numbers of goblet cells (denoted by black arrows) are visible in 14 day cultures following the addition of IL-13 (Figure 4.C and D). Immunohistochemistry with antibodies against LPLUNC1 was performed only on day 14 cells cultured in the presence of IL-13 and showed positive staining in goblet cells (Figure 4.6E) with no staining in the control section (Figure 4.6F).

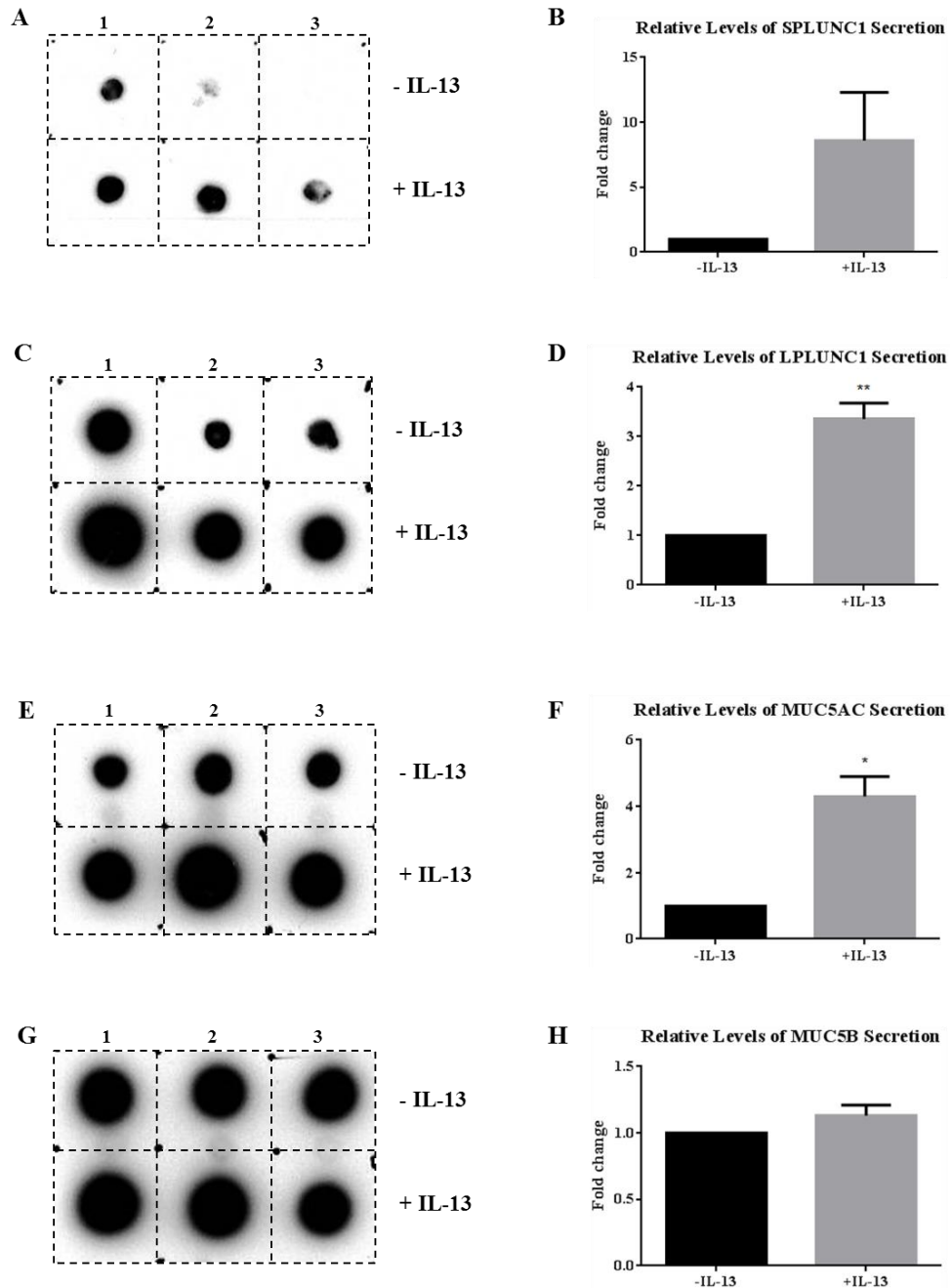


Figure 4.5: Dot blot to compare SPLUNC1, LPLUNC1, MUC5AC and MUC5B in cells cultured in the presence and absence of IL-13.

2 μ l of secretions was spotted onto nitrocellulose membrane and probed with antibodies to SPLUNC1 (A), LPLUNC1 (C), MUC5AC (E) and MUC5B (G) to compare protein levels between ALI cells cultured in the presence and absence of IL-13. Densitometry analysis was used to quantify levels of SPLUNC1 (B), LPLUNC1 (D) and MUC5AC (F) and MUC5B (H) expression in cells cultured with IL-13 than those without. A ratio paired *t* test performed on the raw data values showed significant increases in LPLUNC1 ($p < 0.01$) and MUC5AC ($p < 0.05$) ($n = 3$). Error bars show SEM. (Secretions from differentiations performed using Donor 3 cells).

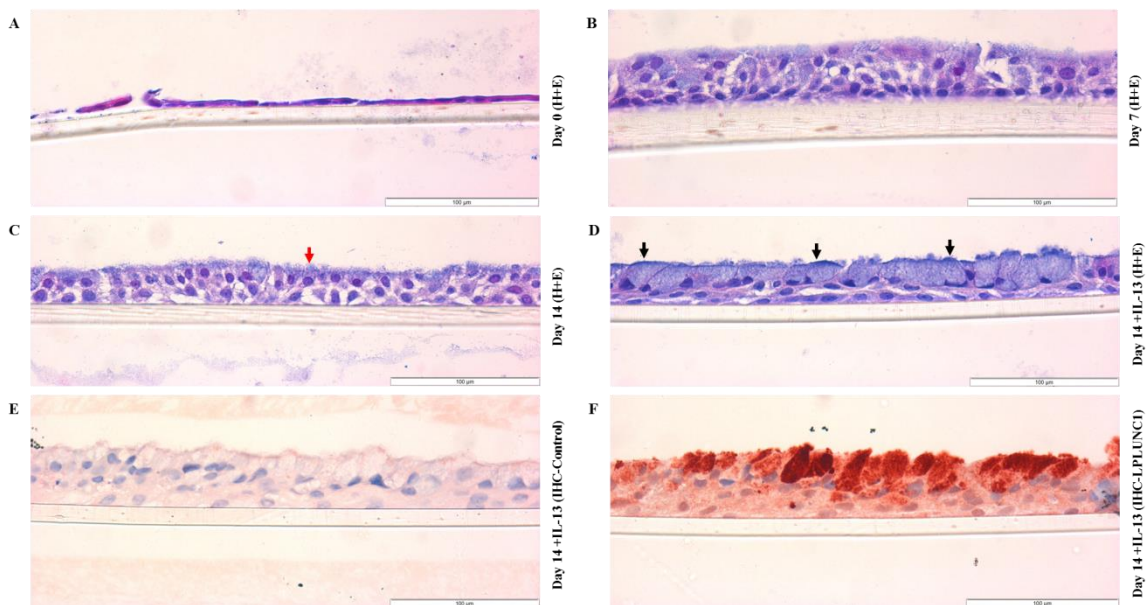


Figure 4.6: Staining of ALI cultures at Day 0, 7 and 14 cultured in the absence and presence of IL-13

Haematoxylin and eosin staining showed that Day 0 (A) cultures are a single cell mono-layer. By day 7 (B) the cell layer appears thicker and by day 14 (C and D) have developed ciliated and goblet cells. Cells cultured for 14 days in the presence of IL-13 (D) visibly have more goblet cells than those without (C). Sections from cells cultured in the presence of IL-13 showed positive (red) staining for LPLUNC1 (F). The control shows no positive staining for LPLUNC1 (E). The red arrow shows a ciliated cell and the black arrows show goblet cells. (Images are of cells from Donor 1)

Immunofluorescence and confocal microscopy allowed closer examination of the differentiated cells. β -tubulin (Figure 4.7A and B) and MUC5AC (Figure 4.7C and D) stained cilia and mucus, respectively, which allowed for comparisons between the cells cultured in the presence (Figure 4.7B and D) or absence (Figure 4.7A and C) of IL-13. Higher levels of MUC5AC were found following the addition of IL-13 but no difference was detected in the level of β -tubulin staining. The differentiation status of the cells was confirmed by immunofluorescent staining for ZO-1 (Figure 4.8A), which stains the tight junctions formed between cells, α -tubulin (Figure 4.8B) staining of ciliated cells, MUC5B positive (Figure 4.8C) goblet cells, and SPLUNC1 positive (Figure 4.8D) serous cells. Z-stack images were not taken of these sections. Controls for all staining were section stained incubated with secondary antibody only (Figure 4.7E and F and Figure 4.8E)

To obtain more quantitative of the effects of IL-13 on the cells, RNA from differentiated cultures was reverse transcribed and qPCR performed using primers for SPLUNC1, LPLUNC1, MUC5AC and MUC5B and actin as an endogenous control. IL-13 treatment had no effect on levels of SPLUNC1 (Figure 4.9A) or MUC5B (Figure 4.9D) while LPLUNC1 and MUC5AC was increased (0.75 and 14-fold difference respectively) in IL-13 cultured cells (Figure 4.9B and C); a ratio paired *t* test performed on the raw data values (fold change data shown in graphs for better comparison) showed the increase in MUC5AC was significant ($p < 0.05$).

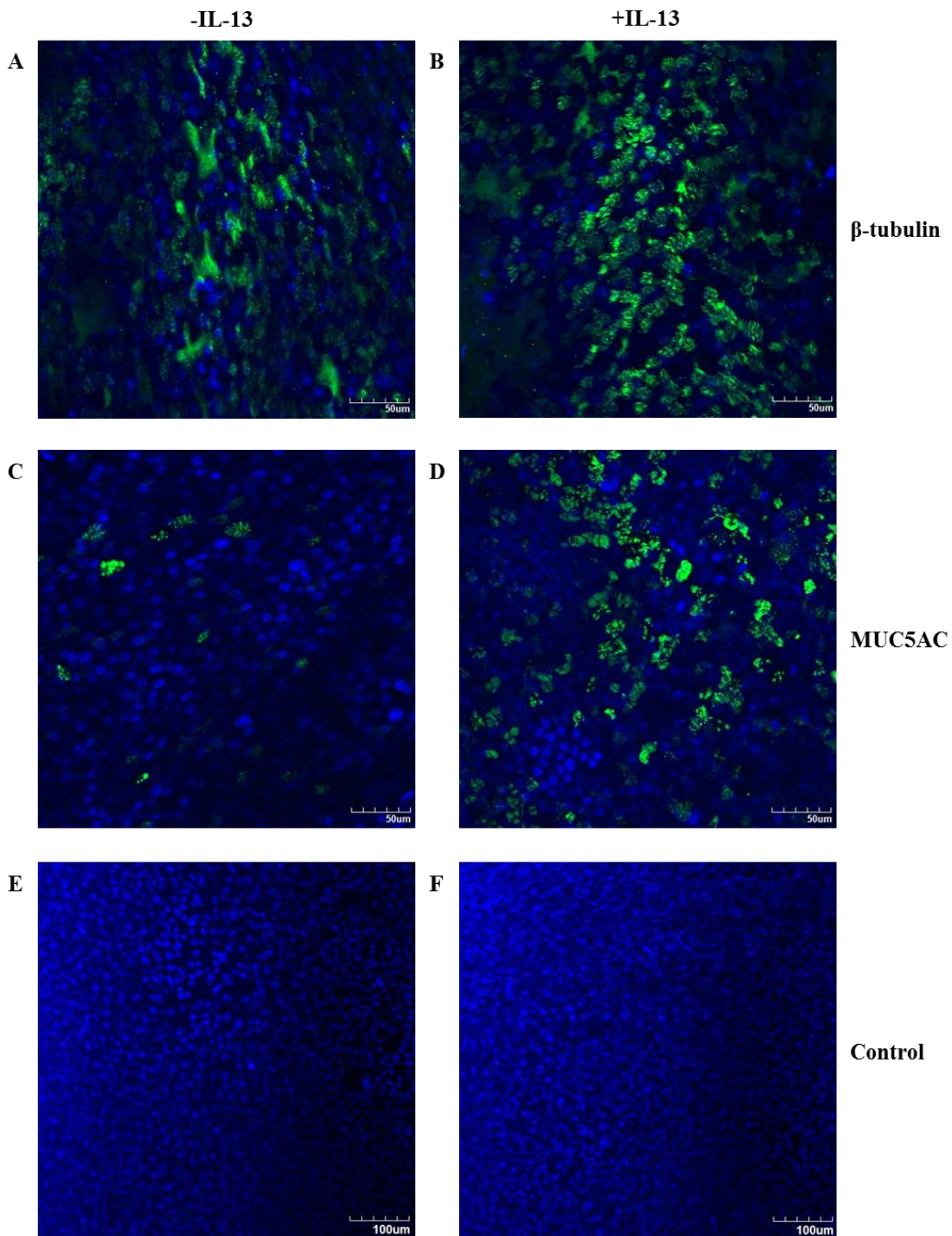


Figure 4.7: Immunofluorescent staining of 21-day differentiated cell cultures to compare effects of IL-13

Positive (green) immunofluorescent staining was seen for β -tubulin (A and B) and MUC5AC (C and D) in cells cultured in the absence and presence of IL-13. Controls were stained with Anti-mouse secondary antibody only (E and F). Nuclei were stained blue with DAPI. (Cells from Donor 2)

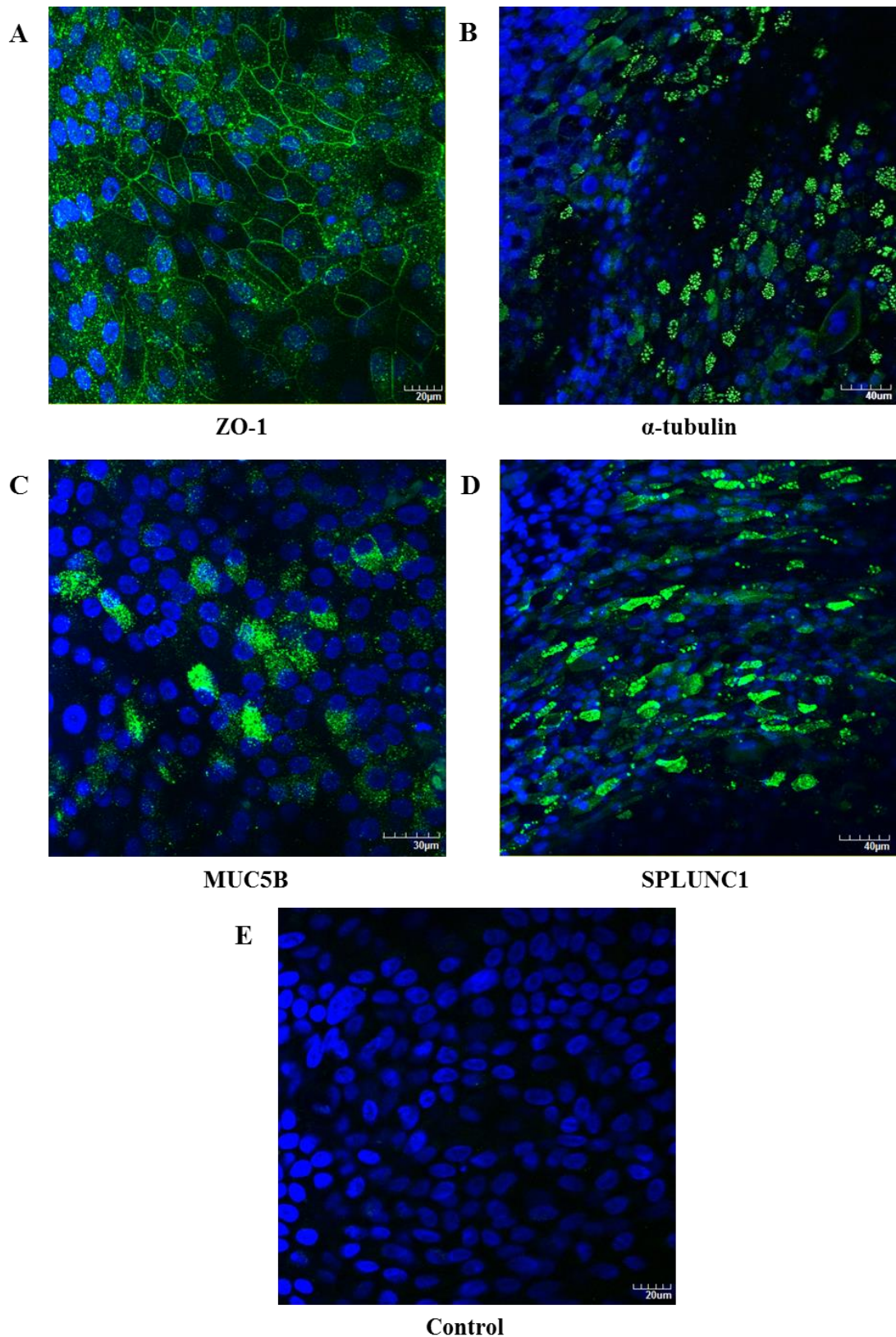


Figure 4.8: Immunofluorescent staining of 21-day differentiated cultures with other markers

Positive (green) immunofluorescent staining was seen for ZO-1 (A), α -tubulin (B), MUC5B (C) and SPLUNC1 (D). Control was stained with Anti-rabbit secondary antibody only (E). Nuclei were stained blue with DAPI. (Images of cells from Donor 3, but are representative of all differentiations).

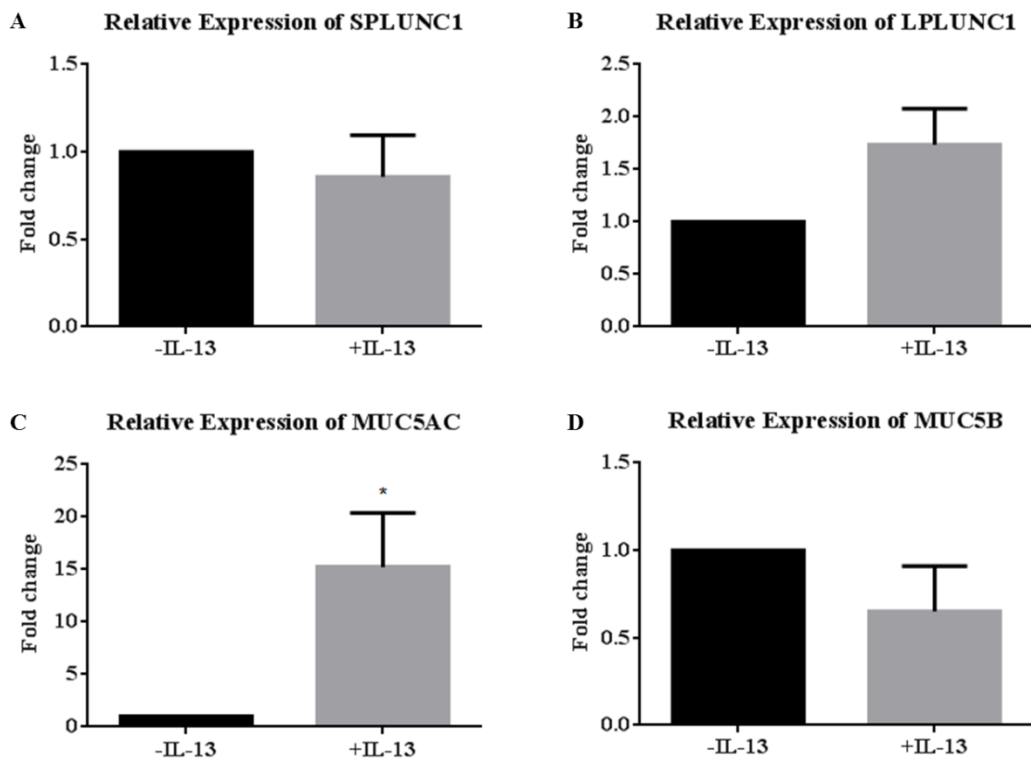


Figure 4.9: Comparison of SPLUNC1, LPLUNC1, MUC5AC and MUC5B in cells cultured in the presence and absence of IL-13 by qPCR.

Cells cultured in the presence of IL-13 appear to show lower levels of expression of SPLUNC1 (A) and MUC5B (D). They show higher levels of LPLUNC1 (B) and much higher levels of MUC5AC (C). A ratio paired *t* test performed on the raw data values showed significant increases in MUC5AC ($p < 0.05$) ($n = 3$). Error bars show SEM. (Donor 3 cells).

4.3.3. IFN- γ stimulation of ALI cells

After establishing the ALI model it was important to test the responsiveness to additional stimulus that could be measured easily. IFN- γ was used as Britto, Liu et al. (2013) showed a reduction of SPLUNC1 expression in a murine airway epithelial model when stimulated with IFN- γ , a finding which was partially corroborated in humans using the NCI-H292 cell line. Due to their putative functions in immune responses and our previous use of PLUNCs as differentiation markers we sought to test these findings in human ALI cells. We treated 21 day differentiated ALI cells with IFN- γ for 18 hours based on the study by Britto, Liu et al. (2013). Apical surface washes were collected, cells lysed in TRI reagent and RNA extracted. Due to the limited number of cells available we only used a single dose of IFN- γ for a single time period.

Apical secretions were separated using SDS-PAGE, Western blotted and probed with antibodies against LPLUNC1 (Figure 4.10A) and SPLUNC1 (Figure 4.10C). The results show bands at the expected sizes for both proteins. Densitometry analysis showed difference 4-fold increase in LPLUNC1 (Figure 4.10B) secretion and a 2-fold increase in SPLUNC1 (Figure 4.10D) secretion when stimulated with IFN- γ .

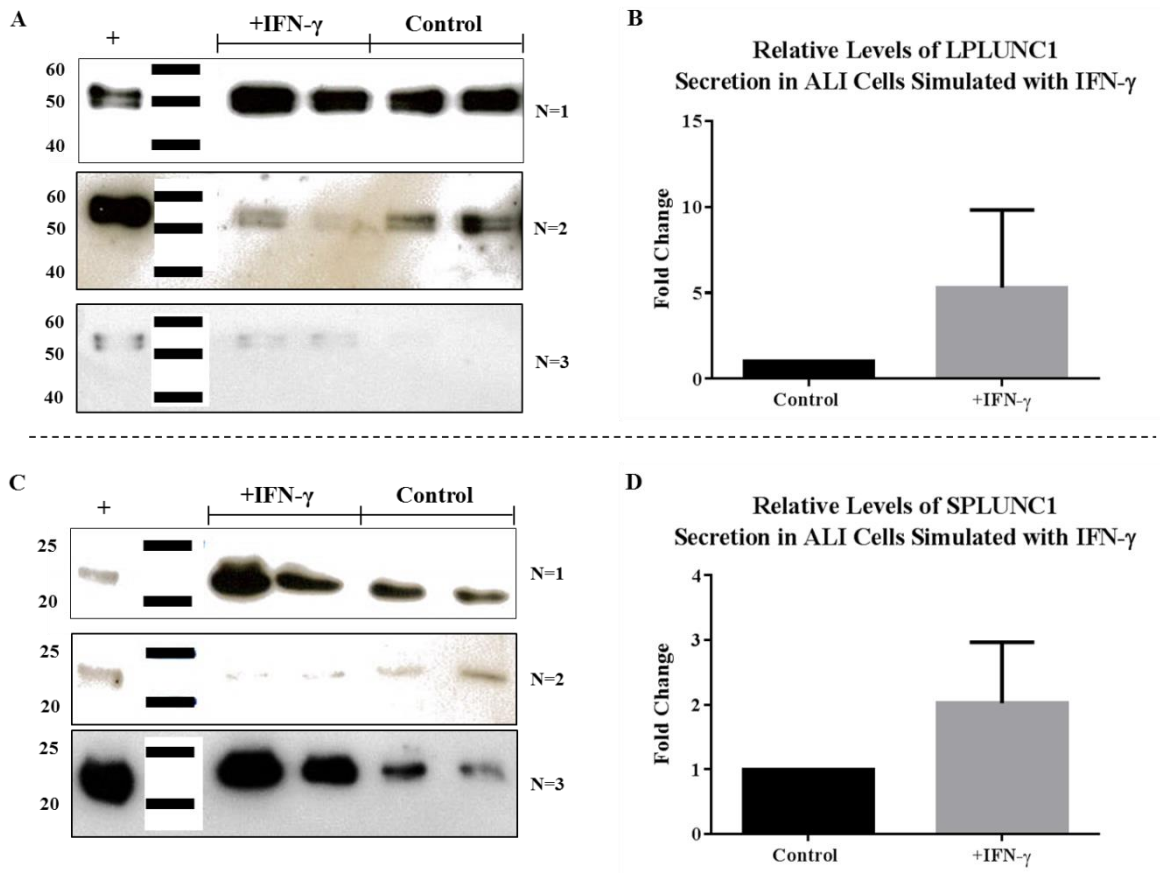


Figure 4.10: Western blot for LPLUNC1 and SPLUNC1 in ALI cells stimulated with IFN- γ .

10 μ l of secretion was separated by SDS-PAGE and Western blotted for LPLUNC1 (A) and SPLUNC1 (C). Bands appear at the appropriate sizes for both proteins in the samples and positive control (an apical secretion from ALI cells cultured by Synairgen and provided to us for a previous study). Densitometry analysis was performed to show induction of LPLUNC1 (B) and SPLUNC1 (D) after stimulation with IFN- γ . A ratio paired *t* test shows no significance (n=3). Error bars show SEM. (All IFN- γ stimulations performed on differentiations using Donor 3 cells).

qPCR generated quantitative data on the expression of SPLUNC1, LPLUNC1 and IP-10 following IFN- γ treatment. No significant differences in SPLUNC1 (Figure 4.11A) and LPLUNC1 (Figure 4.11B) were noted but marked differences (average 1250-fold difference) were seen in the induction of IP-10 following IFN- γ stimulation (Figure 4.11C), this was not significant upon statistical analysis with a paired *t* test. This change confirmed that the stimulation of the cells did exert an IFN- γ effect but that this stimulation had no effect on expression of SPLUNC1 or LPLUNC1. The fold change data from each replicate is shown in Appendix 4.

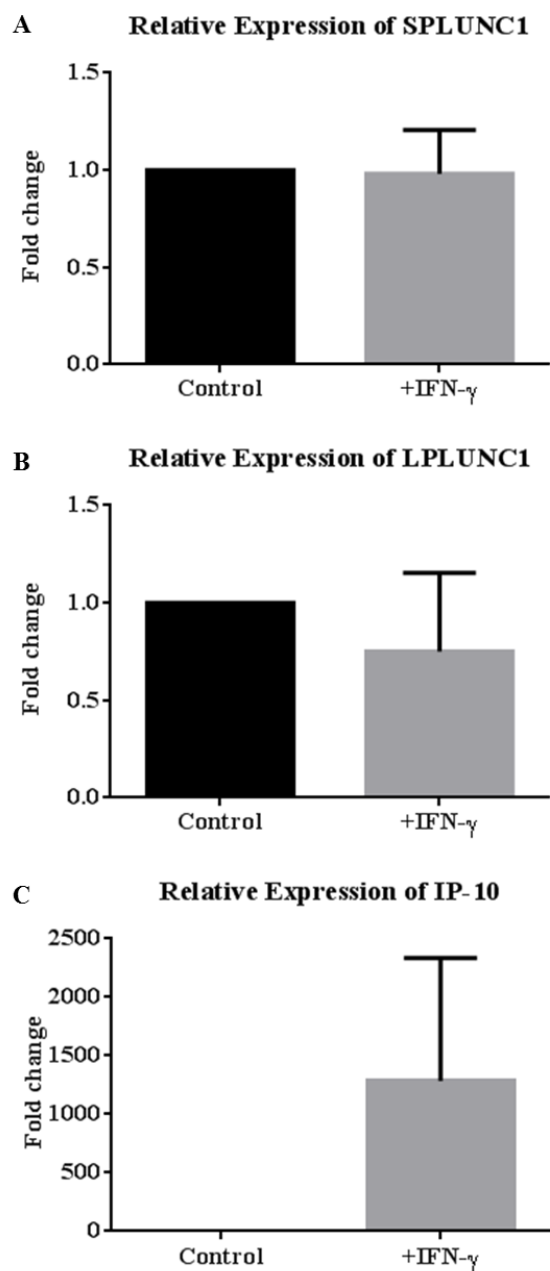


Figure 4.11: Comparison of SPLUNC1, LPLUNC1 and IP-10 in cells stimulated with IFN- γ by qPCR

Quantitative data was generated using qPCR performed with primers against SPLUNC1 (A), LPLUNC1 (B) and IP-10 (C) in samples from cells stimulated with IFN- γ and unstimulated controls. A paired *t* test showed no significance (n=3). Error bars show SEM.

4.4. Discussion

The aim of this chapter was to reproducibly establish ALI cell cultures in our laboratory. Previously, work from the lab with such cells was done on a collaborative basis with ALI cell RNA and secretions being provided by a number of different collaborators (Bingle, Cross et al. 2006; Bingle, Barnes et al. 2007; Bingle, Wilson et al. 2010). We were able to successfully differentiate primary HBE cells using the ALI culture method numerous times using cells from five different donors and modulate this phenotype with the addition of IL-13 to generate a more goblet cell rich phenotype.

The ALI technique was established in 1990 (Wu, Martin et al. 1990) and has been replicated and utilised by many groups since, using a number of differing protocols that have become increasingly more complex, including modulation of cellular phenotype with growth factors and use of a variety of culture tools with transwell inserts resulting in effective differentiation of mucus-secreting cell types. The process of differentiation causes the cells to take on a pseudostratified appearance (Ross, Dailey et al. 2007) and has been shown to be a good representation of the *in vivo* airway epithelial transcriptome (Dvorak, Tilley et al. 2011). We considered that this differentiation process and the associated complex secretory products produced would not only closely mimic the airway *in vivo* but would also help to overcome the issues of cell death and the limited responses seen in cell lines and uHBE cells infected with RSV and NTHi and described in the previous chapter and be a useful tool for studying the responses of the airway epithelium to a variety of stimuli.

In principle, airways cells, whether extracted from patients or purchased commercially, are cultured in transwells with a source of growth medium available below the transwell in the basolateral compartment, while the cells on the apical surface of the transwell are exposed to the air. The transwells can be coated with a number of extracellular matrix proteins, e.g. collagen, for cells to adhere to (Nlend, Bookman et al. 2002; Ross, Dailey et al. 2007; Parker, Sarlang et al. 2010). This is more commonly used in cases where cells are limited, for example when cells are obtained from bronchial brushings, as it aids in cellular adherence in order to aid growth of the cells. The commercial cells used in this project grew well in flasks that were not collagen coated so were also cultured on non-collagen coated transwells. There is some suggestion in the literature that collagen coating is critical for ciliated cell differentiation (Ross, Dailey et al. 2007), however this was not the case for our studies, and the cells differentiated into ciliated and goblet cells

with proportions visibly altered when cultured in the presence of IL-13 to increase goblet cell numbers (Laoukili, Perret et al. 2001; Turner, Roger et al. 2011). Modification of the phenotype through IL-13 provides a model for studies of airway diseases that are associated with mucous cell metaplasia and increased production of mucus, as *in situ* diseased airways, such as those from patients with COPD and asthma contain significantly increased numbers of goblet cells. Thus our aim was to establish these cultures for our own studies, including modulation of the phenotype by changing the culture conditions, and to validate these against those already established by other groups.

Differentiation to a mucociliary phenotype, results in the induction of a significant number of genes which can then act as markers for different epithelial cell types within the cultures. These markers are easily accessible in the mucus-rich secretions of the mucociliary phenotype. Transcriptional analysis performed by Ross, Dailey et al. (2007) showed that two of the most induced genes during the process of mucociliary differentiation are SPLUNC1 and LPLUNC1. The proteins encoded by these genes are members of a larger family known as the PLUNC (BPIF) family (Bingle and Craven 2002; Bingle, Bingle et al. 2011) and are found abundantly in the mucus of the upper airways and nasal passages (Bingle and Bingle 2000; Bingle, Wilson et al. 2010). The induction of PLUNC proteins as a direct result of differentiation of primary airway epithelial cells *in vitro* (Bingle, Barnes et al. 2007; Ross, Dailey et al. 2007; Bingle, Wilson et al. 2010), and the ease in which it is possible to obtain samples (by washing the apical surface of the cultures) make them ideal candidates to use as markers for tracking differentiation during the establishment of ALI culture. Neither of these genes is significantly expressed in undifferentiated primary airway cells (Ross, Dailey et al. 2007). As our group works extensively with these proteins, they were chosen as markers of differentiation for use in this project. It was important to identify and validate antibodies against PLUNC family members in order to use them as markers.

In the absence of readily available commercial antibodies at the start of our studies we generated antibodies against a number of family members using two epitopes per protein to increase the chances of successful antibody generation. The founding PLUNC family member, SPLUNC1 was identified by a number of groups (Weston, LeClair et al. 1999; Bingle and Bingle 2000; Iwao, Watanabe et al. 2001; Di, Harper et al. 2003) and has become one of the most characterised family members since then. During our

studies commercial antibodies did become available and we used one of these as a positive control for the antibodies we had generated. As the single discrete bands for each SPLUNC1 antibody, including the commercial antibody, appeared to be the same size on the gel we concluded that they were identifying SPLUNC1. In other studies in the laboratory we have also validated these same antibodies against recombinant SPLUNC1 protein (Bingle, Cross et al. 2005). The LPLUNC1 gene was identified after conducting BLAST and PSI-BLAST searches to identify expressed sequence tags (Bingle and Craven 2002). Our recent publication (Bingle, Wilson et al. 2010) was the first to focus on LPLUNC1. Our in-house antibodies were validated using recombinant LPLUNC1 expressed in Chinese hamster ovary cells in the absence of a commercially available antibody.

Western blotting performed using the LPLUNC1B antibody showed a discrete banding pattern with a double-band suggesting that LPLUNC1 is post-translationally modified by N-linked glycosylation, an observation supported by the identification of glycosylated LPLUNC1 isoforms in saliva (Ramachandran, Boontheung et al. 2008). Deglycosylation studies showed a reduction of the double band to a single band and further supported this conclusion. We were also able to show that BAL fluid samples contained bands with the same molecular mass as seen in the ALI secretion, suggesting that the ALI cells produce native LPLUNC1 essentially identical to that seen in lung secretions. In other studies the antibodies have been used to localise LPLUNC1 in a population of goblet cells *in situ* as well as in ALI cultures (Bingle, Wilson et al. 2010).

Data produced from our cell cultures supports our suggestion that they were able to differentiate into a mucociliary phenotype. Cells were cultured for 21 days as 14 to 21 days of culture has previously been shown to be sufficient for full differentiation to occur (Ross, Dailey et al. 2007; Turner, Roger et al. 2011). Western blotting for both SPLUNC1 and LPLUNC1 confirmed that both proteins were increasingly detected in the cell secretions during this period of differentiation. As expected, prior to differentiation, cell secretions were negative for both proteins. Undifferentiated cells have a more basal cell phenotype and do not express the differentiated cell markers. Our data did exhibit some variation in the time at which each protein was detected which is in agreement with previous results from our lab using cell samples prepared by the Campos lab in Miami. It does appear however that cells cultured for this study secrete the proteins at a later stage of culture than we have seen previously with LPLUNC1

being secreted by day 5 (Campos, Abreu et al. 2004; Bingle, Wilson et al. 2010) whereas we didn't see it until day 14 in this study. This may reflect differences in the origin of the cells, their passage number and the exact culture conditions that are used. Our cells were from a commercial supplier whereas the Campos lab uses cells isolated from unused donor material, cells are cultured on collagen-coated transwells and the media used is slightly different (Campos, Abreu et al. 2004). LPLUNC1 was secreted as a double band on Western blot illustrating differential glycosylation, which was removed by treatment with deglycosylation reagents (Bingle, Wilson et al. 2010). This confirms that our cells were secreting a similar protein to that previously reported and that is seen *in vivo*. As gene transcription always precedes the production of secreted proteins perhaps unsurprisingly our PCR data showed activation of gene transcription for both LPLUNC1 and SPLUNC1 by day 2 of the differentiation period. This confirms that cells used in this study begin to undergo the process of differentiation soon after being moved to the ALI and that perhaps the lack of secreted protein may reflect a sensitivity issue in the blotting procedure. Importantly, we were able to show that both LPLUNC1 and SPLUNC1 were always detectable in secretions from cells after 21 days of ALI culture and in all of our subsequent studies we used cells that had been cultured for this period of time prior to use. Washes were collected throughout all successful differentiations with induction of both PLUNCs proteins being a consistent occurrence.

Our data also showed that the ALI cultures expressed a number of additional marker proteins. Examining the cellular composition by immunofluorescence staining demonstrated an increase in the number of goblet cells following stimulation with IL-13 during differentiation. The histological sections from similar samples clearly demonstrated the presence of ciliated and goblet cells, as well as the multi-level arrangement that gives the epithelium its pseudostratified appearance. These images are in keeping with those produced by Ross, Dailey et al. (2007), and confirmed that we had been able to establish successful differentiation in our cultures. On the basis of all of these observations we felt confident in using these cultures in subsequent infection studies.

One of the major issues associated with the use of primary cells is that of donor variation. This may in part have been the cause of the temporal differences in secretion of the proteins discussed above. To attempt to overcome this variation we studied multiple samples from eighteen separate differentiation experiments using cells from

five distinct donors. We also generated quantitative data through use of qPCR, dot blotting and densitometry analysis to compare differences between cells cultured in the absence and presence of IL-13. Donors from all cells successfully differentiated into a pseudostratified layer with a mucociliary phenotype. All showed induction of SPLUNC1 and LPLUNC1 upon differentiation although this was consistent within individual donors it was clear that the level of induction was variable between donors.

Immunofluorescence staining for β -tubulin, a marker of ciliated cells identified in the array studies of Ross, Dailey et al. (2007), and a classical ciliated cell marker confirmed the presence of cilia on the surface of cells, although surprisingly there appeared to be no clear difference between those cultured in the presence and absence of IL-13. This contrasts with changes seen previously where there was a marked decrease in the number of ciliated cells in IL-13 positive cultures (Laoukili, Perret et al. 2001), however they utilised an antibody against glutamylated tubulin (Wolff, de Nechaud et al. 1992), a post-translational modification found in ciliary axonemes and essential for airway ciliary function (Ikegami, Sato et al. 2010), which identifies more than just one specific class of tubulins providing a higher level of detection/positive staining. We used the same antibody as Villenave, Touzelet et al. (2010) as they had shown it to detect cilia in cultures, staining with the more specific antibody may have identified a higher amount of staining as well as confirming that the cilia are functional, and should be considered for staining of cilia in the future. A later study showed a decrease in expression of FOXJ1, a transcription factor expressed in ciliated cells, resulting in loss of cilia in cultures treated with IL-13 as IL-13 was shown to regulate the *foxj1* promoter (Gomperts, Kim et al. 2007). Although we have detected FOXJ1 in our cells (Varsha Ganesan, unpublished data) we have not studied differential expression with and without IL-13 treatment.

Marked differences in MUC5AC expression, consistent with previous studies (Laoukili, Perret et al. 2001; Atherton, Jones et al. 2003; Turner, Roger et al. 2011) was demonstrated by immunostaining, qPCR and immunoblotting. These studies utilised immunostaining or fluorescent labelling to examine the morphology of the epithelium and was then used to approximate proportions of goblet cells in comparison to ciliated cells at varying times through the differentiation period. These were estimated to be around 50% secretory and 10% ciliated cells after 21 days of treatment with IL-13 in comparison to the 15% secretory and 25% ciliated cells in cultures that weren't treated

(Laoukili, Perret et al. 2001). Array studies were performed by Ross, Dailey et al. (2007) at differing time points throughout a 28 day differentiation period which focussed on genes that showed up-regulation of more than 10-fold. These studies identified large induction of MUC5B, confirming the study of Bernacki, Nelson et al. (1999). However, initial array studies did not show MUC5AC as undergoing large induction however qPCR studies did show a large induction of both MUC5AC and MUC5B by day 12. Our qPCR studies showed expression of PLUNC proteins, MUC5AC and MUC5B in agreement with Ross, Dailey et al. (2007) and comparison of the cells cultured in the presence and absence of IL-13 showed a significant difference in MUC5AC expression at day 21, consistent with previous studies (Laoukili, Perret et al. 2001; Atherton, Jones et al. 2003). Our data showed a 15-fold increase in MUC5AC expression, which is supported by the study conducted by Turner, Roger et al. (2011) which utilised TaqMan qPCR to compare expression of MUC5AC between cells cultured in the absence and presence of IL-13. They showed large increases in mRNA expression in cells cultured in the presence of IL-13 by day 14. IL-13 has also been shown to induce MUC18, which appeared to aid adherence of NTHi, suggesting action as a receptor for NTHi (Simon, Martin et al. 2011).

A study conducted using cells isolated from nasal polyps and cultured at an ALI in the presence of differing doses of IL-13 showed down-regulation of SPLUNC1 expression (Yeh, Lee et al. 2010). This is not consistent with our study using HBE cells. The down-regulation seen in the nasal cells was dose responsive, using 0, 1, 10 and 100ng/ml and as we used 20ng/ml of IL-13 this may account for the small decrease in our study, or it could equally be a result of the differing cell types used for culture.

Having successfully established the ALI technique the responsiveness of the model was tested by stimulating the cells with IFN- γ as it was reported by Britto, Liu et al. (2013) that IFN- γ stimulation of ALI differentiated mouse tracheal epithelial cells (mTECs) inhibits the transcription of SPLUNC1. This data was further confirmed by Britto, Liu et al. (2013) using the human NCI-H292 cell line. This data complements previous studies on the NCI-H647 cell line using northern blotting (Bingle and Bingle 2000). When we performed these studies in ALI-differentiated cells we anticipated that standard PCR for LPLUNC1 and SPLUNC1 would show a decrease in gene expression in accordance with the data from Britto, Liu et al. (2013). However my findings, of no significant change in SPLUNC1, do not match that of Britto, Liu et al. (2013) or Bingle

and Bingle (2000); both groups showed a reduction of SPLUNC1 upon IFN- γ stimulation. Interestingly, we have been unable to readily detect SPLUNC1 expression in the NCI-H292 cells in our lab (unpublished results). The differences in responses could be due to the differing cell types used or could be due to the length of time the cells were stimulated for. We stimulated for only 18 hours in comparison to the Britto, Liu et al. (2013) study which stimulated cells for 24-48 hours. It seems unlikely that our data was due to lack of activity of the IFN- γ treatment as the large induction of IP-10, the IFN- γ inducible protein, shows that the cells are eliciting a response to the IFN- γ . These results need to be strengthened using both a dose and time course study before any firm conclusions can be drawn relating to the role of IFN- γ in the regulation of SPLUNC1 and LPLUNC1 gene expression in human ALI cells.

As suggested previously, a limitation in the use of primary airway cells is the difficulty in obtaining sufficient cell numbers for replicates of experimental exposures. This certainly limited the number of studies we were able to perform in this validation work. Development of methods that would allow increased cell numbers to be isolated/cultured would allow more studies to be performed from a single donor. Recently published data shows that treatment of ALI cells with a Rho-Kinase (ROCK) inhibitor enhances basal cell proliferation without any subsequent effects on differentiation at the ALI (Horani, Nath et al. 2013). This treatment may provide an effective solution to issues of low cell numbers, allowing seeding of cells at lower densities which would be beneficial in future work to obtain more wells of cells for differentiation. We performed a simple experiment to show that this treatment does indeed appear to increase cell proliferation (Appendix 5) but time limitations did not allow for a systematic analysis of the differentiation capacity of these cells. However, subsequent work performed in the laboratory with primary mouse TBE cells has confirmed increased cell numbers following ROCK inhibitor treatment (Dr Khondoker Akram, personal communication).

In conclusion, ALI culture was successfully and reproducibly established through eighteen differentiations using cells from five different donors. Our data confirmed induction through use of several markers. Modification of the phenotype was also achieved, with culture in the presence of IL-13 resulting in increased goblet cell numbers. Stimulation with IFN- γ did not appear to inhibit SPLUNC1 expression, as had been shown previously; This could be due either to the length of time that cells were

stimulated for or due to the dose chosen. However, having established a differentiated *in vitro* model of 'normal' and 'diseased' airways we were then able to undertake infection studies as outlined in the next chapter.

5. Establishment of infection models using ALI-differentiated primary cells

5.1. Introduction

The ALI differentiation process results in development of a mucociliary phenotype, that includes ciliated, serous and goblet cells as well as the secretion of products from these cells. ALI cells have been shown to provide a good *in vitro* model of the respiratory epithelium, and thus a more representative tool to study the interaction of pathogens (Dvorak, Tilley et al. 2011). Cells become tightly packed, developing tight junctions and a pseudostratified appearance. We considered that the increased cell densities seen in these cultures as well as the multiple innate defence proteins that they release into the apical secretions may prevent the cell death and destruction of the cell-mono layers seen in the studies undertaken in the established cell lines and described in Chapter 3.

As previously mentioned RSV is a common pathogen causing recurring infection in the airway. It has previously been shown that RSV specifically infects ciliated cells in ALI cultures (Zhang, Peeples et al. 2002). This specificity of infection provides an alternative tool to check the integrity of the ALI differentiation model as well as characterise the responses elicited by an RSV infection. NTHi is associated with airway colonisation and exacerbations of symptoms in airway diseases. No key feature has been identified that is necessary of NTHi to establish an infection. However, NTHi strains are known to possess protease activity, in the form of IgA1 protease (Plaut, Qiu et al. 1992), that is utilised to impair the immune response (Kilian, Mestecky et al. 1979; Male 1979). IgA1 protease functions as an autotransporter in bacterial aggregation mediating the formation of micro colonies (Plaut, Qiu et al. 1992) and the protease is found near the top of the biofilm structure, leading to the suggestion that it has a protective function against antibody attack (Webster, Wu et al. 2006). Examination of proteolytic activity exhibited by NTHi during the infection process may also provide insight into early infection and biofilm formation processes.

5.1.1. Aims

The aims of this study were to:

- (1) Establish and characterise RSV infections in ALI cells
- (2) Establish and characterise NTHi infections in ALI cells
- (3) Investigate the proteolytic activity of NTHi on epithelial host defence products

5.2. Methods

5.2.1. Infection of ALI cells with RSV

ALI cells were infected with either 5×10^5 or 1×10^6 pfu/50 μ l of RSV; both doses were used in cell line infections. After initial infections, 1×10^6 pfu/50 μ l was used for infections to ensure infection of the more complex pseudo-stratified ALI cultures. The surface of the cells was washed after 48 hours and the wash frozen. At 5 days post infection, the experiment was terminated by washing the surface of the cells with 180 μ l of PBS (as above) and fixing for 10 minutes with 10% phosphate-buffered formalin. Fixative was replaced with PBS for storage until they were immunofluorescently-stained.

5.2.2. Infection of cells with NTHi

Primary cells differentiated at the ALI were already on transwells, and were cultured in antibiotic free media for at least three days prior to infection. The surface of half of the ALI wells was washed with 180 μ l of PBS 24 hours prior to infection; the other half was washed immediately before infection. Cells were infected with approximate MOIs of 5, 25 and 50 diluted to final volumes of 50 μ l in PBS for 1 hour, 3 days and 7 days. After which, a gentle wash was taken from the surface of the transwell and stored. Wells were washed in the basolateral compartment with PBS to remove excess media and fixed for 10 minutes in 10% phosphate-buffered formalin (basolateral and apical compartments). After 10 minutes the formalin was removed and replaced with PBS for longer term storage. Before imaging, membranes were cut from the transwell using a scalpel and mounted with Vectashield containing DAPI, sealed with nail polish and imaged using the Olympus FV-1000 Confocal Microscope.

5.2.3. Co-infection of RSV and NTHi

ALI cells that had been differentiated for 21 days were infected with previously established doses of both RSV and NTHi. Cells were initially infected with 1×10^6 pfu/50 μ L of RSV for 3 days, after which the surface of the cells were washed and basolateral media (antibiotic free) changed prior to a secondary infection with NTHi at an MOI of 25 for 3 days and 7 days. Cells were infected with RSV first as it took longer to establish an infection than with the NTHi. Single pathogen infections of RSV at 1×10^6 pfu/50 μ L and NTHi at an MOI of 25 were performed alongside for the durations stated in the co-infections so as to act as controls. At the end of the

experimental exposures nuclei were counter-stained with DAPI, mounted and imaged using confocal microscopy.

5.3. Results

5.3.1. Infection of ALI cells with RSV

Differentiated ALI cells were infected with recombinant red RSV at a dose of 1×10^6 pfu/50 μ l for 5 days. This dose had previously been validated in the laboratory in a related project (Ugonna, Bingle et al. 2014). Infections were performed on cells that had been cultured for 21 days in either the absence (Figure 5.1A and Figure 5.2A) or presence (Figure 5.1B and Figure 5.2B) of IL-13. This allowed us to study the effects of the differing phenotypes as IL-13 treated cells have an increased number of goblet cells. After fixing, membranes were immunofluorescently stained for β -tubulin (Figure 5.1) and MUC5AC (Figure 5.2). Membranes were then counter-stained with DAPI and staining was visualised and Z-stack images captured using confocal microscopy. Control staining was performed using secondary antibody only, these were not routinely imaged after initial images showed no background staining. Imaging of secondary antibody only controls can be seen in figures 4.7E and F.

Overlap of green positive β -tubulin with the red virus infected cells was demonstrated (Figure 5.1) (white arrows). Staining for MUC5AC did not overlap with virus infected cells; rather the green staining of MUC5AC was seen around infected cells (Figure 5.2) (white arrows). Z-stack imaging allowed for a composite to be made of z-axis views, with the same patterns as the z-axis views (Figure 5.1 and Figure 5.2) (red arrows). These observations suggest that RSV selectively infected ciliated cells. These results are representative of n=3 individual experimental infections.

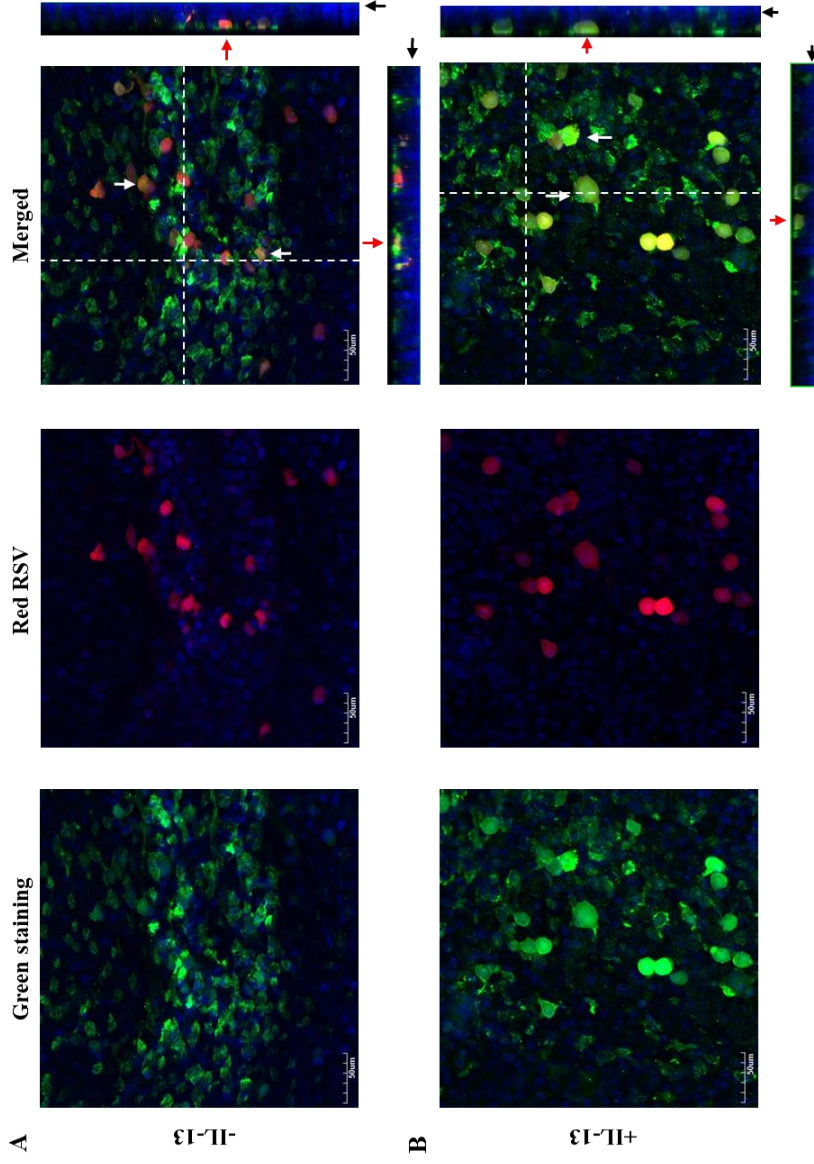


Figure 5.1: Immunostaining for ciliated cells in cultures infected with RSV for 5 days

Cells cultured in the absence and presence of IL-13 were infected with red recombinant RSV. Red fluorescence shows infected cells, blue fluorescence shows nuclei staining with DAPI and green fluorescence shows staining for β -tubulin. Black arrows show nuclei staining and red fluorescence indicate the location of basal cells. White arrows show overlap of β -tubulin green staining and red infected cells. Red arrows show this overlap in the side views. (Images from Donor 1 cells, representative of $n=3$)

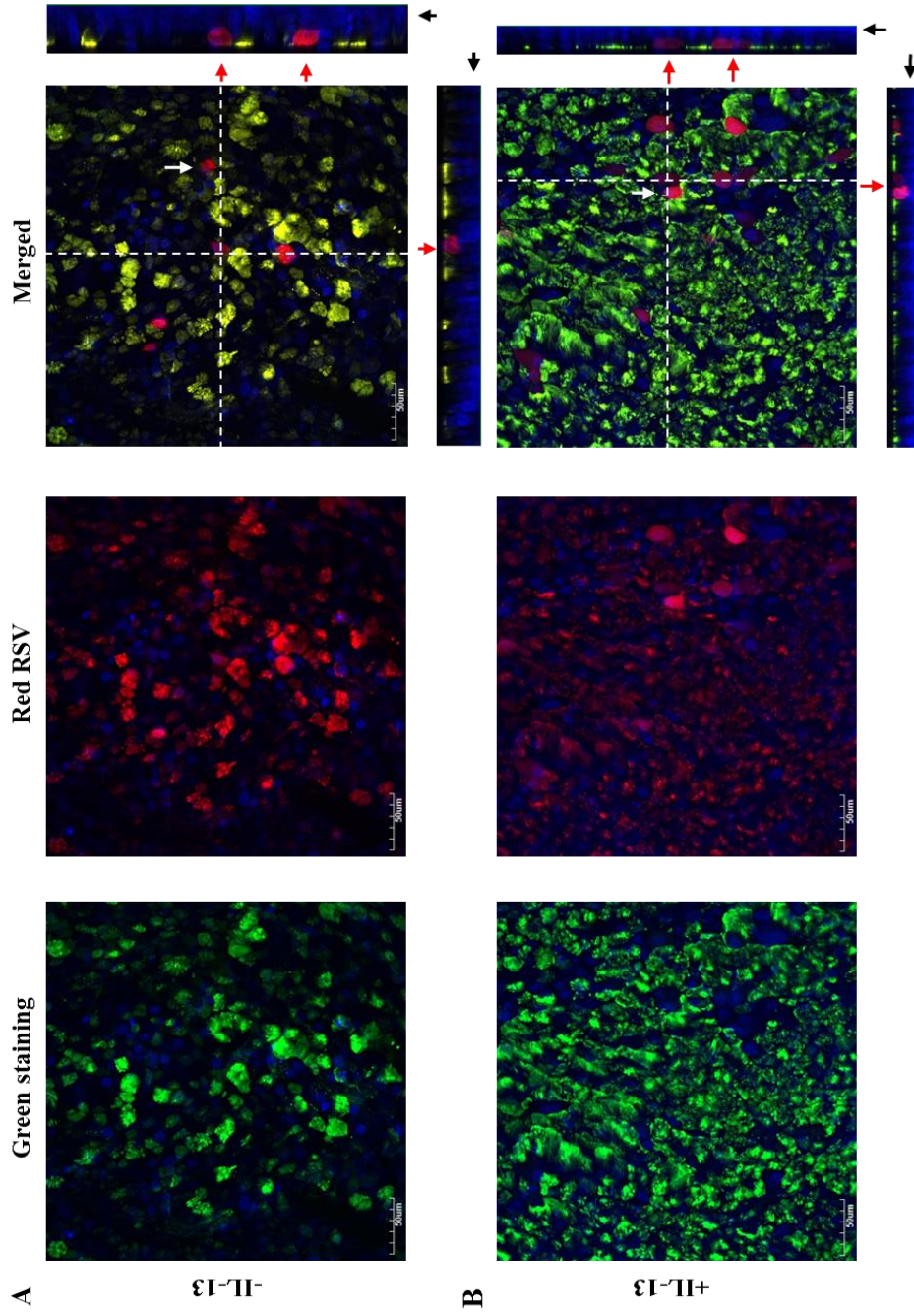


Figure 5.2: Immunostaining for mucus in cultures infected with RSV for 5 days

Cells cultured in the absence and presence of IL-13 were infected with red recombinant RSV. Red fluorescence shows infected cells, blue fluorescence shows nuclei staining with DAPI and green fluorescence shows staining for MUC5AC. Black arrows show nuclei staining and indicate the location of basal cells. The white arrow shows overlap of MUC5AC green staining and red infected cells. Red arrows show this overlap in the side views. (Images from Donor 1 cells, representative of n=3).

To obtain a snapshot of the cytokine profile produced by infected cultures, apical washes from day 4 and 5 of the RSV infected and control wells were combined and cytokine levels were assessed (Figure 5.3). The arrays were all performed at the same time and densitometry was performed on a single exposure of the films to allow comparison of the infected cells with controls. Washes from cells cultured in the absence of IL-13 showed increases in G-CSF (8-fold), IL-1ra (6-fold), and IL-6 (4-fold) alongside induction of IP-10 (Figure 5.4A). Washes from the RSV infected cells cultured in the presence of IL-13 showed induction of RANTES as well as IP-10, alongside increases in G-CSF (2.75-fold), GM-CSF (1.5-fold), IL-6 (1.5-fold) and MIF (2-fold) (Figure 5.4B) although the magnitude of these increases was less than was seen in the IL-13 negative cultures.

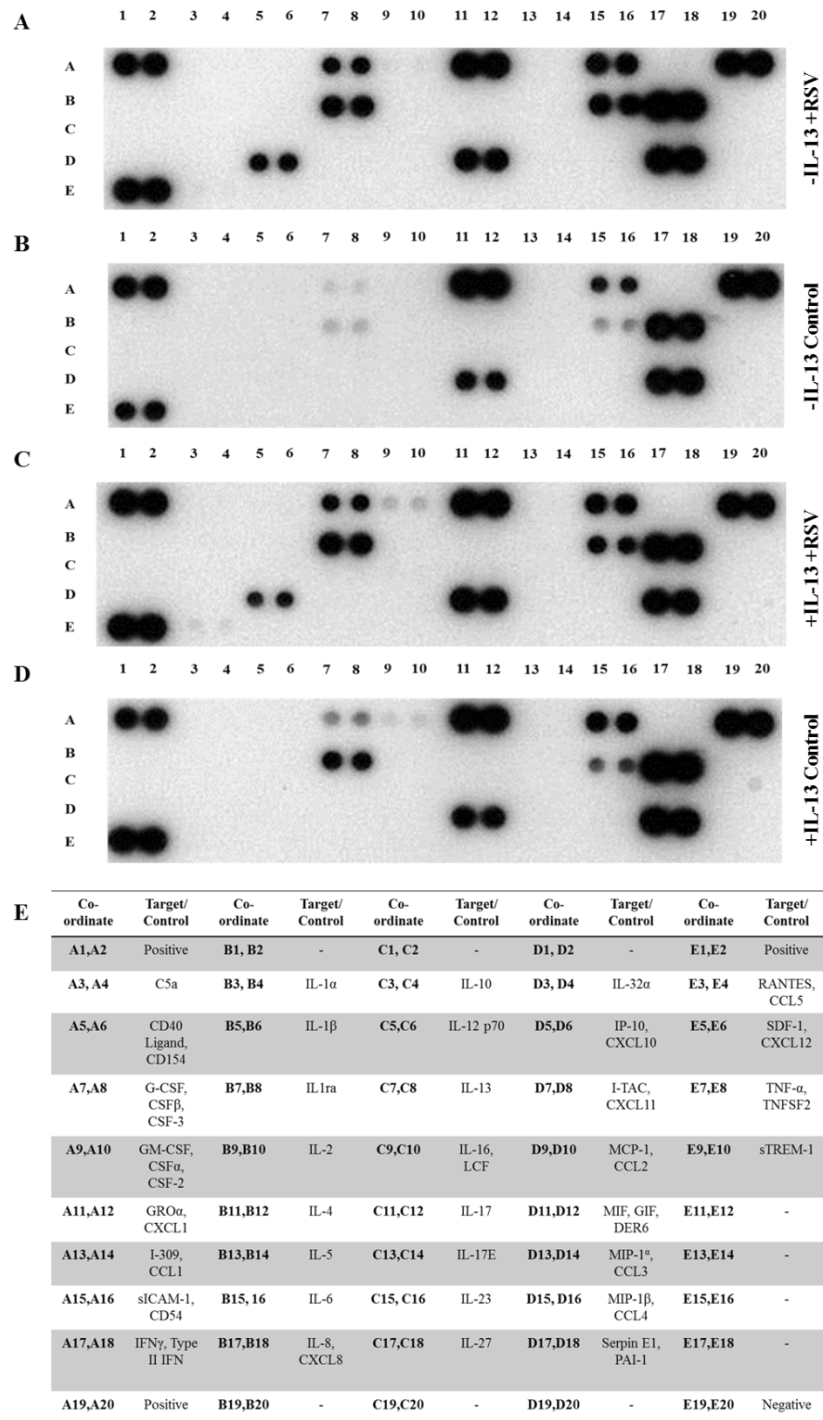


Figure 5.3: Cytokine Array of RSV infections

Cells cultured in the absence (A and B) and presence (C and D) of IL-13 were infected with RSV (A and C) for 5 days. Part E shows the data table for the cytokine array. (Secretions from infections of Donor 1 cells) (n=1).

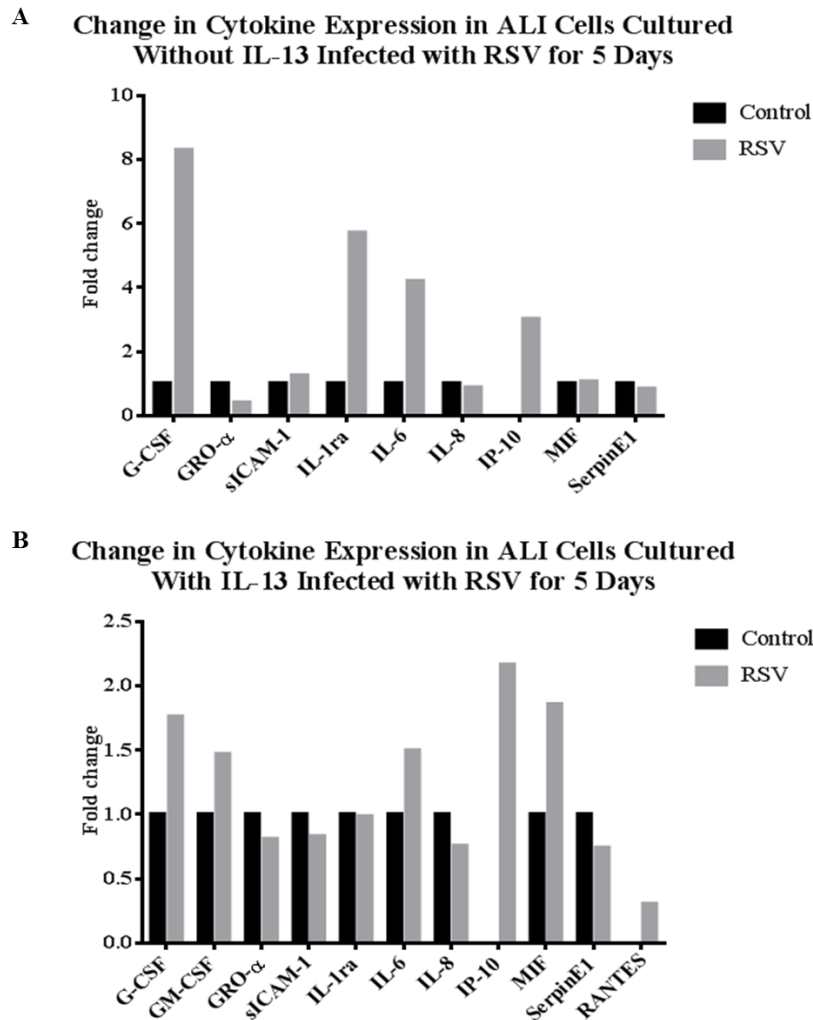


Figure 5.4: Densitometry performed on Cytokine Array of RSV infections

Cells cultured in the absence (A) and presence (B) of IL-13 were infected with RSV for 5 days and washes used to perform a cytokine array (Figure 5.3) and densitometry analysis performed to quantify and show the differences between cytokine responses (n=1).

5.3.2. Infection of ALI cells with NTHi

Infections of ALI cells with NTHi were performed to attempt to establish chronic infections on the surface of the cells with the ultimate aim of developing biofilms. To directly examine the effect of surface secretions on infection and biofilm formation experiments were performed with the apical surface of cell cultures being washed either 24 hours or immediately prior to the infection of the cultures.

Preliminary studies determined numbers of NTHi required to establish infections on the ALI cells. We used the same doses (MOI of 5, 25 and 50) and time points (1 hour, 3 days and 7 days) as in the cell line infection studies outlined in Chapter 3. After infection with a GFP-tagged strain of NTHi confocal microscopy allowed visualisation of resulting infections.

All doses of NTHi used to infect cells in the presence and absence of apical secretions were compared at 7 days (Figure 5.5). There were visible differences between the two conditions with an MOI of 5 (Figure 5.5A and B). The presence of apical secretions appeared to have had an effect on rates of infection, with many fewer green bacteria being present in the infections performed in the absence of secretions. This suggested the absence of mucus may have prevented the NTHi from establishing a successful infection at this lower dose. This difference was much less noticeable in the MOI of 25 (Figure 5.5C and D) and 50 (Figure 5.5E and F) infections with images showing presence of higher numbers of individual bacteria. Thus the MOI of 25 was used as the representative dose for presenting imaging data throughout the remainder of this chapter.

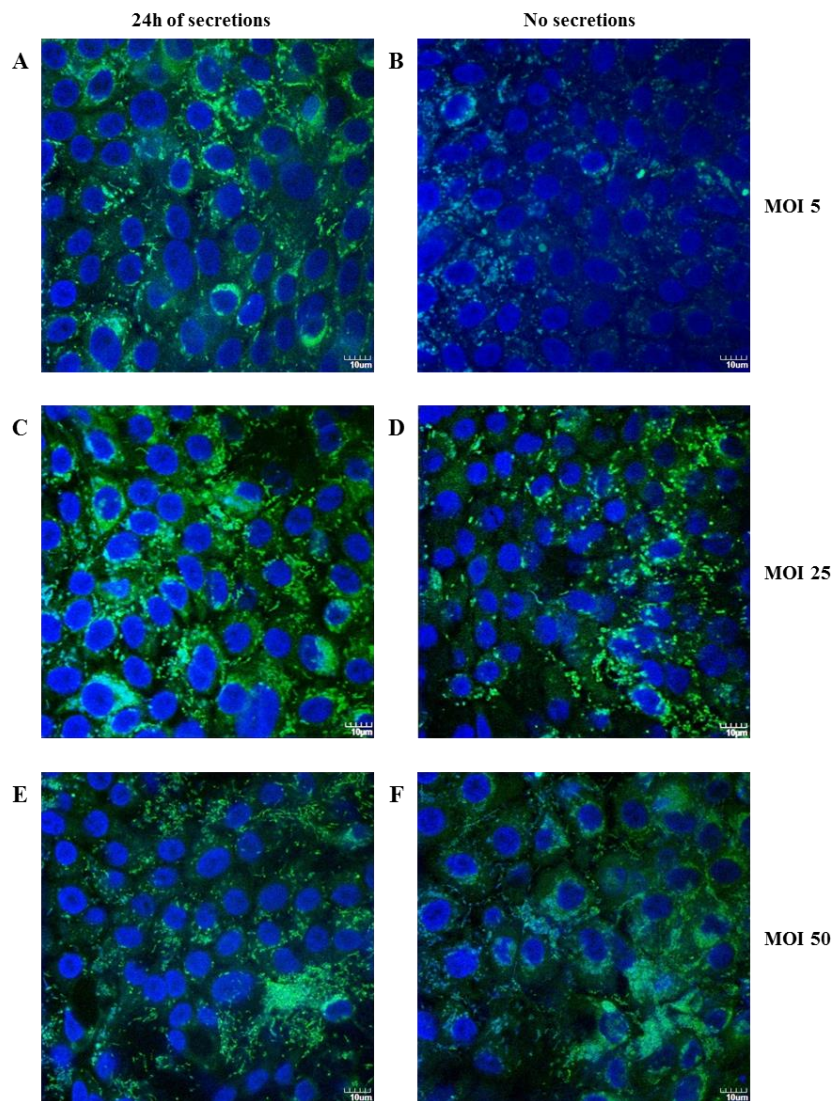


Figure 5.5: NTHi infections of ALI cells cultured with and without surface secretions with three doses of NTHi for 7days

ALI cells were infected with a GFP-tagged strain of NTHi at an MOI of 5 (A and D), 25 (C and D) and 50 (E and F) for 7 days. The apical surface of cells was washed either 24h (A, C and E) or immediately prior (B, D and F) to infection. Nuclei are stained blue with DAPI, and green fluorescence confirms the presence of bacteria in infected wells. (Images from Donor 3, representative of n=3 infections, including Donors 4 and 5).

Using representative images from infections at MOI of 25 we showed the progression of the infection through the selected time points of 1 hour (Figure 5.6A-C), 3 days (Figure 5.6D-F) and 7 days (Figure 5.6G-I); we utilised the one hour time point to check for viable bacteria. As we were interested in chronic infections and biofilm formation we also utilised longer time points for infections. Green fluorescence showed the presence of small amounts of bacteria at 1 hour, which increased by 3 days and further increased by 7 days. As expected uninfected control wells showed no green fluorescence. DAPI staining of the nuclei showed that the majority of cells remained in the cultures at day 7, suggesting that the confluent layer of cells was maintained throughout. There was no significant difference in the amount of bacteria between infection of the cells washed immediately prior to infection and those with apical secretions (mucus, washed 24 hours before infection), however analysis of the green fluorescence channel showed an increase in green fluorescence in cells infected with NTHi for 7 days in the presence of apical secretions (Figure 5.6J).

Bacterial viability was assessed 7 days after infection using apical washes from all 3 MOIs (Figure 5.7). There was no significant difference and the presence of apical secretions on the cultures prior to infection also had no influence on viable bacteria counts.

Infection did not have a significant impact on cell viability or bacterial numbers, therefore additional experiments were performed in which the infection was extended to 10 (Figure 5.8A-C) and 14 days (Figure 5.8D-F). Representative images with MOI 25 are shown (Figure 5.8). Green fluorescence at the 10 and 14 day time points was clearly below that seen in the shorter infections. DAPI staining of nuclei showed that cell numbers were reduced, with spaces being visible in the 10 day image and this was more apparent in the 14 day image when compared to the mock infected control.

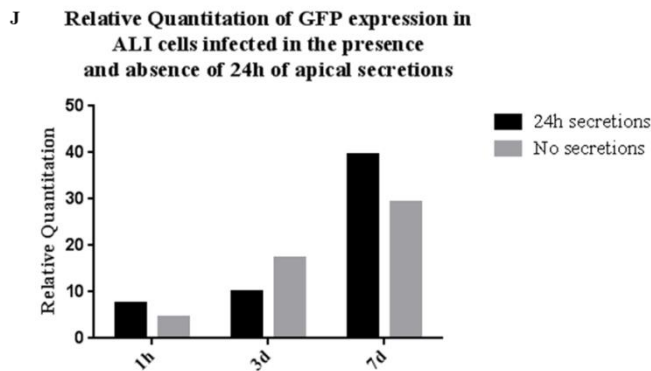
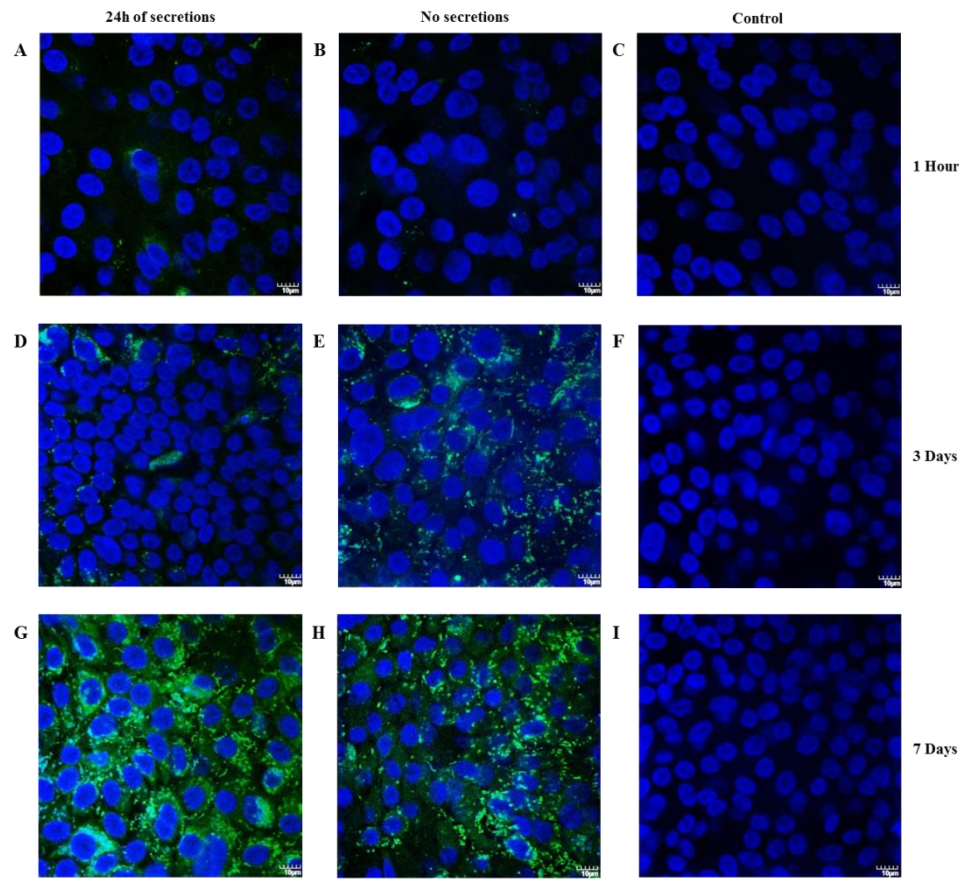


Figure 5.6: NTHi infections of ALI cells cultured with and without surface secretions for 1hour, 3days and 7days

ALI cells infected with a GFP-tagged strain of NTHi for 1h (A-C), 3d (D-F) and 7d (G-I). The apical surface of cells was washed either 24h (A, D and G) or immediately prior (B, E and H) to infection. Nuclei are stained blue with DAPI, and green fluorescence confirms the presence of bacteria in infected wells. There was no green fluorescence in the controls (C, F and I). Images are from wells infected with an MOI of 25. Mean green fluorescence was measured using ImageJ to quantitate differences between the presence and absence of apical secretions (J). (Images from Donor 3, representative of n=3 infections, including Donors 4 and 5).

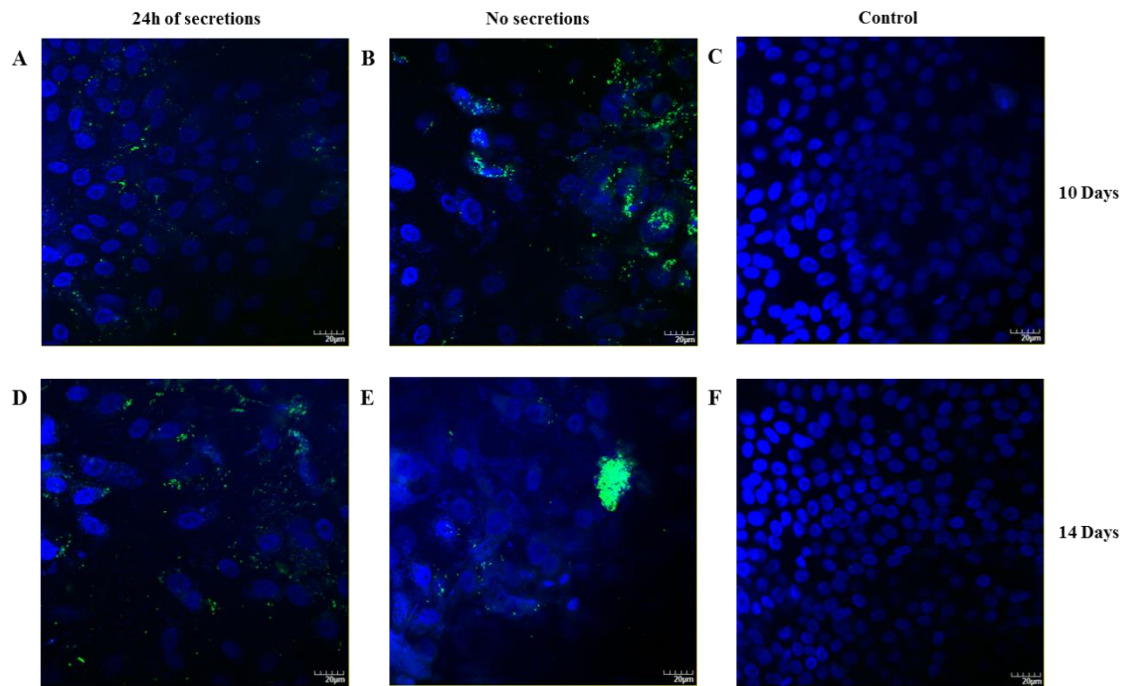


Figure 5.7: NTHi infections of ALI cells after 14 days

ALI cells were infected with a GFP-tagged strain of NTHi for an additional 10 (A-C) and 14 days (D-F). The apical surface of cells was washed either 24h (A and D) or immediately prior (B and E) to infection. Nuclei are stained blue, and green fluorescence confirms the presence of bacteria in infected wells. There was no green fluorescence in the controls (C and F). Images are from wells infected with an MOI of 25. (Images from infection of Donor 5 cells for n=1).

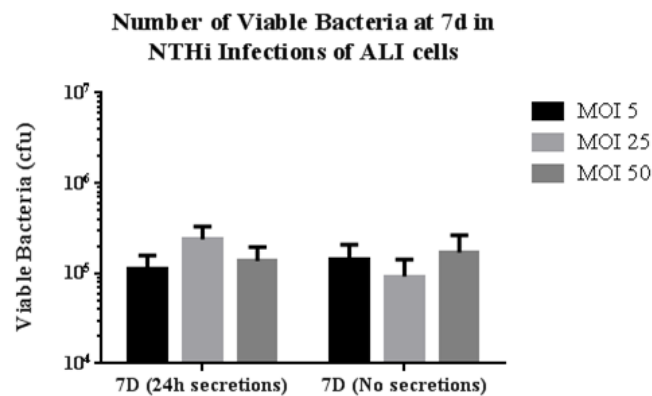


Figure 5.8: Viable Counts of 7 day NTHi infections of cells infected with and without removal of apical secretions

Viable counts were taken for each of the three doses, MOI 5, MOI 25 and MOI 50 at 7 days, from infections that were washed 24 hours and immediately prior to infection (n=3). Errors bars show SEM.

Bacterial viability was also determined in the 10 and 14 day cultures for all 3 MOIs with and without apical secretions prior to infection (Figure 5.9). Bacteria remained viable until 14 days of infection; however there is some variance between the doses by this time point, especially in those with 24 hours of apical secretions prior to infection and bacterial numbers reduced in comparison to the 7 day cultures.

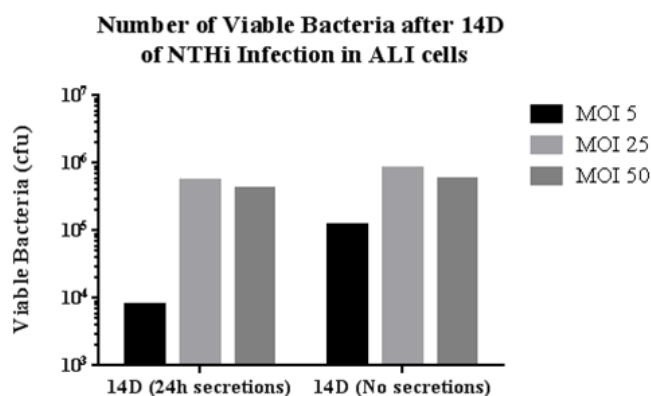


Figure 5.9: Viable bacterial counts of NTHi after infections for 14 days with and without secretions

Viable counts were taken for each of the three doses, MOI 5, MOI 25 and MOI 50 at 14 days, from infections that were washed 24 hours and immediately prior to infection (n=1).

To compare the responses elicited from the different airway models, washes from 7 day infections of ALI cells and the A549, NCI-H292 and uHBE cells with GFP-tagged NTHi were used to perform a cytokine array. Control samples were not analysed in this array as this was a comparison of the complexity of the responses elicited in response to NTHi. In the infection of A549 cells (Figure 5.10A) GM-CSF, GRO- α , IL-8, MIF and SerpinE1 were detected whereas infection of NCI-H292 (Figure 5.10B) shows the presence of GRO- α , IL-1ra, IL-8, MIF and SerpinE1. Infection of uHBE (Figure 5.10C) cells results in increased GM-CSF, GRO- α , sICAM-1, IL-1ra, IL-6, IL-8, MIF and SerpinE1. Infection of ALI (Figure 5.10D) cells show presence of G-CSF, GM-CSF, GRO- α , sICAM-1, IL-1ra, IL-6, IL-8, IP-10, MIF and SerpinE1 showing the more complex responses elicited by differentiated cells.

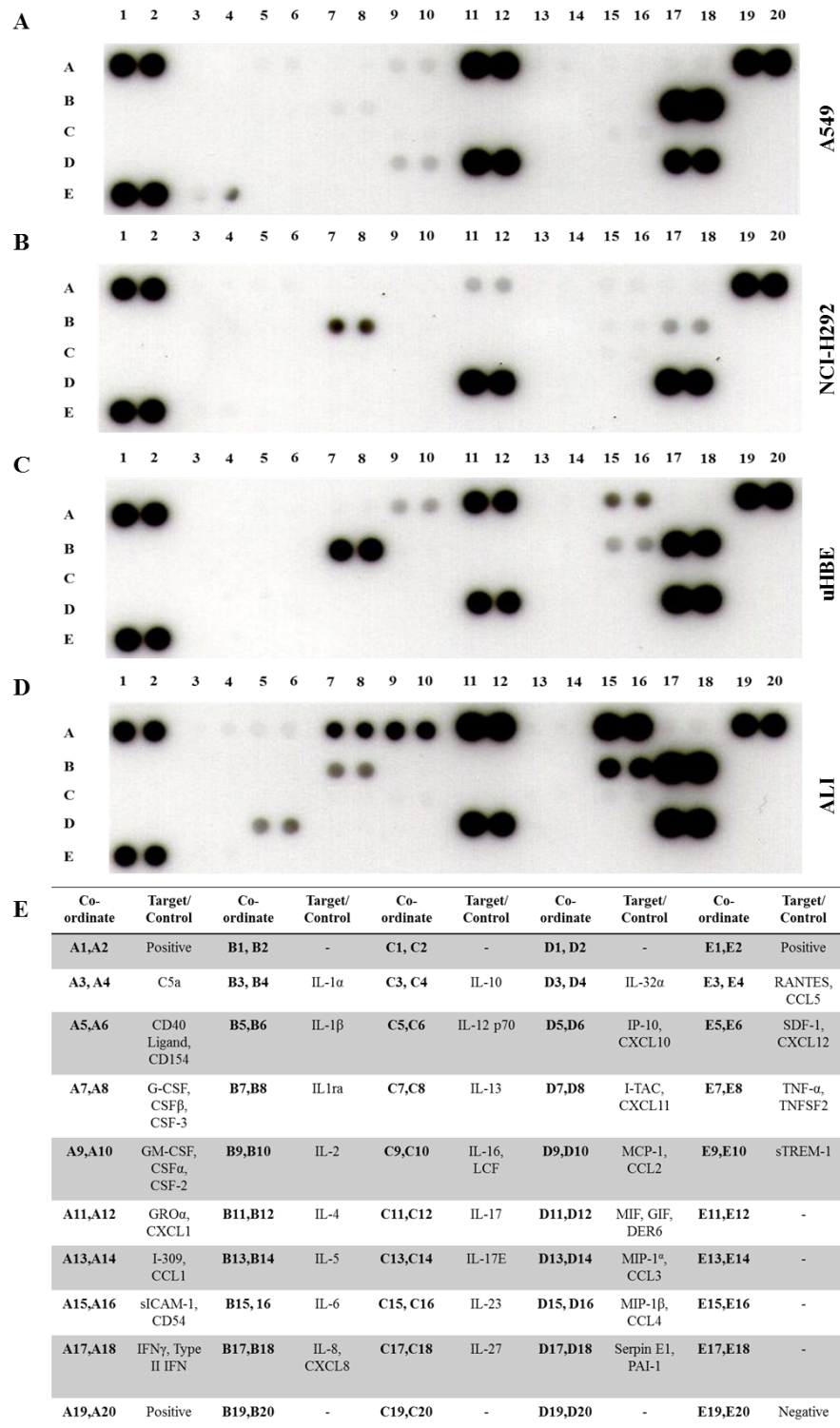


Figure 5.10: Cytokine Array of washes from all 4 different cell types infected with NTHi for 7 days

A549 (A), NCI-H292 (B) uHBE (C) and ALI (D) cells were infected for 7 days with NTH and washes used to perform a cytokine array. (Donor 3 ALI and uHBE cells) (n=1).

The GFP strain used in these studies is a well-established experimental strain but in order to investigate inter-strain differences a clinical isolate was used to infect ALI cultures for 7 days following differentiation in the absence (Figure 5.11B and C) and presence of IL-13 (Figure 5.11D and E) and cytokine array performed. The clinical isolate was provided by the Medical Microbiology department at the Royal Hallamshire Hospital, Sheffield. The isolate had been identified as part of a diagnostic work-up in a single patient and sub-cultured and a frozen stock prepared. We were provided with a plate cultured from this frozen stock. As in previous experiments the arrays were all performed at the same time and densitometry (Figure 5.12) was performed on a single exposure so as to compare the infected cells with the controls. The cytokine array performed using GFP-tagged NTHi (from Figure 5.10) is included in this figure for comparison. Cells cultured in the absence of IL-13 infected with a clinical isolate of NTHi (Figure 5.11B) shows the induction of GM-CSF, IL-1 α , IL-1 β and IP-10 as well as all cytokines identified in the control (Figure 5.11C). Densitometry analysis confirmed this and showed a large increase in G-CSF and sICAM-1 and a small increase in IL-1ra (Figure 5.12A). Infected cells cultured in the presence of IL-13 (Figure 5.11D) show the induction of G-CSF, sICAM-1, IL-1 α and IL-1 β alongside all cytokines identified in the control (Figure 5.11E). Densitometry analysis confirmed this and showed a large increase in MIF expression (Figure 5.12B). Comparing the responses elicited by the clinical isolate (Figure 5.11B) and the GFP-tagged strain (Figure 5.11A) of NTHi showed some similarities, with the presence of G-CSF, GM-CSF, GRO α , sICAM-1, IL-1ra, IL-8, IP-10 and MIF common to both strains; however comparisons with the controls for the clinical isolate show GM-CSF and IP-10 to be induced. When comparing differences, the GFP strain shows the presence of IL-6 as part of the response it stimulates; however there was no control for this as the cytokine array was conducted to compare the responses of the differing cell types so it may not be a response to the GFP strain, but IL-6 is not present in the -IL-13 control for the clinical isolate cytokine array either. The clinical isolate stimulated a very robust induction of IL-1 α , IL-1 β and IL-1ra.

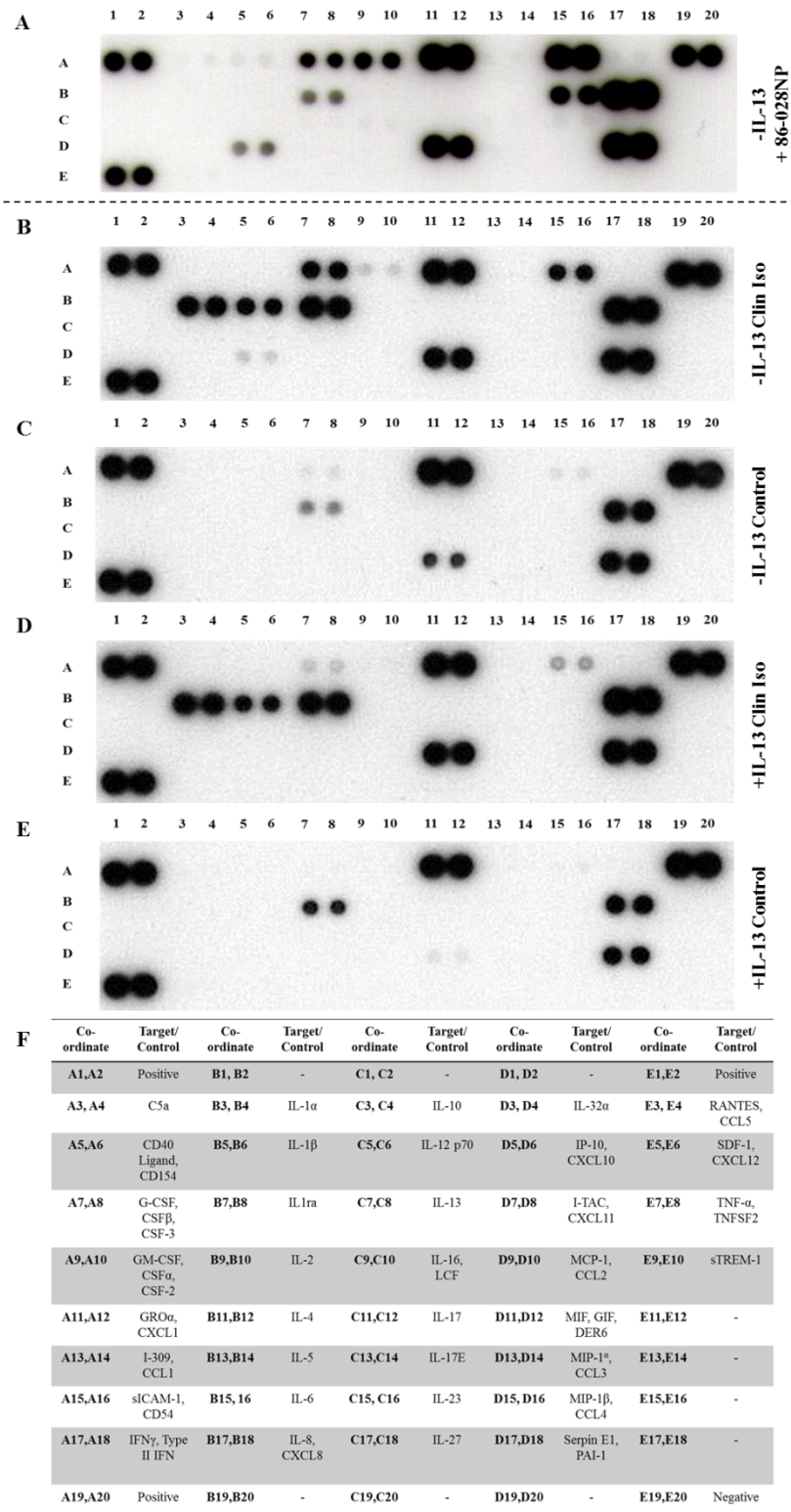
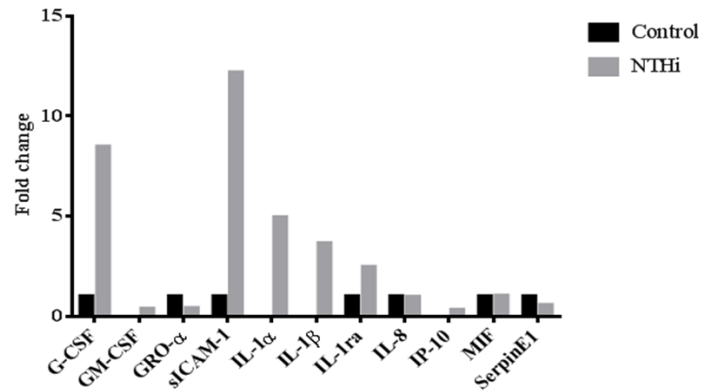


Figure 5.11: Cytokine Array of washes from ALI cells infected with a clinical isolate of NTHi for 7days

Cells cultured in the absence (B and C) and presence (D and E) of IL-13 were infected with a clinical isolate of NTHi (B and D) for 7 days. The cytokine array from ALI cell infected with GFP-tagged NTHi (Figure 5.10) is shown for comparison (A). The data table for the cytokine array is also shown (F). (Donor 2 ALI cells) (n=1).

A Change in Cytokine Expression in ALI Cells Cultured Without IL-13 Infected with a Clinical Isolate of NTHi for 7 Days



B Change in Cytokine Expression in ALI Cells Cultured With IL-13 Infected with a Clinical Isolate of NTHi for 7 Days

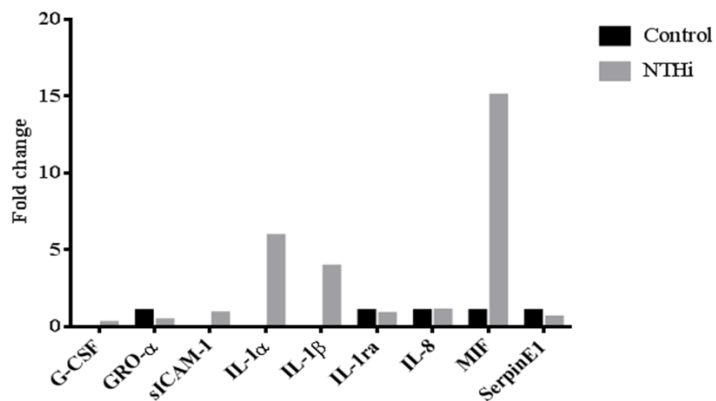


Figure 5.12: Densitometry performed on Cytokine Array of Clinical Isolate NTHi infections

Cells cultured in the absence (A) and presence (B) of IL-13 were infected with a clinical isolate of NTHi for 7 days and washes used to perform a cytokine array (Figure 5.11) and densitometry analysis performed to quantify and show the differences between cytokine responses (n=1).

5.3.3. Base line cytokine profile comparison in ALI cells differentiated in the absence and presence of IL-13

The control cytokine array data from RSV and clinical isolate infections of ALI cells differentiated in the absence and presence of IL-13 enabled comparison of the cytokine profiles exhibited by these two culture conditions. Additionally, the RSV infection was performed in cells from Donor 1 and the NTHi infection in cells from Donor 2 allowing comparisons between donors. Donor 2 (Figure 5.13B) showed a more complex baseline cytokine profile than donor 1 (Figure 5.13A), with donor 2 expressing GM-CSF and IL-6 in addition to G-CSF, GRO- α , sICAM-1, IL-1ra, IL-8, MIF and SerpinE1. The graphs for both donors showed an increase in IL-1ra and decreases in GRO- α and MIF expression, although the decrease in MIF expression was higher in donor 1. In contrast to each other, IL-8 and SerpinE1 appear to decrease upon addition of IL-13 in donor 2, whereas it increases donor 1. Donor 2 showed a complete induction of GM-CSF in cells cultured with in the presence of IL-13. It also showed induction of G-SCF and sICAM-1, however in donor 1, these two cytokines are only expressed in cells cultured in the absence of IL-13. The two data sets were grouped together (Figure 5.13C) to show the average relative expression of cytokines the two donors have in common.

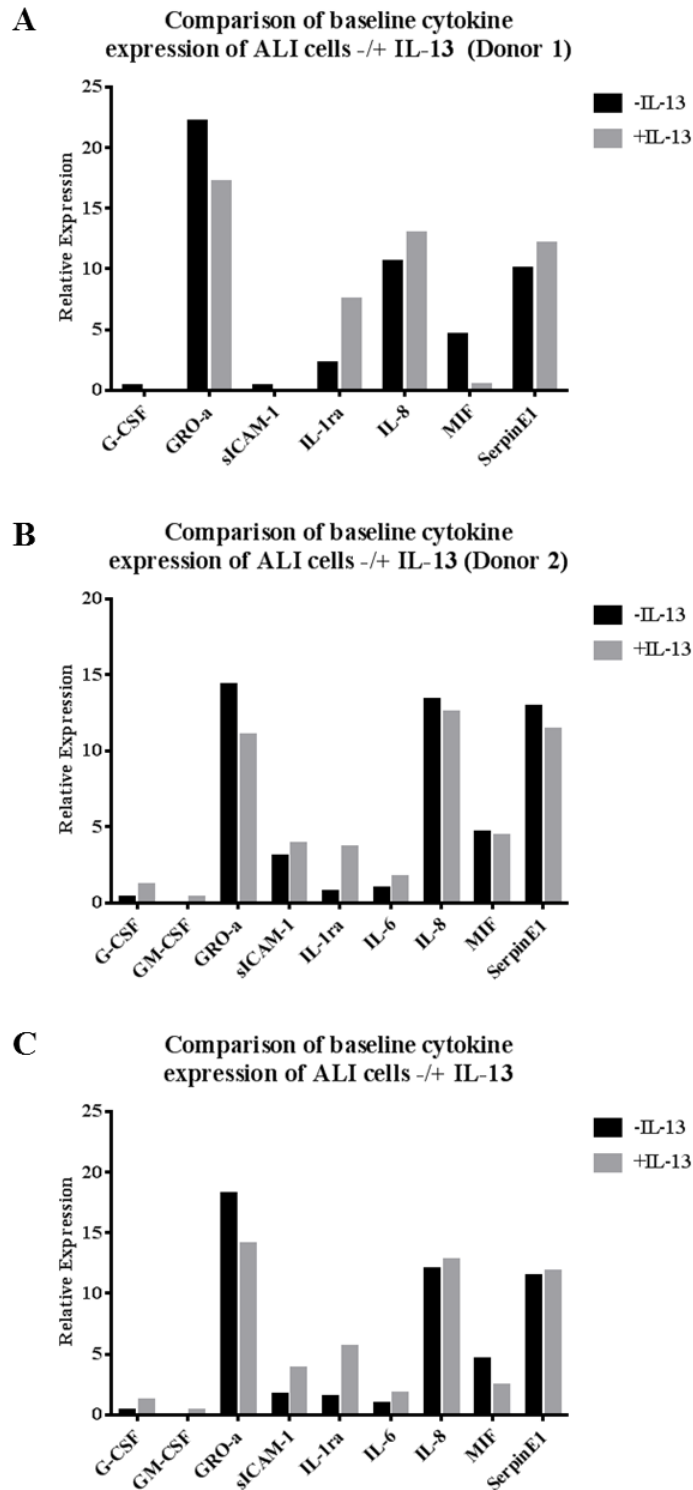


Figure 5.13: Comparison of Baseline Cytokine expression in ALI cells cultures in the absence and presence of IL-13

Densitometry performed on control cytokine array data for ALI infection from Donor 1 (A) and Donor 2 (B) was compared to examine the differing cytokine profiles between cells culture in the absence and presence of IL-13. The values were averaged (C) for further comparison. (n=2 datasets).

5.3.4. Investigating the proteolytic cleavage of PLUNC markers in secreted products

Analysis of the NTHi viability following longer periods of infection showed that both bacterial numbers and cell viability were reduced suggesting the innate defences of the cells were likely impaired following prolonged periods of infections. NTHi possesses an IgA1 protease which may be utilised to evade host defence mechanisms by cleaving host defence proteins. We therefore utilised the readily accessible PLUNC proteins as markers to study proteolytic cleavage of immune molecules using apical secretions from cells following NTHi exposure. These samples came from our long-term infections and through direct interaction for shorter time periods. We initially began these experiments with an examination of exogenous proteases as we wanted to see how PLUNC proteins could be broken down by trypsin and neutrophil proteases. These would then act as controls for studying degradation by NTHi proteases.

5.3.4.1. Investigating the proteolytic activity of neutrophil products and trypsin

In order to investigate the effect of neutrophil proteolytic activity on SPLUNC1, human peripheral blood neutrophils were lysed by multiple freeze-thaws in either PBS or in PBS containing a pan-protease inhibitor solution. An ALI apical secretion sample was incubated with the equivalent of approximately 2.5×10^5 lysed neutrophils for 15 and 60 minutes to examine the effect of the proteolytic activity of whole neutrophil lysate. These samples were then separated by SDS-PAGE and Western blotted for SPLUNC1 using both antibodies we generated (to two different epitopes) SPLUNC1A (Figure 5.14A) and SPLUNC1B (Figure 5.14B). Probing with SPLUNC1B showed a reduction in full-size protein as time progressed, however there was no indication of cleavage fragments to explain the reduction. This was not the case for the blots probed with SPLUNC1A antibody, where the reduction in full size protein coincided with an increase in the number of smaller bands which are likely to be fragments of degraded protein. Neutrophils lysed in the pan-protease inhibitor appeared to cleave the proteins much more slowly, with cleavage fragments appearing at T=60min (Figure 5.14A) alongside a reduction in full-size protein. This was most clearly seen in the blots probed with the SPLUNC1B antibody (Figure 5.14B). The different antibodies recognise different antigens in either the C- or N- terminus of the protein, which is reflected in the difference in the appearance of cleavage products.

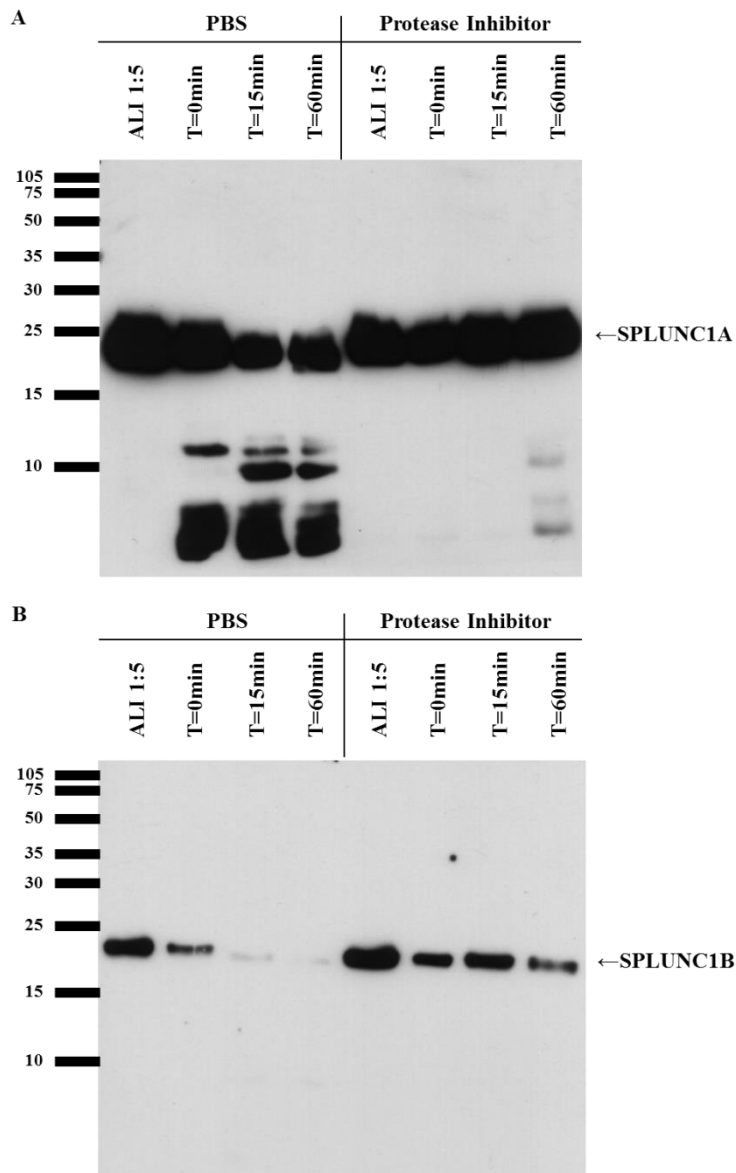


Figure 5.14: Incubation of ALI secretion with Neutrophil Lysate to examine proteolytic degradation

An ALI sample provided by Synairgen was incubated with 250000 neutrophils lysed by repeated freeze-thawing in PBS or pan-protease inhibitor solution for 0, 15 and 60 minutes. These samples were then Western blotted alongside a 1:5 dilution of the ALI sample and probed with the antibodies generated by our lab against SPLUNC1. Replicate blots were probed with SPLUNC1A (A) and SPLUNC1B (B) (n=1).

Trypsin is a naturally occurring protease found in the digestive system, produced by the pancreas, where it functions to break down proteins as part of protein absorption in the digestive system. It is routinely used as a proteolytic agent in experimental studies. Therefore, we decided to use this as an alternative proteolytic agent to investigate the cleavage of LPLUNC1 and SPLUNC1. ALI samples were incubated with trypsin for 20, 40 and 60 minutes, and Western blotted with antibodies against SPLUNC1 and LPLUNC1 (Figure 5.15). The LPLUNC1 blot (Figure 5.15A) showed a small reduction in size whilst still maintaining the double band representing glycosylated protein. Levels of the cleaved product appeared to remain stable for up to 60 minutes of treatment. The SPLUNC1 blot (Figure 5.15B) showed bands for full size protein in all samples, with band intensity decreasing as incubation was extended. This decrease in intensity was coupled with increased intensity of smaller bands of cleavage fragments.

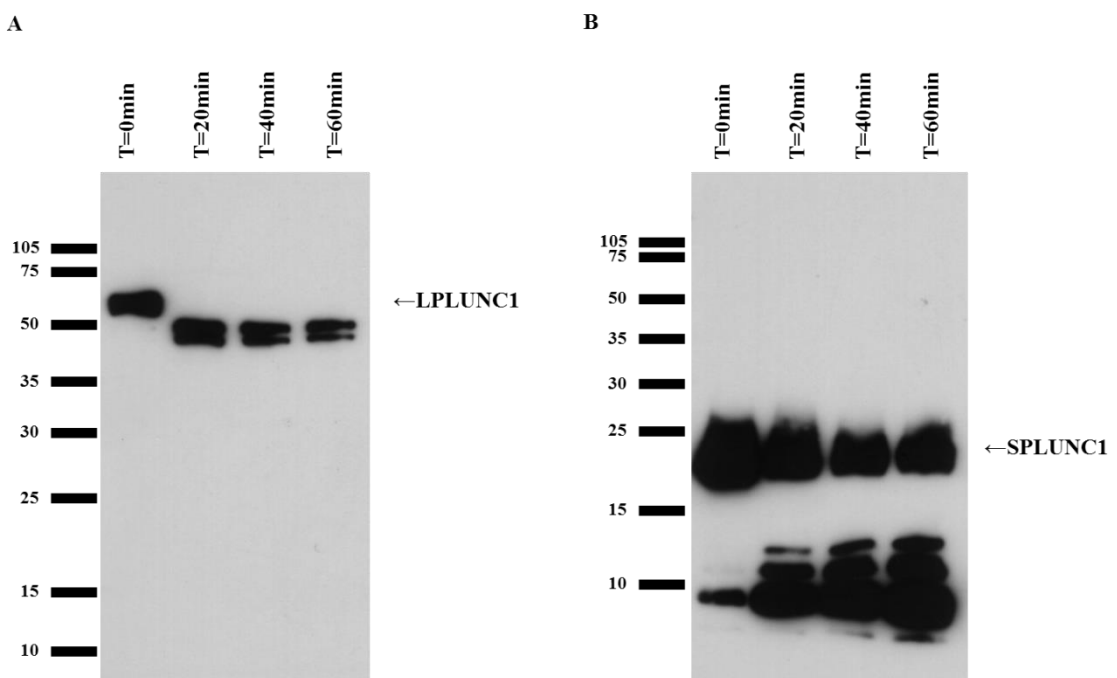


Figure 5.15: Incubation of ALI secretion with trypsin to examine proteolytic degradation

An ALI sample provided by Synairgen was incubated with trypsin for 20, 40 and 60 minutes. Samples were then Western blotted and probed with antibodies against LPLUNC1 (A) and SPLUNC1 (B) (n=1).

5.3.4.2. Investigating the proteolytic activity of NTHi

To directly study the proteolytic effects of NTHi products on airway secretions we incubated apical secretions from non-infected differentiated ALI cultures with approximately 200000 cfu NTHi for 1, 4 and 24 hours. These samples were then separated using SDS-PAGE and Western blotted for LPLUNC1 (Figure 5.16A) and SPLUNC1 (Figure 5.16B). The 0 hour time point was taken prior to addition of NTHi and contains bands at the appropriate size for both LPLUNC1 and SPLUNC1. These results (Figure 5.16) show significant bands at small sizes which appear to be degradation products. Only one dose and three time points were chosen to gain a quick insight into the effects of incubating NTHi with airway secretions. There are also faint larger bands present in the blots (approx. 55-60kDa), which appear to be non-specific; however these could be a result of PLUNC proteins binding to bacterial products as SPLUNC1 has been shown to bind LPS (Zhou, Li et al. 2008). SPLUNC1 has also been shown, through examination of its crystal structure (Garland, Walton et al. 2013), to exist as a dimer (PDB Structure ID: 4KGG/4KGO), and the large bands (approx.55kDa) appear to be at the appropriate size for this to be a potential explanation.

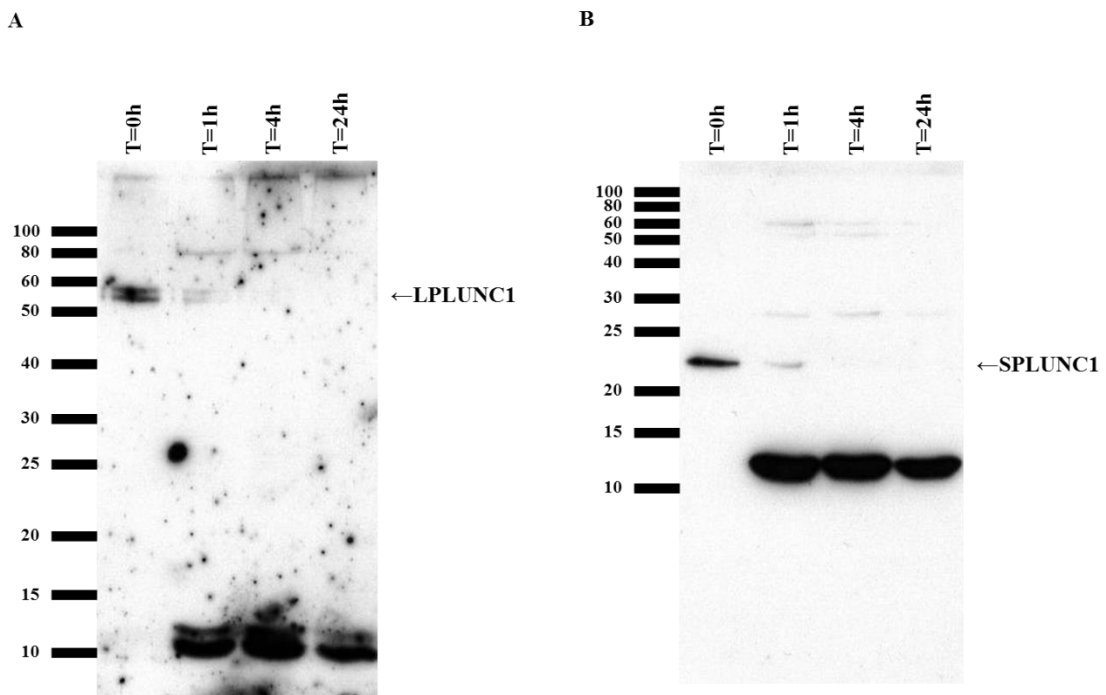


Figure 5.16: Incubation of ALI secretion with NTHi to examine proteomic degradation

Apical secretions incubated with NTHi for 1, 4 and 24h show bands at the appropriate size for LPLUNC1 (A) and SPLUNC1 (B) in the T=0h time point. Bands in the other samples are degradation products (n=1).

We had previously used ALI samples, provided by Synairgen, in other studies in our lab and found them to contain higher levels of total protein in a smaller volume than the samples we had generated during our own ALI cultures. We used one of these samples to generate more intense bands and increase the chance of seeing full-length protein alongside degradation products as there were more proteins to degrade. Also, in an attempt to combat the rapid loss of full-length protein, (Figure 5.16) and so that we could study the effects of the bacteria on protein degradation, we used different numbers of bacteria for a set time. We incubated approximately 1000, 2000, 5000, 10000, 25000 and 50000 cfu NTHi with secretion for 1 hour. As previously the 0 hour time point was taken prior to addition of NTHi. The samples were separated using SDS-PAGE and Western blotted for LPLUNC1 (Figure 5.17A) and SPLUNC1 (Figure 5.17B). The bands in the T=0h lanes show bands of the correct size for LPLUNC1 and SPLUNC1. As the number of bacteria used increases, a proteolytic band appears in both at approximately 10kDa increased in density as the bacterial numbers increase. Taken together, these blotting results show that both SPLUNC1 and LPLUNC1 are targets for

proteolytic degradation and that both are directly degraded by NTHi derived proteases. Furthermore, secretions from infected ALI cultures appear to contain predominantly degraded SPLUNC1 and LPLUNC1 indicating that unrestrained proteolytic activity is present within the cultures.

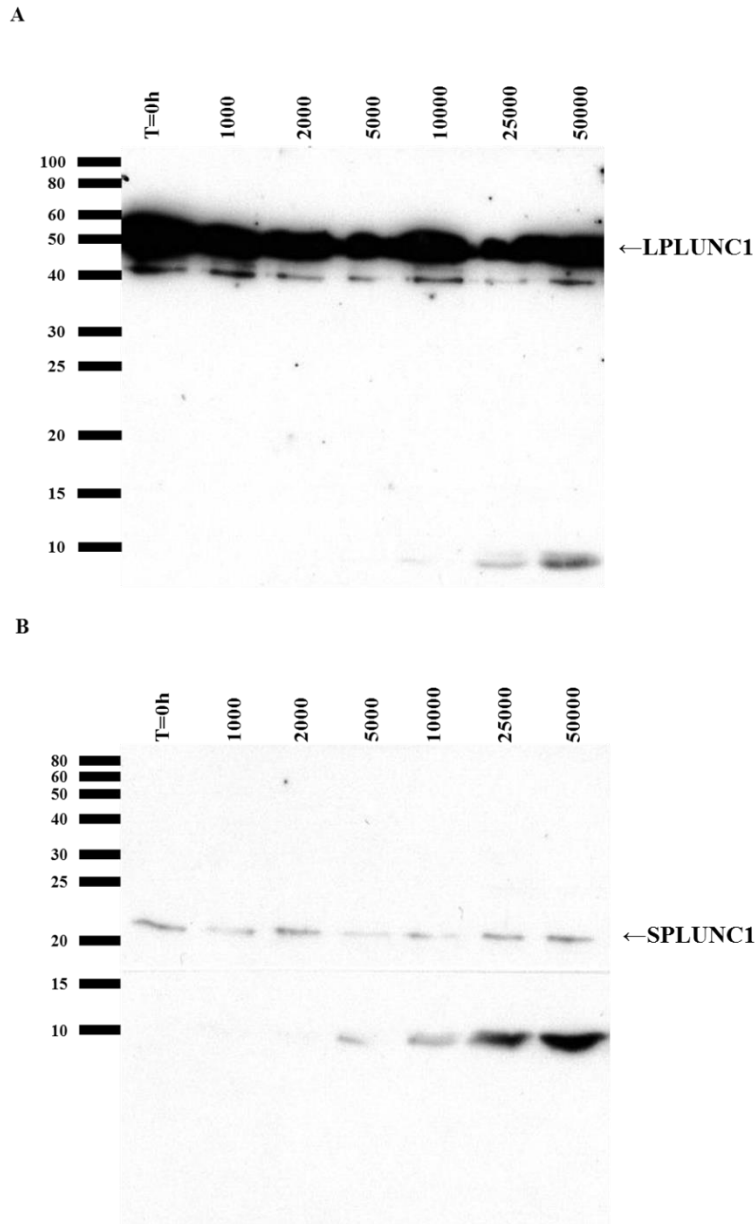


Figure 5.17: Incubation of an ALI secretion with NTHi to examine proteomic degradation

Apical secretion from Synairgen, incubated with varying numbers of NTHi for 1 hour and Western blotted for LPLUNC1 (A) and SPLUNC1 (B) show bands at the appropriate size for both proteins (n=1).

5.3.5. Establishing co-infection of ALI cells with RSV and NTHi

RSV and NTHi can both be associated with co-infection or secondary infection, particularly in diseased airways, and this may result in severe exacerbations of symptoms. It has been suggested that RSV enhances NTHi attachment in the airway through the use of fimbria-binding receptors on the outer surface membrane of the NTHi (Jiang, Nagata et al. 1999). As we had successfully established individual infections, the next logical step was to use 21 day differentiated transwells cultured in the absence of IL-13 to attempt establishment of a co-infection.

Single pathogen infections were performed alongside the co-infections to act as controls (Figure 5.18A and B). 21 day ALI cells were infected with RSV for 3 days, prior to a secondary infection with NTHi for 3 days (Figure 5.18C) and 7 days (Figure 5.18D). The presence of red fluorescence shows successful establishment of an RSV infection and green fluorescence shows that NTHi are present. Nuclei were counter-stained with DAPI when mounted. There was a large amount of destruction in the control RSV infection (Figure 5.18A) with the presence of RSV alongside what appear to be fragments of nuclei. The control NTHi infection (Figure 5.18B) showed clustering of bacteria, however there are spaces beginning to form in the cell layer. There was a large amount of destruction in co-infection wells, with very few cells remaining.

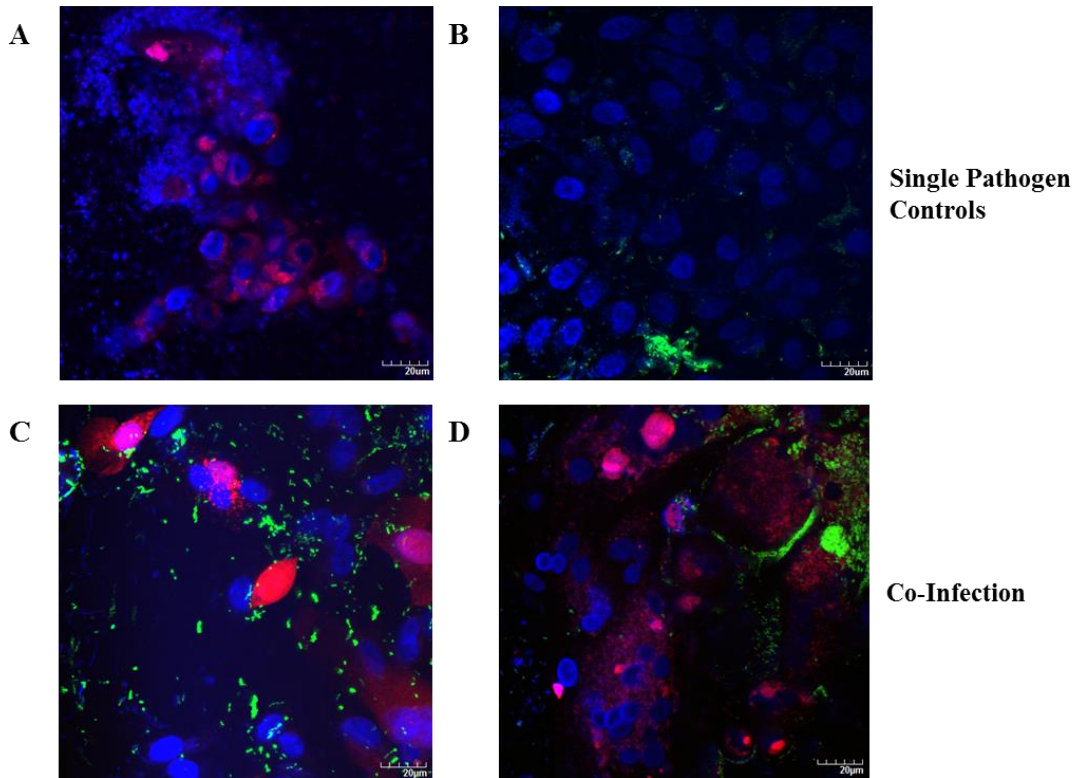


Figure 5.18: Co-infection of ALI cells with RSV and NTHi

Single pathogen control infections were performed in ALI cells (Donor 5) with RSV (A) and NTHi (B) for the longest combined infection period, 10 days, alongside cells which were first infected with RSV for 3 days, followed by infection with NTHi for 3 days (C) and 7 days (D). Red fluorescence shows the presence of virus infected cells. Green fluorescence shows the presence of NTHi. The blue nuclei showed positive staining with DAPI. (n=1).

5.4. Discussion

To more faithfully represent the airway epithelium *in vivo* and overcome the cell death issues experienced when using cell lines and uHBE cells outlined in Chapter 3, the aim of this chapter was to establish and characterise infection models using both RSV and NTHi in ALI-differentiated cells. Our ultimate aim was also to establish long-term infection of cultures and to attempt to establish dual infections. As described in detail in Chapter 4 the ALI cultured cells are more complex as a result of the differentiation process that results in the development of a mucociliary phenotype and the secretion of mucus. This is likely to allow the cells to mount a more complex response to foreign material. We postulated that this complexity would aid cell survival, post infection and provide a more accurate representation of the human airway.

RSV is a common respiratory pathogen, predominantly affecting children and the elderly and is a frequent cause of COPD exacerbations. Previous research has shown that RSV preferentially infects the ciliated cells of the airway epithelium (Zhang, Peeples et al. 2002; Villenave, Thavagnanam et al. 2012; Smith, Kulkarni et al. 2013) although the virus has also been shown to infect other cell types and non-differentiated cells. We monitored infection of the ALI cells with a red fluorescent tagged strain of RSV, followed by immunofluorescence staining for MUC5AC (goblet cells) and β -tubulin (ciliated cells) confirming that RSV targeted ciliated cells. Limited infection was seen in other cell types within the culture and so our data confirms that the airway model has differentiated successfully, generating ciliated and goblet cells which are secreting mucus.

We reasoned that altering the phenotype of the epithelium through the use of IL-13 treatment would result in the cells mounting a different response to viral infection. IL-13 treated ALI cultures have increased goblet cells (and reduced ciliated cell). Our cytokine array data suggested that there were limited differences between the different culture conditions. In both conditions a gene shown to be induced by viral infections, IP-10 was induced as expected, (Becker, Reed et al. 1997; Oshansky, Barber et al. 2010). A recent publication by Smith, Kulkarni et al. (2013) utilised ALI culture cells to study RSV infected cells, also showed a significant increase in IL-6 and IP-10 expression. RANTES (regulated by IL-13) was only seen in infections performed in the presence of IL-13 (Tekkanat, Maassab et al. 2002) but has previously been associated with RSV infections; one study found increased RANTES expression during RSV

infections when performed during smoke-induced COPD exacerbations in mice (Foronjy, Dabo et al. 2014) and RSV induced RANTES expression has been linked to exacerbations of airway disease with RANTES being identified in samples from patients with bronchiolitis (Hull, Rowlands et al. 2003). Another study characterised responses to RSV infection in mice and used immunohistochemistry analysis to identify high numbers of eosinophils in the lungs of RSV infected mice (John, Berlin et al. 2003). A study conducted by Bukreyev, Belyakov et al. (2001) showed that GM-CSF attenuated viral replication and increased the number of pulmonary antigen-presenting cells. Another study, using a BEAS2B cell line, showed accumulation of IL-6 and GM-CSF in infections that took place for longer than 96 hours, with IL-8 being a predominant cytokine released in the first 24 hours (Noah and Becker 1993). As these cytokine arrays were performed on cells that had been infected for 5 days, we would possibly expect to see GM-CSF in the responses of cells from both culture conditions, although it is possible that levels may have dropped by 5 days post infection, and remained for longer in the cultures differentiated in the presence of IL-13 as they are more representative of 'diseased' cells.

One of the objectives of this project was to establish long-term NTHi infections on the ALI cells with a view to using these for biofilm formation. Our work with the cell lines (presented in Chapter 3) had shown that it was not possible to establish such cultures in cell lines. This was probably because they did not mount robust enough innate defence responses to counter the effect of the infections. We were, however, able to show that longer term infections could be established in the ALI cultures. The maximum length of time infections could be tolerated by the ALI cell was around 7 days as viable bacterial counts performed using 7 day washes show that the bacteria were still viable and IF microscopy showed abundant bacteria on the cultures. Viable bacterial counts were also performed at 14 days and show more variation in viable bacteria between starting doses and the presence of secretions on the surface prior to infection. Removal of surface secretions (containing mucus and host defence proteins secreted by the cells) prior to infection did not have a major impact on the development of infection. There also was no major impact on the cell layer at this time however extending the infection period to 14 days resulted in the cell layer becoming disrupted, with gaps beginning to appear in the cell layer. Only one previous study has used differentiated airway epithelial cells for extended infection with NTHi (Ren, Nelson et al. 2012). They infected cells with NTHi for periods of up to 10 days, collected internalized bacterial numbers, used trans-

epithelial electrical resistance and performed ELISAs and electron microscopy to characterise their infections. Comparing out cytokine array data with their ELISA data we identified IL-1 β , IL-6, IL-8 and GM-CSF but no TNF- α . Out of the 5 cytokines they performed ELISA for they showed high amounts of IL-8 and IL-6 with exposures on the cytokine array showing large amounts of both cytokines. The exposures for GM-CSF and IL-1 β appear to show more GM-CSF than IL-1 β which was not the case in the ELISA data. These differences could be due to donor or culture technique variability.

Our data suggests that infecting for 7 days is best for both the cell and the bacterial survival. By 7 days the bacteria appear to cluster around cells in small patches and comparing this to biofilm studies performed on NTHi and other bacterial species, this appears to be consistent with the initiation of a biofilm structure (Starner, Zhang et al. 2006; Hall-Stoodley, Nistico et al. 2008; Ma, Conover et al. 2009; Juneau, Pang et al. 2011). Examination of the side view image from Z-stack imaging indicates bacteria are located between and below nuclei, although this appears to be in close proximity to the nucleus suggesting that they could be inside the cells. A study conducted using cell lines showed that NTHi are able to use paracytosis to migrate through epithelial layers (van Schilfgaarde, van Alphen et al. 1995); which could allow invasive infections or evasion of the immune response; NTHi has also been shown to invade the airway epithelium (Ketterer, Shao et al. 1999; Murphy and Kirkham 2002). The extended infections, of up to 10 days, performed by Ren, Nelson et al. (2012) found that NTHi is located paracellularly. As we were only able to work with limited numbers of ALI cell cultures, we were unable to performing studies to establish the location of the bacteria in the cultures. Staining the tight junctions that form when these cells differentiate with an antibody against ZO-1 would provide an outline of the cells in order to locate the bacterial location as being inside or outside of the cell.

The cytokine array data comparing infected primary and ALI cells to cells lines shows that primary and ALI cells elicit a much more complex response to NTHi infection. This might be expected as the ALI cells are more complex cultures, representing the multiple cell types seen in the airway epithelium as well as the products they secrete. Controls for these infections were not analysed by cytokine array we were unable to identify cytokines induction/up-regulation upon infection.

To investigate issues relating to the strain specific responses to infection we also used a clinical isolate of NTHi obtained from the Medical Microbiology department at the Royal Hallamshire Hospital, Sheffield. This could not be visualised in the same manner as the GFP-tagged strain so we limited our analysis to the cytokine array to study the differences in the immune response. Use of a clinical isolate elicited a more complex response from the cells than the GFP-tagged strain. The most visible differences between the strains are the presence of IL-6 in the GFP-strain infections and IL-1 α and IL-1 β in the clinical isolate samples. This data clearly shows that cellular responses differ between individual strains and the different cytokines produced suggest differences in responses by the immune system to different strains. This showed that one model is not enough; and a model needs to be generated for each strain. Some strains of NTHi are more successful at colonising the airway, and different responses may contribute to this, particularly due the variable nature of virulence factors present between strains. Laboratory strains often become attenuated and so clear differences in the number/range of cytokines elicited by the two isolates were observed. NTHi has previously been shown to induce NF- κ B dependent transcription of TNF- α , IL-1 β and IL-8 in normal HBE cells (Watanabe, Jono et al. 2004) and IL-1 α , IL-6 and IP-10 in human middle ear epithelial cells (Moon, Lee et al. 2006). A more recent study investigating the effects of extended culture of NTHi on ALI cells shows presence of IL-1 β , IL-6, TNF- α , GM-CSF and IL-8 (Ren, Nelson et al. 2012). The differences that we have seen in the cytokine array data (particularly relating to IL-1) may be mediated by differences in the LOS molecules on the surface of the bacteria inducing innate defence pathways through TLRs (Clemans, Bauer et al. 2000), however this n=1 data set is the only data to show induction of IL-1 α . IL-1 β and IL-1ra and with limited literature on available requires further investigation.

Cytokine array results from both strains we used do not show presence of TNF- α , which based on the studies mentioned above, was somewhat surprising. Studies have suggested that TNF- α expression is induced earlier than IL-6 and IL-8 (Foxwell, Kyd et al. 1998; Clemans, Bauer et al. 2000). This combined with the variation between strains, may have resulted in our time point of 7 days being too late to pick up the peak of TNF- α expression that this strain elicits. The studies by Ren, Nelson et al. (2012) and Watanabe, Jono et al. (2004) used different strains to our study so may have elicited different responses at different times. TNF- α stimulates an acute response and regulates immune cells so should be detectable in response to a bacterial infection, and our

inability to detect this could mean immune cells are not activated and any response mounted is ineffective.

The cytokine array data generated for the infections in this chapter enabled comparison of the baseline cytokine profiles between cells cultured in the absence and presence of IL-13. This showed the variability between donors, with one donor exhibiting a slightly more complex response than the other. Differentiations modulated with IL-13 generally showed similar cytokine profiles to those cultured in the absence of IL-13 but showed higher or lower expression of cytokines they had in common. This expression was not consistent between donors.

Host-pathogen interactions both *in vivo* and *in vitro* result in the generation of a complex milieu which is often characterised by alterations in the protease/anti-protease balance. Dis-regulated proteases can impair host responses due to degradation of specific host defence molecules and cause tissue damage. Abnormalities of protease/anti-protease balance are associated with many pulmonary infection and inflammatory diseases. Such shifts in this balance can significantly affect epithelial host defence protein function with many being known to be inactivated by the action of unrestrained proteases (Greene and McElvaney 2009; Kukkonen, Tiili et al. 2013). Infection with NTHi *in vivo* leads to recruitment of immune cells to the site of infection, and their proteolytic activity could result in degradation of immune molecules. Neutrophil products, specifically neutrophil elastase, have been shown to degrade SPLUNC1 (Hobbs, Blanchard et al. 2013; Jiang, Wenzel et al. 2013). This degradation of SPLUNC1 resulted in an increase in bacterial load of NTHi, which was reduced upon addition of recombinant SPLUNC1. We examined degradation of proteins with neutrophil lysate, trypsin and NTHi. Neutrophil lysates were prepared in the presence and absence of a pan-protease inhibitor. Our data showed rapid degradation of SPLUNC1 into a number of fragments, with smaller bands appearing after 15 minutes and further loss of full-length protein after a 1 hour incubation period. This degradation was slowed by the addition of a pan-protease inhibitor as degradation did not begin until at least 1 hour incubation. This is only partially consistent with the data of Jiang, Wenzel et al. (2013) who showed cleavage of a 3kDa fragment. This could be due to their use of human neutrophil elastase, as opposed to the lysed whole neutrophils we used, which contains a variety of other proteases and also to the use of different antibodies designed against different epitopes of the proteins

Trypsin has been shown to activate the epithelial sodium channel ENaC, which is the rate limiting step in sodium adsorption in the regulation of airway surface liquid and mucociliary clearance (Donaldson, Hirsh et al. 2002; Haerteis, Krappitz et al. 2014). Our incubations with trypsin showed a similar degradation pattern for SPLUNC1 as that achieved when samples were incubated with neutrophil lysate, with a reduction of whole protein and the appearance of multiple smaller bands, however this degradation was slower in trypsin digestions. Truncation of SPLUNC1 has been reported *in vivo*, with samples from nasal lavage of patients with RSV (Fornander, Ghafouri et al. 2011) suggesting that proteolytic inactivation of the protein may be an important determinant of its function in airway disease. Currently there is no similar information on the degradation of LPLUNC1 but our data shows that this protein is also a target for protease digestion, again suggesting that its function may be impaired in diseases associated with protease imbalance.

As SPLUNC1 and LPLUNC1 were degraded by exogenous proteases we then looked for evidence of protein degradation in apical secretions from cells infected with NTHi. The rationale for this being that impairment of host defence proteins might be associated with inadequate cellular responses to *in vitro* infections. Proteolytic activity of NTHi is due to the IgA1 protease (Plaut, Qiu et al. 1992) which is used to aid evasion of the host defences (Kilian, Mestecky et al. 1979; Male 1979). Recent work has shown that NTHi expresses two different types of *iga* gene that are adapted for infection and colonisation (Murphy, Lesse et al. 2011). Our data shows increasing amounts of degradation with increasing bacterial numbers, with bands appearing at the appropriate size for the whole protein and also smaller bands just below 10kDa. As both of these proteins have putative functions in host defence (Bingle and Craven 2002; Bingle and Craven 2004), degradation may result in negative effects on the immune response. This degradation can be seen in other defence proteins and with other bacterial species (as reviewed by Potempa and Pike (2009) and Koprivnjak and Peschel (2011)). As previously mentioned degradation of SPLUNC1 reportedly leads to an impaired epithelial defence against NTHi (Jiang, Wenzel et al. 2013). The direct degradation of these proteins by NTHi may also be a mechanism employed by NTHi to diminish the epithelial defences in order to establish a successful infection.

As RSV and NTHi co-infect airways, particularly in COPD exacerbations (Bandi, Jakubowycz et al. 2003; Sethi, Mallia et al. 2009) we attempted to study co-infection in ALI cells. Previous studies have shown that a prior RSV infection, as this has been identified as the virus most commonly associated with paediatric otitis media development due to NTHi, can facilitate a potential NTHi infection by aiding attachment of the P5 fimbriae of NTHi to the cell (Jiang, Nagata et al. 1999) and causing dysregulation of a mucosal β -defensin in order to enhance colonisation by NTHi. In our studies ALI cells infected with RSV for 3 days, prior to a secondary NTHi infection for 3 and 7 days showed successful infection with both pathogens; however we saw large amounts of cell death, with very few cells remaining. This was the only attempt at establishing this type of infection as establishment of a successful co-infection requires more extensive optimisation than the time allowed in this project.

During the latter part of the project we encountered difficulties with maintaining the differentiation status of the ALI cultures which limited studies. Cultures would differentiate successfully (as judged by microscopy and Western blotting of SPLUNC1) but then lose confluence developing 'holes' in the differentiated cell layer between 14-21 days after ALI conditions were imposed. This 'failure to thrive' was investigated ruling out degradation of retinoic acid and not collagen coating the transwells. We were unable to identify a cause for this prior to the end of the study.

The aims of this chapter were to establish and characterise RSV and NTHi infections in the ALI model. We were able to successfully establish infections with RSV and NTHi, however, while many infections were performed, further infections need to be performed to generate a reproducible infection model and completely characterise the responses. The infections need to be performed with cells obtained from many more donors and using a number of strains of the pathogens in order to generate a more robust data set that is representative of the airway epithelium and responses elicited upon exposure to pathogens.

6. Discussion

The main aim of this study was to establish an *in vitro* model of the airway epithelium, to use as a tool to understand pulmonary innate defence mechanisms following exposure to pathogens, particularly RSV and NTHi. The secondary aim was to establish NTHi cultures, in the form of biofilms, on the surface of the airway epithelium, as these are frequently identified in the airways of patients with pre-existing airway disease.

6.1. Major Findings

Initial work using immortalised cell lines A549 and NCI-H292 cells and primary uHBE cells showed that they are not a representative model for the airway epithelium and are not robust enough to withstand infection with RSV and NTHi. Long term infections did appear to show that biofilms were achievable. The cells exhibited poor survival rates and limited cytokine responses and comprise of a single cell type, unable to differentiate to recapitulate the features of the airway epithelium. We were able to successfully establish the ALI cell culture technique in our lab using primary HBE cells. These cells develop a pseudostratified epithelium complete with mucociliary phenotype providing a good representation of the airway epithelium. These cultures showed induction of SPLUNC1 and LPLUNC1, and positive staining for a number of markers including tubulin and mucins. Stimulations with IFN- γ showed induction of the IFN-inducible protein IP-10 showing the cells were able to mount a cytokine response. Infection of these cells with RSV and NTHi showed them to be much more robust and responsive when exposed to pathogens. Infections using two different strains of NTHi showed the difference in responses elicited by different strains of bacteria.

6.2. Establishing an airway infection model

The first step in this work was to establish a representative airway model. Initially cancer derived cell lines, A549 and NCI-H292, were investigated alongside primary uHBE cells, which were used to introduce donor variability. The limited responses from cancer derived cell lines and undifferentiated primary cells, and poor cell survival upon infection showed that they are not the most effective tool for conducting studies aimed at characterising the immune response due to the limited number of cytokine and chemokines produced and that cells do not produce the full range of molecules seen in the airway.

The ALI culture technique is becoming widely used for airway epithelial studies. We successfully established this differentiation of primary airway epithelial cells in our lab, with cytokine array showing that ALI cells elicited a much more complex response and proved to be much more resilient upon infection. This increased robustness allowed for longer term experiments to be performed in order to establish NTHi biofilms. Modulation of the phenotype with IL-13 increased the proportions of goblet cells, enabling study of the diseased airway epithelium.

6.2.1. NTHi infection

An ALI model, called the EpiAirway™ model was utilised by Ren, Nelson et al. (2012) to study long-term NTHi infections. They saw clustering of bacteria at focal sites of the epithelium during an infection was also reported by. Our studies also showed clusters of bacteria towards the apical surface of cells with the bacteria remaining in our wells appearing to cluster around surviving cells. This observation coupled with our unsuccessful attempts to grow bacteria in cell media alone, suggests that NTHi may require cells to be able to grow (results not shown), contributing to the suggestion that clustering aids survival. Confocal imaging of a 3 day uHBE infection showed a mesh-like structure around the clusters of bacteria, which could represent the glue-like matrix observed by Moxon, Sweetman et al. (2008). This could also be seen in small amounts in 7 day infections of cell lines and resembles the formation of biofilms seen in both NTHi and other bacterial species (Starner, Zhang et al. 2006; Hall-Stoodley, Nistico et al. 2008; Ma, Conover et al. 2009; Juneau, Pang et al. 2011). It has also suggested that MUC18 is a putative receptor for NTHi (Simon, Martin et al. 2011), aiding adherence to cells. This suggests that IL-13 modulated cultures may have higher levels of bacterial adherence, which was not the case in the study, however these infections were performed for long periods of time and this may have more of an impact during establishment of infection and when trying to infection.

Preliminary studies were performed to investigate the role of neutrophils in the formation of biofilms (the role of which is outlined in section 1.4.2). We investigated the effect of the ALI cell media on neutrophils, as this is not the standard media for neutrophil culture work. We were able to establish that there is no significant effect on neutrophil survival when neutrophils are cultured in ALI medium as opposed to the standard RPMI based medium used in neutrophil culture (data is shown in Appendix 4).

6.2.2. RSV infection

We were able to successfully establish RSV infections in ALI cells, and showed that RSV was specifically targeting ciliated cells. The ALI model has previously been shown to be an effective tool for studying RSV infections (Villenave, O'Donoghue et al. 2011; Villenave, Thavagnanam et al. 2012; Guo-Parke, Canning et al. 2013). The study by Guo-Parke et al 2013 also showed use of nasal epithelial cells instead of bronchial epithelial cells cultured at the ALI to be an effective tool. These three studies have used the ALI model extensively to begin to characterise RSV infection, including utilisation of multiple RSV strains and the responses the cells elicited, which includes induction of IP-10, RANTES, IL-6 and IL-8 (Villenave, Thavagnanam et al. 2012). We wanted to establish an RSV infection model for use in co-infections in order to study the effects of multiple pathogens.

6.2.3. Co-infection

A number of studies have begun to look at RSV and NTHi co-infections. A number of studies show that NTHi colonisation is aided by an initial RSV infection (Jiang, Nagata et al. 1999; Fukasawa, Ishiwada et al. 2009; McGillivray, Mason et al. 2009). A recent study showed that NTHi increases the airways susceptibility to RSV infections and amplifies the inflammatory response (Gulraiz, Bellinghausen et al. 2015).

6.3. Signalling

To confirm the airway model functioned like an airway, and ensure exposure to pathogens elicited responses from the cells and stimulated inflammatory mediator production, semi-quantitative arrays were performed. Within experiments, arrays were performed at the same time, and data generated using the same exposure time per experiment. Our initial aim for using the cytokine arrays was to not to identify novel expression patterns or quantify the responses elicited, but ensure that the cells were responding to stimulus, therefore all cytokine arrays were only performed once and were not replicated to generate such data. However, a summary of the cytokine profiles pre- and post- infection in all cell types can be found in table 6.1 with an indication from the available data as to level of increased/decreased expression. In general, the results show that infection of ALI differentiated cells generated a more 'significant' response than that seen in cell lines. It also demonstrates that the clinical isolate stimulates a more pro-inflammatory response than laboratory strains.

The results from the cytokine arrays, alongside published data, were used to identify targets for qPCR on samples from cells infected with NTHi and RSV for 24 hours with primers against IP-10 and IL-6 (Clemans, Bauer et al. 2000; Gaschler, Skrtic et al. 2009; Oshansky, Barber et al. 2010; Smith, Kulkarni et al. 2013). Additionally, based on published data (Wang, Lim et al. 2002; Jono, Xu et al. 2003; Chen, Lim et al. 2004; Shen, Yoshida et al. 2008; Jono, Lim et al. 2012), primers against MUC5AC were used with samples from NTHi infected cells.

An obvious target to study, in both the ALI cells and cell lines would have been IL-8, however, the cytokine arrays performed on ALI cultures showed that readily detectable amounts of IL-8 were present in our control cultures, suggesting constitutive expression of IL-8 in normal cultures. This could either be due to the method of cell isolation, or the donors that they were isolated from (as cells are rarely taken in large numbers from healthy patients).

Cytokine	Cell type												
	A549		NCI-H292		uHBE		ALI (-IL-13)		ALI (+IL-13)				
CD40 Ligand													
G-CSF					+	+	++	+++		+		+	
GM-CSF					+	++	+					+	
GRO- α	0		+		-	0		-	-		-	-	
sICAM-1							+++	+		+		-	
IFN- γ													
IL-1 α					+	--	++			++			
IL-1 β							++			++			
IL-1ra							+	++		-		0	
IL-6					0	+		++				+	
IL-8	+++		+++		0	-	0	0		0		-	
IL-23													
IP-10						++	+	++				+++	
MIF	-		0		0	0	0	0		+++		++	
SerpinE1	-		+		++	0	-	-		-		-	
RANTES												+	
Key:													
	Control							RSV infections					
	NTHi 86-028NP-GFP infections							NTHi Clinical Isolate infections					
- = decreased expression			0 = no change				+ = increased expression						

Table 6. 1: Summary of cytokine profiles pre- and post- infection in all cell types.

Our studies showed an induction in IP-10 and IL-6 in response to RSV infection in all cell types; and in IL-13 stimulated ALI cells RANTES was also induced. Studies performed on a variety of cell types show that RSV utilises TLR2 (Murawski, Bowen et al. 2009), TLR3 (Groskreutz, Monick et al. 2006; Huang, Wei et al. 2009), TLR4 (Haeberle, Takizawa et al. 2002; Tulic, Hurrelbrink et al. 2007; Wheeler, Chase et al. 2009; Zhou, Yang et al. 2014) and TLR7 (Huang, Wei et al. 2009) to activate and elicit an immune response. This results in activation of the MyD88 and RIG-I signalling pathways, leading to up-regulation of a variety of molecules, including IL-6, IL-8, IP-10, RANTES, leukocyte inhibitory factor (LIF), MIF, ICAM-1, TNF- α and IFNs, predominantly type III IFNs (Tulic, Hurrelbrink et al. 2007; Okabayashi, Kojima et al. 2011; Lotz and Peebles 2012; Smith, Kulkarni et al. 2013; Villenave, Shields et al. 2013; Foronjy, Dabo et al. 2014). RSV has been shown to activate these cytokines through a number of signalling pathways including NF- κ B, MAPK and JAK/STAT signalling (Bitko, Velazquez et al. 1997; Chen, Monick et al. 2000; Kong, San Juan et al. 2003). RANTES was found to be induced in a time-dependent manner by Hirakawa, Kojima et al. (2013), who also identified upregulation of MMP-10 which was prevented by inhibitors of JAK/STAT, EGFRs and MAPK (Hirakawa, Kojima et al. 2013). RSV has been shown to activate EGFR by phosphorylation in lung epithelial cells, increasing ERK activity to stimulate the immune response and increase survival of RSV infected cells by delaying apoptosis (Monick, Cameron et al. 2005; Krzyzaniak, Zumstein et al. 2013). The induction of RANTES and IL-6 and increased MIF expression in our RSV infected ALI cells shows activation of signalling pathways consistent with that observed in other studies.

NTHi stimulates an immune response predominantly through the activation of TLR2 (Shuto, Xu et al. 2001), and a study investigating the clearance of NTHi in mouse lungs has shown that signalling through TLR4 is also important (Wieland, Florquin et al. 2005). Both receptors activate MyD88-dependent and independent signalling and both the canonical and non-canonical NF- κ B signalling pathways, with NTHi particularly leading to activation of p38 MAPK and thus a plethora of pro-inflammatory cytokines and mucins (Wang, Lim et al. 2002; Jono, Xu et al. 2003; Chen, Lim et al. 2004; Mikami, Gu et al. 2005; Mikami, Lim et al. 2006; Lee, Takeshita et al. 2008; Shen, Yoshida et al. 2008; Xu, Xu et al. 2008; Jono, Lim et al. 2012; Kyo, Kato et al. 2012). Studies have shown increases in expression of IL-1 α , IL-1 β , TNF- α , IL-6, IL-8, MCP-1 and GM-CSF (Clemans, Bauer et al. 2000; Ren, Nelson et al. 2012) which these studies

have also shown. NTHi has been shown to increase ICAM-1 expression (Frick, Joseph et al. 2000), but has also been shown to bind to ICAM-1 (Avadhanula, Rodriguez et al. 2006) suggesting that this increased expression is a tool utilised by NTHi to increased numbers of adherent bacteria. ICAM-1 expression is regulated by the NF- κ B, JAK/STAT and MAPK pathways which release of these cytokines is consistent with. We also observed increased expression of ICAM-1 consistent with these studies. More recently, NTHi has been shown to interact with the type II transmembrane receptor Dectin-1, which is expressed in lung tissues to induce the pro-inflammatory response, however the ligand for this activity is unidentified (Heyl, Klassert et al. 2014).

6.4. Strengths and Weaknesses

Establishment of the ALI model provides a good representation of the complex airway epithelium as all cell types are present and airway lining fluid can be studied. Modulation of the mucociliary phenotype with IL-13 models an airway that is more reflective of diseased airways seen in subjects with CF, COPD and asthma. The infection models we established used different culture conditions, for example we did not collagen coat the transwell prior to initiating ALI culture, differing time periods, cells were differentiated for a minimum of 21 days prior to removal of antibiotics and were exposed to pathogens for longer periods of time and we also used different strains of pathogen, specifically when working with the clinical isolate of NTHi.

One of the limiting factors with the use of primary human ALI cells is the number of cells that can be generated from a single sample/donor. Novel strategies need to be developed to overcome this. A recent publication showed that culture of primary human airway cells with Rho Kinase (ROCK) inhibitors (specifically Y27632) enhanced basal cell survival and proliferation (Horani, Nath et al. 2013). ROCK proteins are involved in cell adhesion, migration, apoptosis, proliferation and differentiation. We have replicated this data using two different doses of ROCK inhibitor, which improved basal cell proliferation, regardless of dose, providing an effective solution to issues surrounding low cell numbers. They also showed that treatment with ROCK inhibitor did not have any effect on cells when they were differentiated at the ALI (Horani, Nath et al. 2013). Again this is an area that should be further addressed in the future. Currently our laboratory is using this technique to increase the yield of primary mouse airway cells for culturing at the ALI.

As it stands the model is not particularly robust as it needs to factor in the high levels of variability between replicates from the same donor, and the variability between donors. A high level of variability was also seen by Gohy, Hupin et al. (2015) during their work on COPD airway cells. This demonstrates an issue with the quality of the cells we are working with as each well of cells is different, i.e. differing cell population proportions, even from cells from the same donor. To increase robustness of the model, many more replicates need to be performed both within the same donor number and additional donor numbers.

One of the limitations of the infection aspect of the model is that lab strains were used throughout, with only a small investigation performed using a clinical isolate. There is suggestion that lab strains are somewhat attenuated, reducing their virulence/pathogenicity. This is particularly important for the use of NTHi as no key virulence feature has been identified for NTHi infection. The small amount of work we performed with a clinical isolate shows that multiple strains of NTHi need to be investigated including lab strains and potentially more virulent clinical isolates. We also worked under the assumption that we started each infection with the same amount of each pathogen; this is yet another variable that contributes to the large amount of variability seen. The infections would also need to be performed with far more replicates than are performed in this project.

Further work performed within our group using similar techniques has since shown that quantifying RNA through use of the Nanodrop is not very precise at quantifying RNA in low concentrations using A280/A260, therefore we may not have obtained accurate concentrations for use in proceeding work. We have also found that use of TaqMan probes as opposed to SYBR-green labelled probes to give more robust qPCR data sets. There is also an issue with potential contaminants remaining after RNA extraction using TRI reagent, which also has an impact upon downstream work. Use of TaqMan probes, an alternative to TRI reagent and a more reliable quantification method would increase the reliability of qPCR data sets.

The high variability introduced by both the differences between donors, between each transwell of cells cultured and load if inoculate used to perform the infection is seen in the quantification data throughout this thesis, large differences can be seen between stimulated/infected cells and controls when data is combined, however individual

replicates (and the error bars) show the high level of variability between experiments. The only way to establish the significance of results reliably is to perform many more replicates, for example the study performed by Gohy, Hupin et al. (2015) used cells from 104 patients. In this project it would improve confidence in statistical analysis performed on quantitative data.

One of the major difficulties we experienced with the nature of establishing this culture technique and establishing infections was the need to plan new experiments prior to being able to fully analyse and understand the results from the experiments already performed, resulting in performance of fewer replicates that we now consider necessary for interpreting data.

6.5. Future Directions

Upon increasing the robustness of the model, a study focussing on identification and quantification of immune responses using a variety of techniques, including ELISA, micro array/cytokine arrays, Western blotting and qPCR would help to understand the role of specific cytokines and also provide information about defence mechanisms and key virulence factors involved in these infections. These can then be studied either by knocking down or silencing expression of selected target genes, or over-expressing specific genes in either the epithelial cells or the pathogen.

Preliminary studies showed that co-infection with multiple pathogens and introducing neutrophils to study their effects on biofilm formation would require more optimisation time than this project allowed; however introducing multiple pathogens into the infection model would increase understanding of multiple pathogen colonisation seen in the airways of CF and COPD patients and further study into biofilm composition would aid understanding of why they are so difficult to treat.

These further characterisation studies could be used to aid our understanding of immune responses, pathogen interactions and biofilm formations and help to develop therapeutics. The model provides an ideal platform to test the efficacy of treatments in eliminating the pathogen. This can also be said for the identification of potential vaccination targets for NTHi. A vaccine already exists for RSV, which is administered to high risk patients to prevent a severe RSV infection but does not completely prevent

infection as it is not very effective; therefore further study may identify a treatment that totally eradicates it from the host.

6.6. Conclusions

The main aim of this thesis was to develop a model of the airway epithelium *in vitro* in order to use it as a tool to understand pulmonary innate defence mechanisms. To establish the airway model we investigated the use of cell lines, primary cells and the ALI culture model to generate a differentiated airway epithelium. To begin to study defence mechanisms we chose two pathogens to focus on, RSV and NTHi which are both frequently identified as causing respiratory infections in healthy and ‘diseased’ airways. My initial objective was to establish the effectiveness of cell lines and uHBE cells for use as a model of the airway, and establish starting doses of both pathogens and begin to optimise them. The cell lines proved unable to cope with infections for extended periods of time, exhibiting significant cell death at longer time points. Infection of the uHBE cell line allowed biofilm formation. None of these cells elicited a response to stimulus with pathogens, showing that they were not an effective tool.

The results of the cell line/undifferentiated primary cell work suggested the use of a more complex model that represented the multiple cell types within the airway epithelium; therefore we chose to establish ALI culture of uHBE cells. As this technique has been established by a number of other groups it was important to characterise the cultures we established to ensure that they were consistent with those of other groups; this was successfully done. After establishing the ALI culture model, the third objective was to expose the more complex and representative cultures to RSV and NTHi, in order to establish infections and investigate the immune responses elicited. These two pathogens have also been implicated in co-infection; especially in ‘diseased’ airways. Therefore, key objectives were to establish RSV and NTHi infections, establish a biofilm-type infection/colonisation in the bacterial infection and establish co-infection models. Although we were unable to conduct any short term infection studies due to development of issues with cell differentiation, long term infections show clustering of bacteria in cell cultures that is consistent with biofilm formation. Washes taken from these infections confirmed that the bacteria present were still viable and that the bacteria appeared to be having some effect on immune molecules. This resulted in direct interaction studies between live NTHi and ALI secretions, showing degradation of the putative immune molecules LPLUNC1 and SPLUNC1. We also attempted a co-

infection using the same doses we had used for individual experiments, however large amounts of cell death showed that this would need lots more optimisation than time allowed, even without the issues we were experiencing with cell differentiation. As such, what we learnt from this thesis relates to the technical issues of working with primary cells and the requirements for generating robust data when working with these cells, rather than contributing any new data.

In conclusion, we have successfully generated a representative model of the airway epithelium established infections on these cells with RSV and NTHi and established NTHi biofilms. Cytokine arrays showed that ALI cells elicited a more complex response, which differs between isolates. Unfortunately, we were unable to establish a model of co-infection; however this could be addressed in a future project. The successful establishment of an airway and infection model provides an effective tool to study pulmonary innate defence mechanisms, mechanisms of infection and identify therapeutic/vaccination targets.

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APPENDICES

Appendix 1 – Materials

1. List of Materials

0.5M Tris pH6.8	Bio-Rad Laboratories
1.5M Tris pH8.8	Bio-Rad Laboratories
10% Phosphate-buffered Formalin	Fischer
20% Sodium Dodecyl Sulphate	Sigma
24-well plate	Costar
40% Acrylamide	Geneflow
4-chloro-1naphthol	Sigma
96-well Plates for qPCR	Starlabs
96-well qPCR plate sealers	Starlabs
Agarose	Sigma
Airway Epithelial Cell Basal Medium	Promocell
AlexaFluor 488 (Anti-Mouse)	Invitrogen
AlexaFluor 488 (Anti-Rabbit)	Invitrogen
AlexaFluor 568 (Anti-Rabbit)	Invitrogen
Ammonium PerSulphate	Fischer
AMV-RT	Promega
Anti-goat IgG peroxidase conjugate	Sigma
Anti-Rabbit HRP	Dako
Anti-V5 HRP Antibody	Life Technologies (R96125)
Applied Biosystems 7900HT Fast Real-Time PCR System	Applied Biosystems
Bio Rad (LU101 imaging system)	Bio Rad
Bio-Rad Mini-PROTEAN 3 Gel Electrophoresis System	Bio-Rad Laboratories
Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell	Bio-Rad Laboratories
Bovine Serum Albumin	Sigma
Brain Heart Infusion Broth	Oxoid
Bromophenol Blue	Phi Bio
ColorPlus Prestained Protein Ladder	New England BioLabs
Columbia Agar Base	Oxoid
Cryo-SFM	Promocell
Developer	Ilford
DMEM	Sigma
DMSO	Sigma
dNTPs	Promega
DPX	Sigma
DTT	AGTC Bioproducts
Eosin	Fischer Scientific
EZ-ECL Chemiluminescence Detection Kit for HRP	Geneflow
Foetal Bovine Serum (FBS) (heat inactivated)	Fischer Scientific
Fixer	Ilford
Formaldehyde	Fischer Scientific
FV10-ASW 2.0 Viewer	http://www.olympus-global.com/en/

Glycerol	Fischer Scientific
Glycine	VWR
Goat Serum	Sigma
GoTaq Flexi DNA Polymerase	Promega
Haematoxylin	Fischer Scientific
Hemin	Sigma
Human Bronchial Epithelial (HBE) Cells	Promocell
Human Cytokine Array Panel A Array Kit	R & D Systems
Hydrogen Peroxide	Sigma
Hypercassette	Amersham
Hyperfilm	Amersham
HyperLadder I	Bioline
HyperLadder IV	Bioline
I-block	Invitrogen
IFN- γ	R & D Systems
IL-13	eBioscience
Incubator	Sanyo O ₂ /CO ₂ incubator
Kanamycin sulphate	Invitrogen
Laminar Flow Hood	MDH Inter Med
Leica DMIRB and Spot Advanced Imaging Software	Leica
L-Glutamine	Sigma
MBF ImageJ for Microscopy	http://www.macbiophotonics.ca/imagej/
MOPS (10x)	Fischer Scientific
Mr Frosty	Nalgene
Muc5AC Antibody	AbD Serotec (1695-0128)
Muc5B Antibody	Santa Cruz (SC20119)
Nanodrop-1200	Core facility-University of Sheffield
Nitrocellulose Membrane	Bio-Rad
Oligo(dT)	Promega
Oligonucleotide Primers	Sigma
Olympus FV-1000 Confocal Microscope	Light Microscopy Facility- University of Sheffield
Oxalated Horse Blood	Oxoid
Penicillin/Streptomycin	Sigma
Phosphate Buffered Saline (PBS)	Gibco
PNGaseF	New England Biolabs
Protease Inhibitor Tablet (EDTA free)	Roche
PVDF	Bio-Rad Laboratories
Quick-diff Blue	Reagen
Quick-diff Red	Reagen
Retinoic Acid	Sigma
RNA Loading Buffer	Sigma
RNasin	Promega
RPMI 1640	Sigma
RSV Antibody	AbD Serotec (7950-0004)
Scott's tap Water	Sigma
SPLUNC1 Antibody	R & D Systems (MAB1897)
Sodium Chloride (NaCl)	Sigma
Sterile Water for injection	Braun
SYBR Green (find out proper name)	Thermo Fischer
T25 & T75 Tissue Culture Flasks	Nunc

TEMED	Fischer
Transwell inserts (transparent PET membrane with 0.4µm pores)	BD Falcon
Tris (powder)	VWR
Tris-HCl pH8.0	Sigma
Triton-X	Sigma
Trypsin-EDTA	Sigma
Tween-20	Sigma
Vectashield Mounting Medium containing DAPI	Vector Laboratories
Vectastain Elite ABC kit	Vector Laboratories
Vector NovaRed Substrate kit	Vector Laboratories
Vivaspin tubes	Sartorius Stedim UK Ltd
Xylene	VWR
Y27632 (ROCK Inhibitor)	Tocris
Yeast Extract	Oxoid
ZO-1 antibody	Invitrogen (40-2200)
α-tubulin antibody	Abcam (ab61226)
β-NAD	Sigma
β-tubulin antibody	Sigma (T5201)

2. List of Primary Cell Donors (Lot Nos.)

Donor 1	9102601.3
Donor 2	9082701.12
Donor 3	9022003.10
Donor 4	1101701.12
Donor 5	6031301.14

3. List of Primers

Primer	Sequence
SP1 F	AGGTCTTCTGGACAGCCTCA
SP1 R	TGTCCAAGCCTCTGAGAACC
LP1 F	TCCCAGTGTCATTGGTGAAG
LP1 R	AGGAGGCTGGAGTAAGCACA
Actin F	GATCATTGCTCCTCCTGAGC
Actin R	ACATCTGCTGGAAGGTGGAC
IP-10 F1	CCAATTTTGTCCACGTGTTG
IP-10 F2	CCACGTGTTGAGATCATTGC
IP-10 R1	ATTTTGCTCCCCTCTGGTTT
IP-10 R2	CCTCTGTGTGGTCCATCCTT
IL-6 F1	CACACAGACAGCCACTCACC
IL-6 F2	AAAGAGGCACTGGCAGAAAA
IL-6 R1	TTTTCTGCCAGTGCCTCTTT
IL-6 R2	TTTCACCAGGCAAGTCTCCT
MCP-1 F1	CCCCAGTCACCTGCTGTTAT
MCP-1 F2	AGCAAGTGTCCCAAAGAAGC
MCP-1 R1	AGATCTCCTTGGCCACAATG
MCP-1 R2	TGGAATCCTGAACCCACTTC
Muc5AC F	GTGTCACCAACACCAGCAAG
Muc5AC R	TCCTGGTGCTTCTCACACTG
Muc5B F	ATGAAACCTGGGTCAACAGC
Muc5B R	TCCTCACAGGAGTAGCAGCA

4. Antibody Details and Dilutions

Western Blotting

Antibody	Species Raised In	Primary Antibody Dilution	Secondary Antibody	Secondary Antibody Dilution
Human LPLUNC1A (in-house)	Rabbit	1:200	Anti-Rabbit HRP	1:2000
Human LPLUNC1B (in-house)	Rabbit	1:500	Anti-Rabbit HRP	1:2000
Human SPLUNC1A (in-house)	Rabbit	1:1000	Anti-Rabbit HRP	1:2000
Human SPLUNC1B (in-house)	Rabbit	1:200	Anti-Rabbit HRP	1:2000
SPLUNC1 (Mono) (R&D, MAB1897)	Mouse	1:200	Anti-Mouse HRP	1:2000
MUC5AC (AbD Serotec, 1695-0128)	Mouse	1:500	Anti-Mouse HRP	1:2000
MUC5B (Santa Cruz, SC20119)	Rabbit	1:500	Anti-Rabbit HRP	1:2000
Anti-V5 HRP (Life Technologies, R96125)	Mouse	1:2000	N/A- Already HRP conjugated	

Immunohistochemistry

Antibody	Species Raised In	Primary Antibody Dilution	Blocking Serum	Antigen Retrieval
Human LPLUNC1B (in-house)	Rabbit	1:600	Normal Goat	Yes

Immunofluorescence

Antibody	Species Raised In	Primary Antibody Dilution	Secondary Antibody	Secondary Antibody Dilution
Human SPLUNC1B (in-house)	Rabbit	1:500	Anti-Rabbit AlexaFluor 488	1:250
MUC5AC (AbD Serotec, 1695-0128)	Mouse	1:200	Anti-Mouse AlexaFluor 488	1:250
MUC5B (Santa Cruz, SC20119)	Rabbit	1:500	Anti-Rabbit AlexaFluor 488	1:250
α-tubulin (Abcam, ab61226)	Rabbit	1:1000	Anti-Rabbit AlexaFluor 488	1:250
β-tubulin (Sigma, T5201)	Mouse	1:500	Anti-Mouse AlexaFluor 488	1:250
ZO-1 (Invitrogen, 40-2200)	Rabbit	1:250	Anti-Rabbit AlexaFluor 488	1:250

5. Cytokine Array Data Reference Table

Co-ordinate	Target/Control	Co-ordinate	Target/Control
A1, A2	Positive	C11, C12	IL-17
A3, A4	C5a	C13, C14	IL-17E
A5, A6	CD40 Ligand, CD154	C15, C16	IL-23
A7, A8	G-CSF, CSF β , CSF-3	C17, C18	IL-27
A9, A10	GM-CSF, CSF α , CSF-2	C19, C20	-
A11, A12	GRO- α , CXCL1	D1, D2	-
A13, A14	I-109, CCL1	D3, D4	IL-32 α
A15, A16	sICAM-1, CD54	D5, D6	IP-10, CXCL10
A17, A18	IFN- γ , Type II IFN	D7, D8	I-TAC, CXCL11
A19, A20	Positive	D9, D10	MCP-1, CCL2
B1, B2	-	D11, D12	MIF, GIF, DER6
B3, B4	IL-1 α	D13, D14	MIP-1 α , CCL3
B5, B6	IL-1 β	D15, D16	MIP-1 β , CCL4
B7, B8	IL-1ra	D17, D18	SerpinE1, PAI-1
B9, B10	IL-2	D19, D20	-
B11, B12	IL-4	E1, E2	Positive
B13, B14	IL-5	E3, E4	RANTES, CCL5
B15, B16	IL-6	E5, E6	SDF-1, CXCL12
B17, B18	IL-8, CXCL8	E7, E8	TNF- α , TNFSF2
B19, B20	-	E9, E10	sTREM-1
C1, C2	-	E11, E12	-
C3, C4	IL-10	E13, E14	-
C5, C6	IL-12, p70	E15, E16	-
C7, C8	IL-13	E17, E18	-
C9, C10	IL-16, LCF	E19, E20	Negative

Appendix 2 – Solutions

1. Cell culture

Complete culture Medium	Concentration	Volume
RPMI		500ml
Heat-inactivated FBS	10%	50ml
Penicillin/Streptomycin	1%	5ml
Penicillin/Streptomycin not added when making up antibiotic-free medium.		

5% Serum HeLa Medium	Concentration	Volume
DMEM		500ml
Heat-inactivated FBS	5%	25ml
Penicillin/Streptomycin	1%	5ml
L-Glutamine	2mM	5ml

2% Serum HeLa Medium	Concentration	Volume
DMEM		500ml
Heat-inactivated FBS	2%	10ml
Penicillin/Streptomycin	1%	5ml
L-Glutamine	2mM	5ml

Freezing Medium	Concentration	Volume
RPMI		35ml
Heat-inactivated FBS	20%	10ml
DMSO	10%	5ml

BEGM	Concentration	Volume
Airway Epithelial Cell Basal Medium		500ml
Penicillin/streptomycin	1%	5ml
Bovine Pituitary Extract	0.004ml/ml	
Epidermal growth factor (recombinant human)	10ng/ml	
Insulin (recombinant human)	5µg/ml	
Hydrocortisone	0.5µg/ml	
Epinephrine	0.5µg/ml	
Triiodo-L-thyronine	6.7ng/ml	
Transferrin (human)	10µg/ml	
Penicillin/Streptomycin not added when making up antibiotic-free medium.		

ALI Media	Concentration	Volume
DMEM		250ml
Airway Epithelial Cell Basal Medium		250ml
Penicillin/Streptomycin	1%	5ml
Bovine Pituitary Extract	0.004ml/ml	
Epidermal growth factor (recombinant human)	10ng/ml	
Insulin (recombinant human)	5µg/ml	
Hydrocortisone	0.5µg/ml	
Epinephrine	0.5µg/ml	
Triiodo-L-thyronine	6.7ng/ml	
Transferrin (human)	10µg/ml	
Retinoic Acid	50nmol	
Penicillin/Streptomycin not added when making up antibiotic-free medium.		

2. Western Blotting

SDS-PAGE Gels

Makes 2 gels.

Resolving Gel	12%	15%
Water	9.9ml	8.1ml
40% Polyacrylamide	6.8ml	8.4ml
1.5M Tris pH8.8	5.7ml	
20% SDS solution	112.5 μ l	
20% APS	225 μ l	
TEMED	9 μ l	

Stacking Gel (5%)

Water	6.0ml
40% Polyacrylamide	1240 μ l
0.5M Tris pH6.8	2520 μ l
20% SDS solution	50 μ l
20% APS	100 μ l
TEMED	10 μ l

2x SDS Lysis Buffer

Reagent	Concentration	Mass/Volume
1M DTT	0.1M	1ml
Glycerol	20%	2ml
0.5M Tris pH 6.8	0.06M	1ml
0.2% Bromophenol Blue	0.02%	200 μ l
(dissolved in ethanol)		
20% SDS Solution	4%	
Protease Inhibitor	2%	1 tablet
Water		Up to 10 ml

Stored in 1ml aliquots at -20°C

10x SDS-PAGE Running Buffer

Reagent	Concentration	Mass/Volume
Tris	25mM	30.3g
Glycine	4mM	2.9g
20% SDS solution	0.1%	50ml
Water		Up to 1 litre

To make 1 litre of 1x solution 100ml of 10x added to 900ml water. Stored at room temperature.

1x Transfer Buffer

Reagent	Concentration	Mass/Volume
Tris	48mM	2.9g
Glycine	38.6mM	1.45g
20% SDS solution	0.037%	925µl
Methanol	20%	100ml
Water		Up to 1 litre

Stored at room temperature.

10x TBS-Tween

Reagent	Concentration	Mass/Volume
1M Tris-HCl pH8.0	0.01M	100ml
NaCl	0.17M	97.3g
Tween-20	0.05%	5ml
Water		Up to 1 litre

To make 1 litre of 1x solution 100ml of 10x added to 900ml water. Stored at room temperature.

10x TBS

Reagent	Concentration	Mass/Volume
1M Tris-HCl pH8.0	0.01M	100ml
NaCl	0.17M	97.3g
Water		Up to 1 litre

To make 1 litre of 1x solution 100ml of 10x added to 900ml water. Stored at room temperature.

Blocking Buffer/ Antibody Buffer

Reagent	Concentration	Mass/Volume
Powdered Milk	5%	5g
TBS-Tween		100ml

Stored at +4°C for 24 hours

3. Gel Electrophoresis

RNA Gel

Reagent	Mass/Volume
Agarose	0.5g
Water	38.5ml
10x MOPS	5ml
Formaldehyde	8.5ml

Makes 50mls.

DNA Gel

Reagent	1% Gel	2% Gel
Agarose	1g	2g
1x TAE	100ml	

Makes 100mls.

4. Immunofluorescent Staining

Permeabilisation buffer

Reagent	Concentration	Mass/Volume
Goat Serum	10%	1ml
PBS	90%	15ml
Triton-x	0.5%	50µl

Makes 10mls. Stored at 4°C.

5. Bacterial culture

Chocolate agar plates / + kanamycin

Reagent	Concentration	Mass/Volume
Columbia Agar Base	3.9%	7.8g
Oxalated Horse Blood	7.5%	15ml
De-ionised Water		200ml
Kanamycin (20mg/ml)	20µg/ml	215 µl

Columbia agar base and water are mixed and autoclaved. Agar is cooled to 70°C in a water bath for 15 minutes. Horse blood was added and put back in the water bath for 5 minutes to lyse the blood until the mixture has turned a chocolate colour. If agar is being supplemented with kanamycin (for 86-028NP GFP strain) it is then cooled to a maximum of 55°C whilst mixing gently before addition of kanamycin. Plates are then poured, allowed to cool and stored at +4°C and dried and warmed before use.

BHI broth

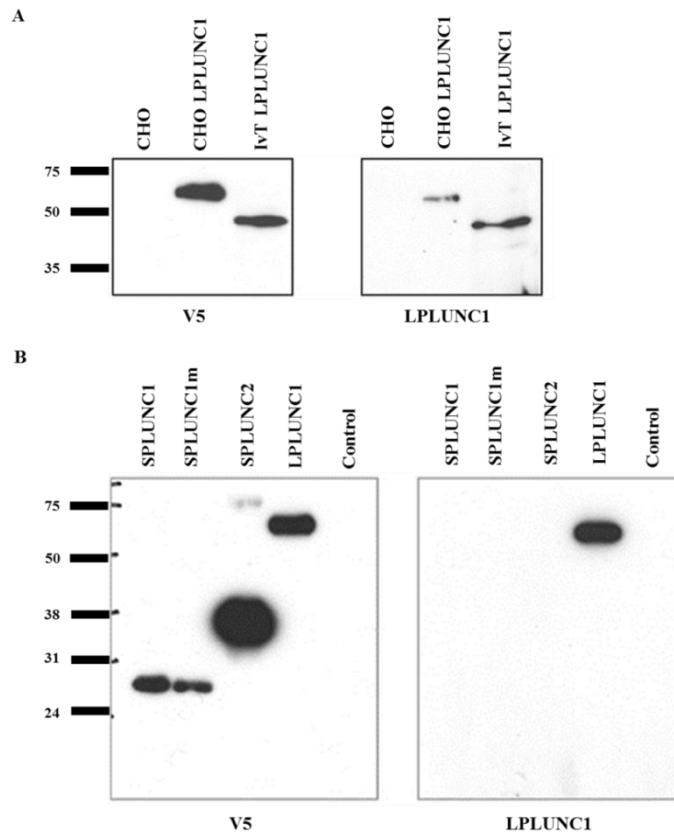
Reagent	Concentration	Mass/Volume
BHI	3.7%	18.5g
Yeast Extract	0.5%	2.5g
De-ionised Water		500ml

BHI, yeast extract and water mixed and placed in universal bottles in 20ml aliquots. These are then autoclaved and stored at room temperature.

Supplement	Concentration	Mass/Volume
Kanamycin (20mg/ml)	20µg/ml	20 µl
Hemin (1mg/ml)	10µg/ml	200µl
β-NAD (200µg/ml)	2µg/ml	200µl

Kanamycin is stored long term at -20°C and at +4°C for up to 1 month. Hemin and β-NAD are stored at +4°C. All are added to 20mls of BHI broth as and when needed.

Appendix 3 - Validation and Characterisation of Antibodies



Appendix Figure 1: Characterisation of the LPLUNC1 antibody by Western blotting

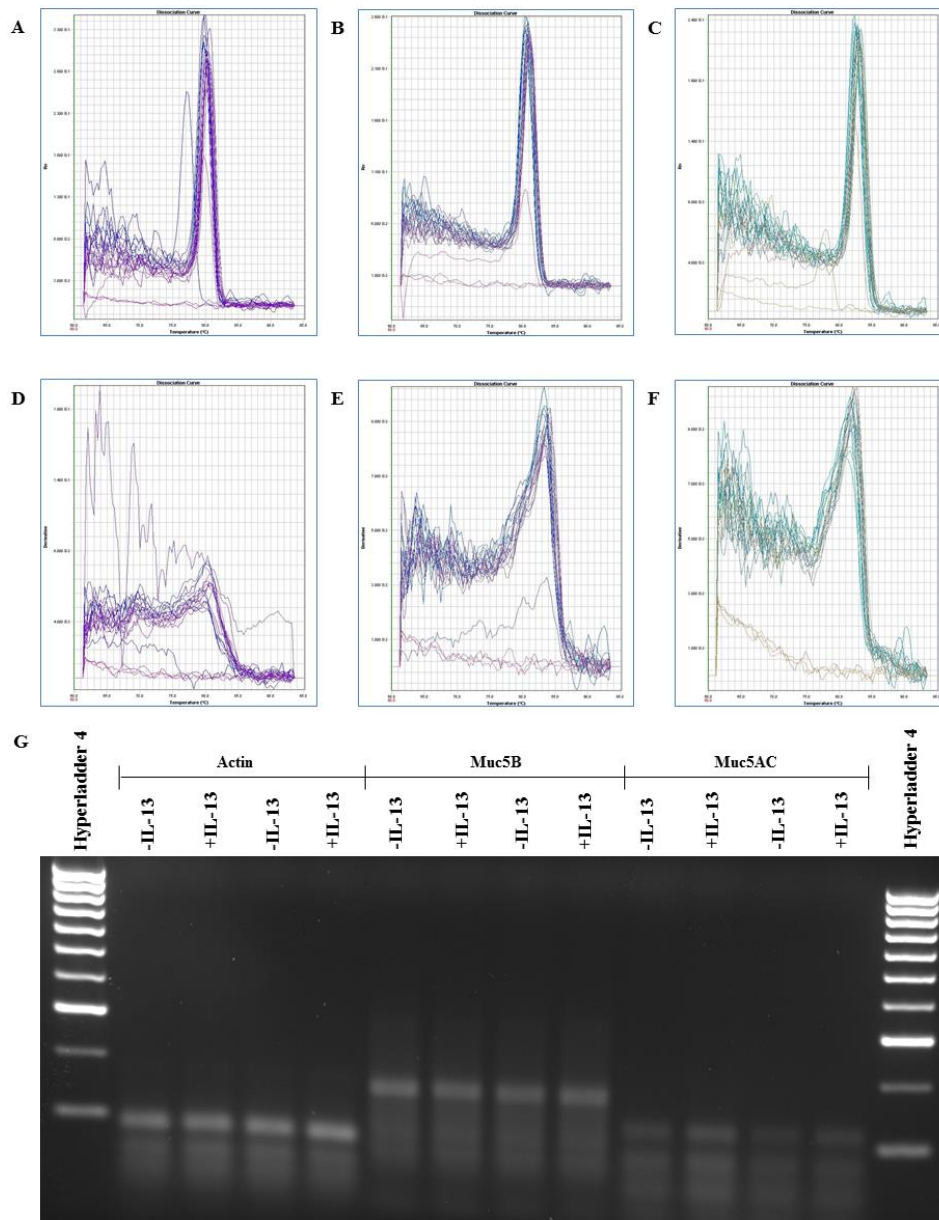
V5-epitope tagged LPLUNC1 proteins were generated by coupled *in vitro* transcription and translations (IVT-LPLUNC1) or from conditioned medium from stable CHO expressing cell lines (CHO-LPLUNC1) or control medium (CHO) as outlined in the materials and methods section. 2 μ l of the IVT reaction or 5 μ l of concentrated conditioned media was resolved on replicate 12% SDS-PAGE gels and Western blotted using the LPLUNC1B polyclonal antibody or an antibody to the V5 epitope as described (A). V5-epitope tagged PLUNC proteins were generated from conditioned medium from stable CHO expressing cell lines, expressing wt- and cys-mutant SPLUNC1, SPLUNC2 or LPLUNC1 (CHO-LPLUNC1) or control medium (CHO) as outlined in the materials and methods section. 5 μ l of concentrated conditioned media was resolved on replicate 12% SDS-PAGE gels and Western blotted using the LPLUNC1B polyclonal antibody or an antibody to the V5 epitope as described (B). All blots show presence of bands at the appropriate size for all samples. Data was generated by Dr Frances Barnes and taken from Bingle et al (2010).

Appendix 4 – qPCR Data

1. Confirmation of Amplification Product of Primer Pairs

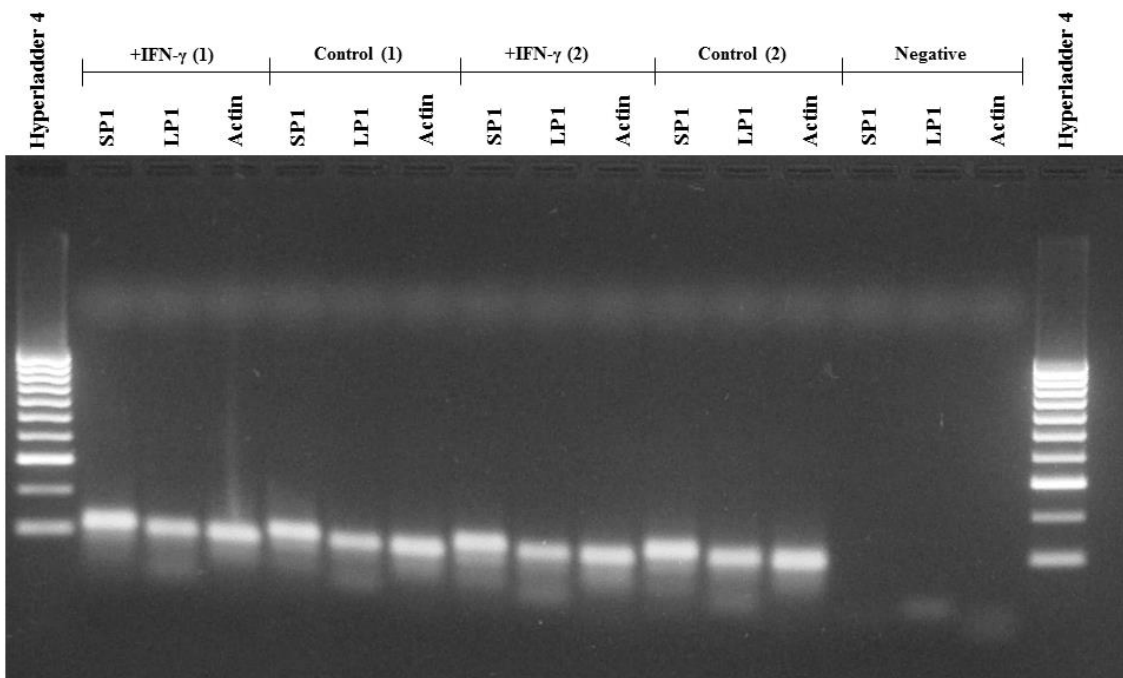
After the qPCR was complete, dissociation curves were performed to ensure that the desired amplicon was detected. SYBR green detects any dsDNA, including primer dimers and contaminating DNA resulting in a curve with multiple peaks, whereas a pure sample has one peak therefore eliminating the need to run a DNA gel. Dissociation curves were generated for SPLUNC1 (Appendix Figure 3A), LPLUNC1 (Appendix Figure 3B), Actin serving as the endogenous control for the PLUNC qPCR samples (Appendix Figure 3C), MUC5AC (Appendix Figure 3D), MUC5B (Appendix Figure 3E) and Actin serving as the endogenous control for the Mucin qPCR samples (Appendix Figure 3F). All curves, except MUC5AC, show a single peak, indicating amplification of a single product. However, the MUC5AC plot appears to show one sample that does not conform to the others so a selection of samples from this plate was run on a 2% agarose gel, including the ‘abnormal’ sample (Appendix Figure 3G). This shows bands for actin, MUC5B and MUC5AC at the expected sizes, confirming amplification of the right product. The sample loaded on the far right, next to the markers, is the ‘abnormal’ sample seen in the dissociations curve, which does appear to be any different to the other samples.

A 1.5% DNA gel was poured and two replicate samples of IFN- γ stimulated and control from the qPCR were resolved to confirm correct product size. The gel showed bands at the expected size for all three primer pairs, SPLUNC1, LPLUNC1 and actin, in all samples (Appendix Figure 4).



Appendix Figure 3: Dissociation Curve and Agarose Gel of qPCR products

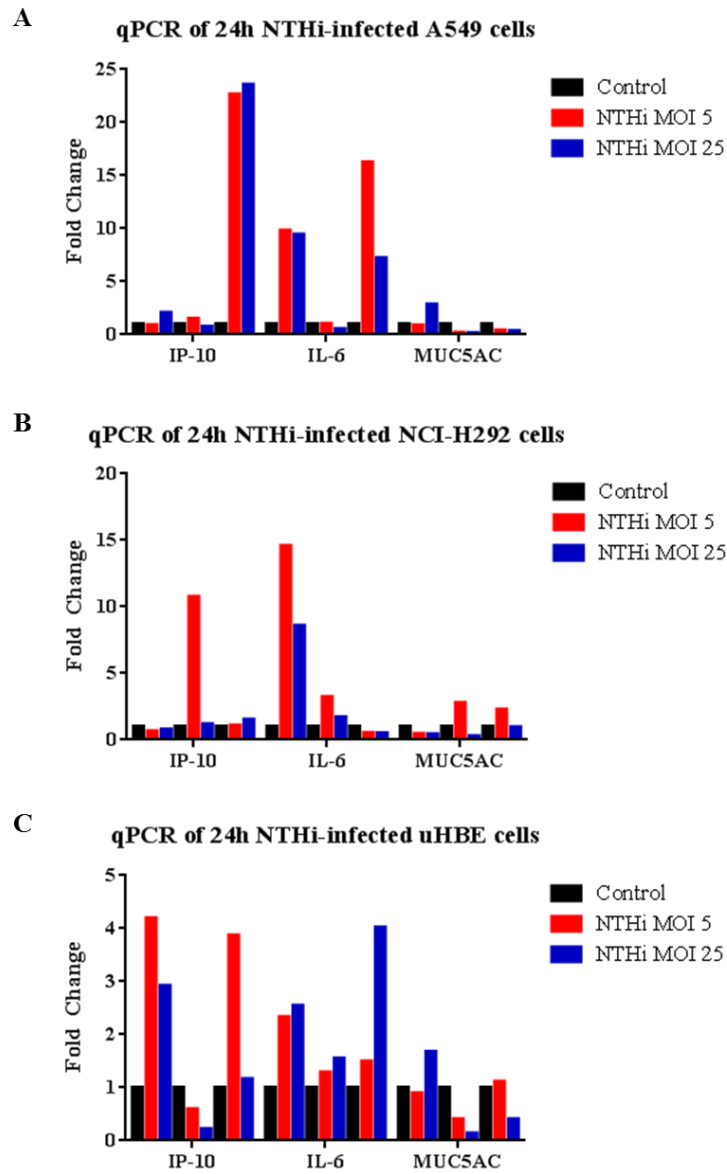
Dissociation curves were performed for SPLUNC1 (A), LPLUNC1 (B), Actin serving as the endogenous control for the PLUNC qPCR samples (C), MUC5AC (D), MUC5B (E) and Actin serving as the endogenous control for the Mucin qPCR samples (F). All curves, except MUC5AC, show a single peak, indicating amplification of a single product. However, the MUC5AC plot appears to show one sample that does not conform to the others so a selection of samples from this plate was run out on a 2% agarose gel, including the abnormal sample (G). This shows bands for actin, MUC5B and MUC5AC at the expected sizes, confirming amplification of the right product. The sample loaded on the far right, next to the markers, is the ‘abnormal’ sample seen in the dissociations curve, which does appear to be any different to the other samples.



Appendix Figure 4: DNA gel of samples from qPCR to confirm correct amplification product

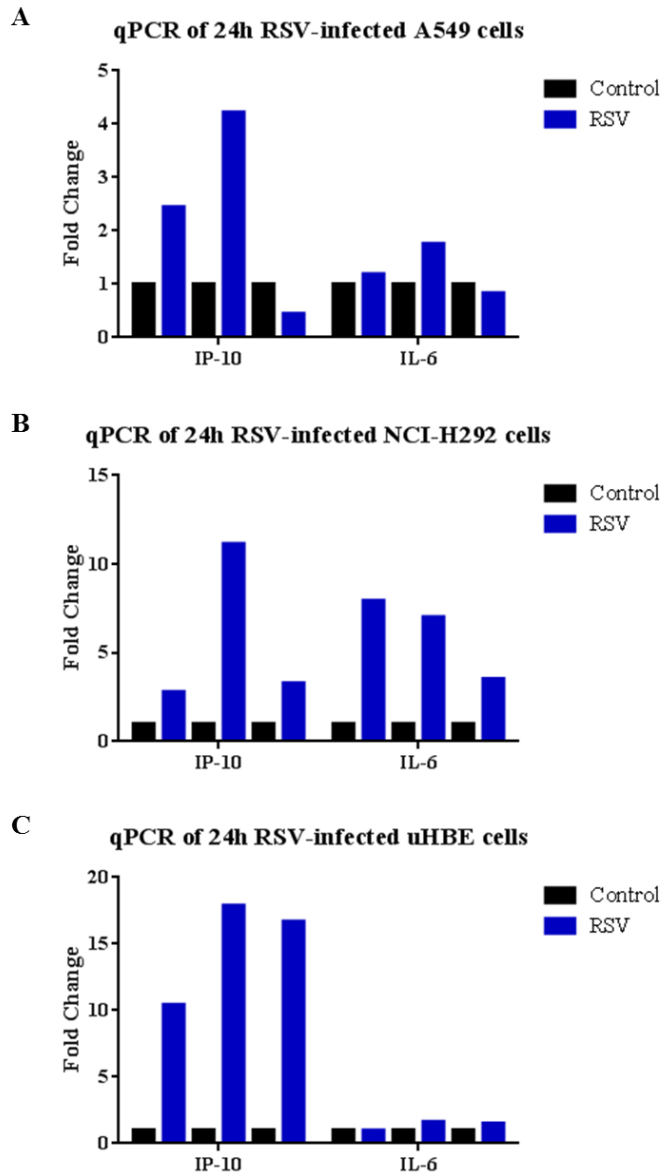
Samples from two repeats of the IFN- γ stimulated and control wells for SPLUNC1, LPLUNC1 and actin were run on a 1.5% agarose gel. The bands were of expected size in each sample.

2. Individual replicates of qPCR data



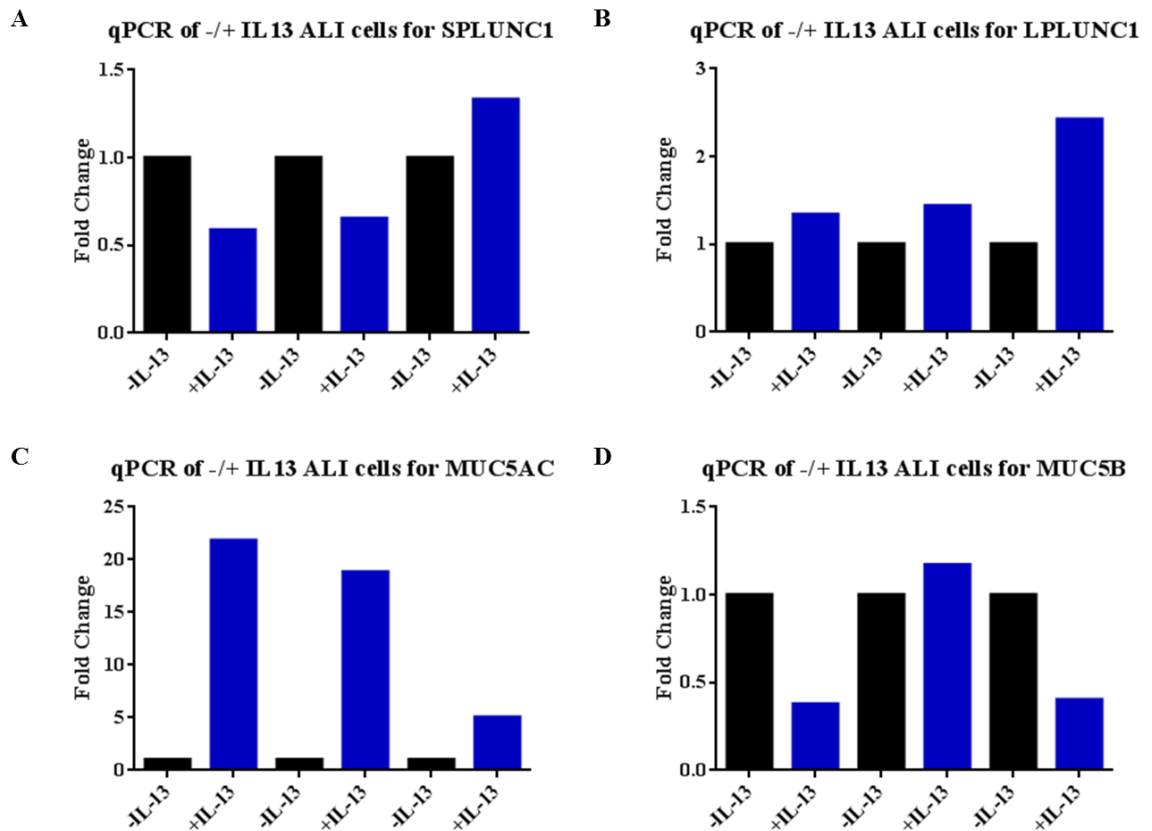
Appendix Figure 5: Fold change qPCR data for each individual replicate in 24h NTHi infection of A549, NCI-H292 and uHBE cells

Data for the individual replicates of qPCRs performed for IP-10, IL-6 and MUC5AC in A549 (A), NCI-H292 (B) and uHBE (C) cells to show the variation between replicates.



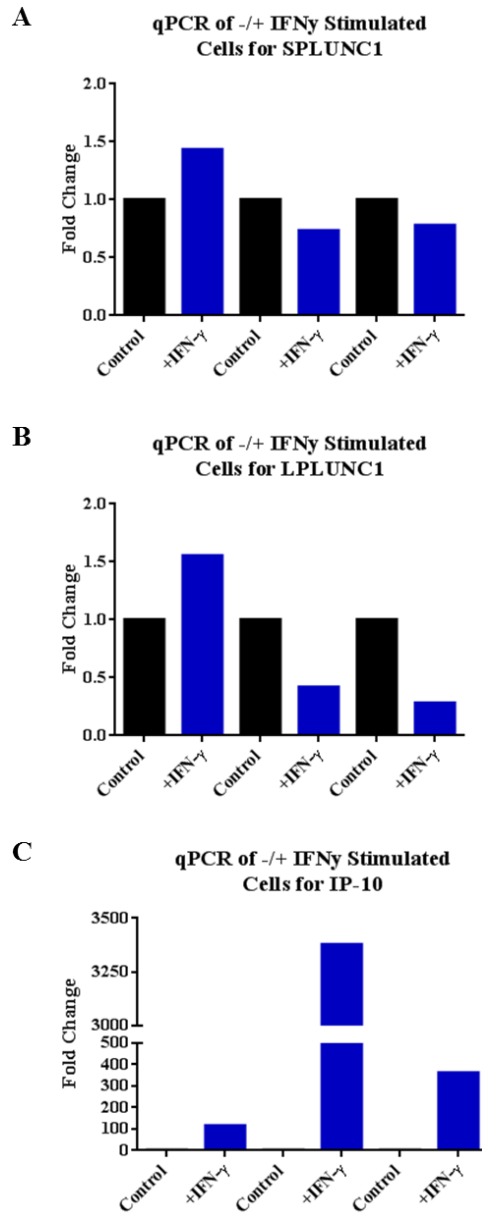
Appendix Figure 6: Fold change qPCR data for each individual replicate in 24h RSV infection of A549, NCI-H292 and uHBE cells

Data for the individual replicates of qPCRs performed for IP-10 and IL-6 in A549 (A), NCI-H292 (B) and uHBE (C) cells to show the variation between replicates.



Appendix Figure 7: Fold change qPCR data for each individual replicate in ALI cells cultured in the absence and presence of IL-13

Data for the individual replicates of qPCRs performed for SPLUNC1 (A), LPLUNC1 (B), MUC5AC (C) and MUC5B (D) in cells cultured in the absence and presence of IL-13 to show the variation between replicates.



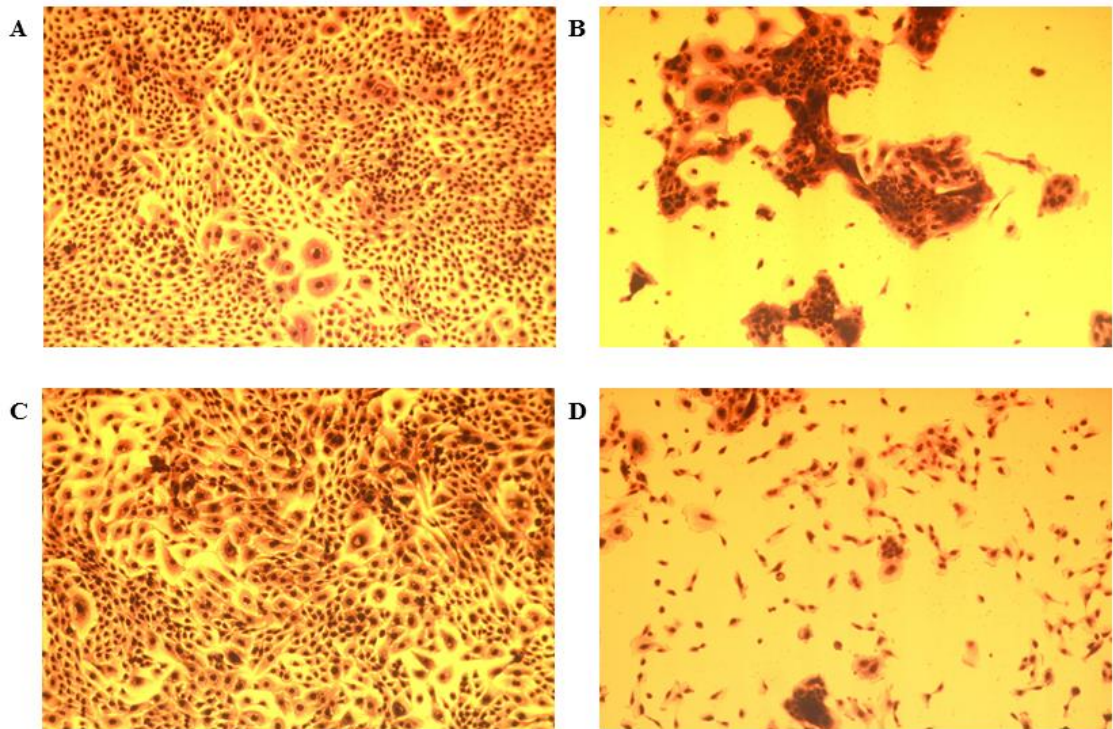
Appendix Figure 8: Fold change qPCR data for each individual replicate in ALI cells stimulated with IFN- γ

Data for the individual replicates of qPCRs for SPLUNC1 (A), LPLUNC1 (B) and IP-10 (C) in ALI cells treated with IFN- γ and controls to show the variation between replicates.

Appendix 5 – Preliminary Studies

1. Effect of ROCK Inhibitor on Cell Numbers

Primary human bronchial epithelial cells were seeded into 24 well plates at a density of 10,000 cells per well. Wells were cultured in the presence of 5 μ M or 10 μ M of the Rho Kinase inhibitor, Y27632, alongside control wells that were cultured in standard media. These were cultured for 3 days, after which they were fixed with methanol and stained using quick-diff stains; quick-diff red for 2 minutes, then quick-diff blue for 1 min and washed with tap water. These were then imaged using a Leica IMDRB microscope. Images of the monolayer primary cell cultures show increased cell numbers in wells treated with the Rho Kinase inhibitor in comparison to the control wells. There appears to be no difference between the 5 μ M and 10 μ M doses of Y27632.

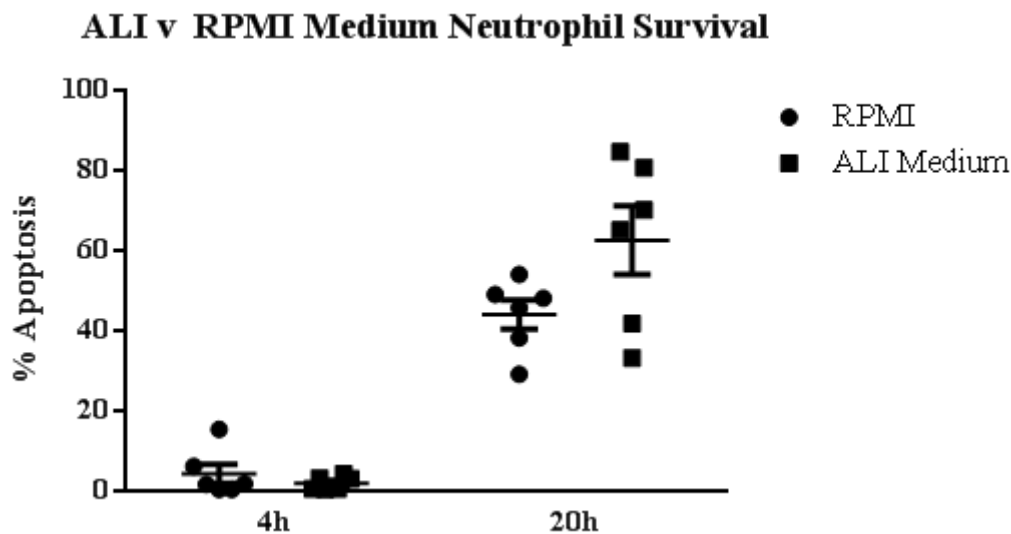


Appendix Figure 9: Culture of primary human bronchial epithelial cells with the Rho Kinase Inhibitor Y27632 increases cell proliferation

10,000 cells per well were cultured in the presence of 5 μ M (A) or 10 μ M (C) of Y27632 for 3 days alongside untreated controls (B and D). Quick-diff staining of cells showed increased cell numbers in treated wells than controls.

2. Neutrophil Studies

Neutrophils were cultured in RPMI medium and ALI medium for 4 and 20 hours. The samples were then cytopspun and stained using Quick-diff staining. The number of live and apoptotic neutrophils were counted and percentage apoptosis calculated. At 4 hours there appeared to be no difference between the two conditions; however at 20 hours the differences are much more visible. This difference was not found to be significant using a paired *t*-test for each time point.



Appendix Figure 10: Effect of ALI medium on neutrophil apoptosis

Neutrophils cultured in the RPMI medium they prefer and ALI medium for 4 and 20 hours showed no difference in levels of apoptosis after 4 hours. There was a more marked difference at 20 hours; however this was not significant when using a paired *t*-test for each time point.