Development of a primary airway epithelial cell culture model and explanted tissue archive to study the role of neutrophilic inflammation and airway remodelling in cystic fibrosis lung disease.

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Abstract

Cystic fibrosis (CF) is the most common inherited life-limiting condition in the United Kingdom. Lung disease, involving retention of mucopurulent secretions, neutrophilic inflammation and endobronchial infection is the major cause of mortality. CF is caused by variants in the CF-transmembrane conductance regulator gene, however the exact pathogenesis of lung disease is not fully understood. Valid experimental models are therefore critical to advance research.

I describe the establishment of a successful method to culture primary bronchial epithelial cells (PBECs) from explanted CF lungs removed at transplantation. This technique has yielded an important resource to further study the pathogenesis of CF lung disease.

The cytokine interleukin-17 orchestrates the activity of neutrophils and increases mucin gene expression in BECs – two key features of CF lung disease. I demonstrate that interleukin-17 is increased in the airway of people with advanced CF lung disease. I also show evidence suggesting that neutrophils themselves may be a source of interleukin-17 potentially leading to an ever-increasing spiral of inflammation.

In a CF mouse model ceramide accumulates in BECs and is associated with neutrophilic inflammation and susceptibility to *Pseudomonas aeruginosa* infection. Furthermore, amitriptyline treatment normalised ceramide, inflammation and susceptibility to infection. The role of ceramide is a complex area, however, with a divergence of opinion in the literature and paucity of human data. I demonstrate using immunohistochemistry that ceramide is increased in the lower airway epithelium in advanced CF lung disease compared to pulmonary hypertension and unused lung donors and is correlated with neutrophilic inflammation and increased in those colonised with *Pseudomonas aeruginosa*. Ceramide species C16:0, C18:0 and C20:0 but not C22:0 are increased in lung homogenates of CF lungs compared to pulmonary hypertension measured using the independent technique of high performance liquid chromatography-mass spectrometry.

Both interleukin-17 and ceramide represent important topics for further translational CF lung disease research.

Dedication

For Ashleigh and Thomas x

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I am indebted to a large number of people who have helped me in the completion of this work and without whom it would have been impossible.

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project and I appreciate the support that I have received from both of these organisations.

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Declaration

I declare that I undertook the work described in this thesis between September 2007 and August 2010 full-time at Newcastle University and the Freeman Hospital, Newcastle upon Tyne. I was directly involved in the planning, initial ethics and funding applications and was responsible for the day-to-day running, organisation and analysis of the results. I personally performed the laboratory work under the supervision of Dr Christopher Ward with the exception of the experiments listed below.

Gail Johnson, Freeman Hospital, performed expert immunohistochemistry; I performed the scoring and image analysis. Michael Hinchcliffe and Professor Jeff Pearson, Institute for Cell and Molecular Bioscience, Newcastle University, performed the slot blot enzyme-linked immunosorbent assay analysis for MUC5B. Dr Mike Gray and Dr Bernard Verdon, Institute for Cell and Molecular Bioscience, Newcastle University, performed the electrophysiology studies. Dr Joe Gray, Pinnacle, Institute for Cell and Molecular Bioscience, Newcastle University, performed the high performance liquid chromatography-mass spectrometry work. Staff of Electron Microscopy Research Services, Newcastle University, performed the scanning electron microscopy work. Reference is made in this thesis to reticular basement membrane measurements made previously by Dr Christopher Ward, Newcastle Univeristy, and interleukin-17 bronchoalveolar lavage measurements by Dr Des Murphy, Cork University Hospital, Republic of Ireland. Routine microbiological cultures were performed by Professor John Perry and Audrey Nicholson, Medical Microbiology, Freeman Hospital.

Awards

During the course of this PhD I have received the following awards:

Inaugural Joint Medical Research Council/Cystic Fibrosis Trust Clinical Research Training Fellowship, 2008

Best oral presentation: Northern Paediatric Research and Development Conference, 2008

British Lung Foundation Travel Fellowship, European Respiratory Society Annual Congress, 2008

Cystic Fibrosis Trust Travel Award, European Cystic Fibrosis Society Basic Science Meeting, 2009

Best poster: Institute of Cellular Medicine and Institute of Ageing and Health Research Day, judged by Professor Steven Holgate, 2009

Best oral presentation: Academic Paediatrics Association Winter Meeting, 2009

European Respiratory Society Bursary for the 8th European Respiratory Society Lung Science Conference, 2010

Publications

I was an author on the following list of manuscripts during the length of this PhD:

Publications directly relating to the work included in this thesis

Brodlie, M., McKean MC, Johnson GE, Gray J, Fisher AJ, Corris PA, Lordan J and Ward, C. (2010) 'Ceramide is increased in the lower airway epithelium of people with advanced cystic fibrosis lung disease', *Am J Respir Crit Care Med,* 182, (3), pp. 369-375.

Brodlie, M., McKean, M. C., Johnson, G. E., Perry, J. D., Nicholson, A., Verdon, B., Gray, M. A., Dark, J. H., Pearson, J. P., Fisher, A. J., Corris, P. A., Lordan, J. and Ward, C. (2010) 'Primary bronchial epithelial cell culture from explanted cystic fibrosis lungs', *Exp Lung Res,* 36, (2), pp. 101-10.

Brodlie, M., McKean, M. C., Johnson, G. E., Anderson, A. E., Hilkens, C. M. U., Fisher, A. J., Corris, P. A., Lordan, J. L. and Ward, C. (2010) 'Raised interleukin-17 is immunolocalised to neutrophils in cystic fibrosis lung disease', *Eur Respir J,* Nov 25, Epub ahead of print.

Publications arising from other related projects

Borthwick, L. A., McIlroy, E. I., Gorowiec, M. R., Brodlie, M., Johnson, G. E., Ward, C., Lordan, J. L., Corris, P. A., Kirby, J. A. and Fisher, A. J. (2010) 'Inflammation and epithelial to mesenchymal transition in lung transplant recipients: role in dysregulated epithelial wound repair', *Am J Transplant,* 10, (3), pp. 498-509.

Borthwick, L. A., Sunny, S. S., Oliphant, V., Perry, J., Brodlie, M., Johnson, G. E., Ward, C., Gould, K., Corris, P. A., De Soyza, A. and Fisher, A. J. (2010) 'Raised interleukin-17 is immunolocalised to neutrophils in cystic fibrosis lung disease', *Eur Respir J,* Sep 16, Epub ahead of print.

Brodlie, M. and McKean, M. C. (2009) ‘Exhaled nitric oxide in the diagnosis of childhood asthma’, *Bmj,* 339, pp. b5418.

Ward, C., Eger, K., Diboll, J., Jones, D., Haniffa, M. A., Brodlie, M., Fisher, A., Lordan, J. L., Corris, P. A. and Hilkens, C. M. (2009) 'Bronchial epithelial cells cultured from clinically stable lung allograft patients promote the development of macrophages from monocytes rather than dendritic cells', *Thorax,* 64, (5), pp. 430-5.

Brodlie, M., Eger, K., Hilkens, C. M. and Ward, C. (2009a) 'Airway epithelial cells as guardians of immune homeostasis?', *Thorax,* 64, (11), pp. 1009.

Brodlie, M., Flood, T. and Spencer, D. A. (2009b) 'Heterogeneity in the clinical manifestations of mannose-binding lectin deficiency', *J Pediatr,* 155, (5), pp. 764-5.

Brodlie, M., Flood, T. J., Black, F. and Spencer, D. A. (2009c) 'Lymphoid bronchiolitis presenting at birth in an immunocompetent child: Chronic interstitial lung disease of unknown aetiology', *Pediatr Pulmonol,* 44, (6), pp. 622-4.

Brodlie, M. and McKean, M. C. (2009) 'Dyspnoea and cough in a toddler', *Bmj,* 339, pp. b4436.

Brodlie, M. and Spencer, D. A. (2009) 'Bronchomalacia occurring in monozygotic twins--further information about its inheritance', *Acta Paediatr,* 98, (9), pp. 1531-3.

Brodlie, M., Chaudhari, M. and Hasan, A. (2008) 'Prostaglandin therapy for ductal patency: how long is too long?', *Acta Paediatr,* 97, (9), pp. 1303-4.

Brodlie, M. and McKean, M. C. (2008) 'Strategies to screen for adrenal suppression in children with asthma: there is no consensus among UK centres', *Thorax,* 63, (9), pp. 841-2.

Chaudhari, M., Brodlie, M. and Hasan, A. (2008) 'Hypertrophic cardiomyopathy and transposition of great arteries associated with maternal diabetes and presumed gestational diabetes', *Acta Paediatr,* 97, (12), pp. 1755-7.

Respiratory disorders in *Illustrated Textbook of Paediatrics* (Fourth edition) Eds. Lissauer T and Clayden G. Mosby, London. In press, 2010.

Presentations

I delivered the following presentations during the length of this PhD.

Invited talks

- *'Reflux and cystic fibrosis lung disease'* Reflux and its consequences – Key opinions in laryngeal, pulmonary and oesophageal manifestations. In conjunction with 9th International Symposium on Human Pepsin. Hull. April 2010.

- *'The culture of primary bronchial epithelial cells from the explanted lungs of people with cystic fibrosis (CF) removed at the time of transplantation – The European Perspective'* EurocareCF - Epithelial Cells From The Lung: Cultivation and Characterisation. July 2008. Lisbon, Portugal.

- *'The culture of primary bronchial epithelial cells from the explanted lungs of people with cystic fibrosis (CF) removed at the time of transplantation – A model to study CF lung disease'* European Cystic Fibrosis Society Basic Science Conference. April 2008. Douro, Portugal.

Talks to learned societies

- *'Ceramide is increased and associated with neutrophilic inflammation in the lower airway of people with cystic fibrosis'* British Thoracic Society, Winter Meeting, December 2009. London.

- **'Ceramide is increased and associated with neutrophilic inflammation in the lower airway epithelium in cystic fibrosis'* Academic Paediatrics Association. November 2009. Manchester. *Winner of best oral presentation.

- *'The cytokine interleukin-17 is raised in the airway of people with cystic fibrosis'* European Respiratory Society. September 2009. Vienna.

- *'Primary bronchial epithelial cells respond to a bile acid challenge by increasing interleukin-8 production'* Physiological Society. September 2009. Newcastle upon Tyne.

- *'Ceramide is a potential biomarker of epithelial injury which is raised in the airway of people with cystic fibrosis.'* European Cystic Fibrosis Society, New Frontiers in Basic Science of Cystic Fibrosis. April 2009. Tavira, Portugal.

- *'Mucus: what controls hydration?'* European Cystic Fibrosis Society, New Frontiers in Basic Science of Cystic Fibrosis. April 2009. Tavira, Portugal.

- *'The culture of primary bronchial epithelial cells from the lungs of people with cystic fibrosis removed at the time of transplantation: A model to study cystic fibrosis lung disease.'* British Thoracic Society, Winter Meeting. December 2008. London.

- *'The culture of primary bronchial epithelial cells from the lungs of people with cystic fibrosis removed at the time of transplantation - A model to study cystic fibrosis lung disease.'* Academic Paediatrics Association. November 2008. London.

- *'Strategies to screen for adrenal suppression in children with asthma – There is no consensus amongst United Kingdom centres'* Royal College of Paediatrics and Child Health, Annual Meeting. March 2008. York.

Poster presentations to learned societies

- **'Ceramide is increased and associated with neutrophilic inflammation in the lower airway of people with cystic fibrosis'* European Respiratory Society 8th Lung Science Conference. March 2010. Estoril, Portugal. *Awarded an ERS bursary

- *'Ceramide is increased and associated with neutrophilic inflammation in the lower airway of people with cystic fibrosis'* Clinician Scientists in Training Meeting, Medical Research Society/Academy of Medical Sciences. February 2010. London.

- *'Reticular basement membrane thickening in end-stage cystic fibrosis lung disease'* British Thoracic Society Winter Meeting, December 2009. London.

- *'Primary bronchial epithelial cell cultures from the explanted lungs of people with cystic fibrosis – a valuable resource for translational research'* European Cystic Fibrosis Society: New Frontiers in Basic Science of Cystic Fibrosis. April 2009. Tavira, Portugal.

- *'The culture of primary bronchial epithelial cells from the lungs of people with cystic fibrosis removed at the time of transplantation – A model to study cystic fibrosis lung disease'* Clinician Scientists in Training Meeting, Medical Research Society/Academy of Medical Sciences. March 2009. London.

- *'High-resolution computed tomography in children with confirmed post-infectious obliterative bronchiolitis.'* British Thoracic Society Winter Meeting. December 2008. London.

- *'Correlation of clinical and radiological findings in children with possible post-infectious obliterative bronchiolitis.*' European Respiratory Society Annual Congress. October 2008. Berlin, Germany.

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AC	acid ceramidase
ALI	air-liquid interface
Amph	amphotericin
ASL	airway surface liquid
ASM	acid sphingomyelinase
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BEGM	Bronchial Epithelial Growth Medium®
BSA	bovine serum albumin
CaCC	calcium-activated chloride channel
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
Ceft	ceftazidime
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
Cl^-	chloride
Colo	colomycin
CoTrim	co-trimoxazole
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Em	emphysema
EM	electron micrograph
ENaC	epithelial sodium channel
FCS	fetal calf serum
FEV_1	forced expiratory volume in 1 second
FOXP3	forkhead box P3
GATA3	GATA-binding protein 3
GM-CSF	granulocyte macrophage colony stimulating factor

H&E	haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC-MS	high performance liquid chromatography-mass spectrometry
IFNγ	interferon gamma
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
IMS	industrial methylated spirits
IRT	immunoreactive trpsinogen
MAPK	mitogen-activated protein kinase
Mero	meropenem
mRNA	messenger ribonucleic acid
MRC	Medical Research Council
Na^+	sodium
NADP	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa B
NHS	National Health Service
p-ANCA	perinuclear anti-cytoplasmic neutrophil antibodies
PAS	periodic acid-Schiff
PBEC	primary bronchial epithelial cell
PBS	phosphate-buffered saline
PDZ	post synaptic density protein, Drosophila disc large tumor suppressor and zonula occludens-1 protein
PH	pulmonary hypertension
PTC124	*Ataluren* compound
RBM	reticular basement membrane
RORγ	retinoic acid receptor-related orphan receptor gamma
RORC2	human orthologue of RORγt
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
S1P	sphingosine-1-phosphate
SEM	scanning electron microscopy
STAT3	signal transducer and activator of transcription 3
TBS	tris buffered saline
TCR	T cell receptor

TGF-β	transforming growth factor beta
TER	trans-epithelial resistance
Tim	timentin
T_H	T helper
Tobr	tobramycin
TNF-α	tumour necrosis factor alpha
T_{REG}	regulatory T cells
UD	Unused donor
Vanc	vancomycin
Vori	voriconazole
VX770	Vertex® 770 compound

1. Chapter 1. Introduction

1.1. Background: cystic fibrosis from 1938 to 2010

Cystic fibrosis (CF) is the most common life-limiting genetically acquired disease of white populations with an incidence of approximately 1 in 2500 newborns. (O'Sullivan and Freedman, 2009) Although less frequent, CF is also increasingly recognised in non-white racial groups. (Kabra et al., 2007, Kabra et al., 2006, Spencer et al., 1994) There are over 7000 individuals who have CF in the United Kingdom (UK), spanning both paediatric and adult age groups. (Davies *et al.*, 2007)

The first clinical description of CF was published in 1938. (Andersen, 1938) A disease entity, separate from infants with coeliac disease, consisting of mucus plugging of the pancreas was described and termed *'cystic fibrosis of the pancreas'*. The early clinical descriptions of CF were characterised by fat and protein malabsorption, steatorrhoea, growth failure and respiratory infection. It was assumed that pancreatic damage and subsequent deficiency of pancreatic enzymes resulted in malabsorption and poor nutrition leading to a predisposition for respiratory infections that were frequently terminal. (Davis, 2006) In 1944 Farber described thick and tenacious mucus obstructing the ducts of mucus glands throughout the body leading to the alternative terms *'mucoviscidosis'* and *'generalised exocrinopathy'*. (Farber, 1944, Di Sant'Agnese, 1956)

During the heat wave of 1948 in New York di Sant'Agnese observed that several infants presenting with dehydration fitted a CF phenotype. He demonstrated that their sweat was abnormal, containing five-fold concentrations of sodium and chloride, and that this abnormality persisted once the high temperatures had subsided. (Di Sant'Agnese, 1953)

The modern day term CF refers to a disease caused by variants in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that codes for a protein that functions primarily as a chloride channel. (O'Sullivan and Freedman, 2009, Sheppard and Welsh, 1999) CFTR is expressed in many epithelial cells, including sweat duct,

airway, pancreatic duct, intestine, biliary tree and *vas deferens*. This may give rise to an elevated sweat chloride concentration, lung disease characterised by bacterial infection and bronchiectasis, pancreatic insufficiency, intestinal obstruction, biliary cirrhosis and congenital bilateral absence of the *vas deferens*, often found in combination. (Davis, 2006)

Over the past two decades the level of understanding of the disease has increased rapidly and this has impacted significantly on the clinical management of people with CF. This has yielded a significant increase in survival. (Dodge *et al.*, 2007) Where CF used to represent a lung and gastrointestinal disease primarily of young children it is now a complex multi-system disorder that extends in to adulthood. Indeed there will soon be more adults than children with CF in the UK. Infants born today with CF have a predicted median survival of greater than 50 years. (Dodge *et al.*, 2007) Figure 1 shows the steady improvement in length of survival for people with CF born in the UK over the last four decades.

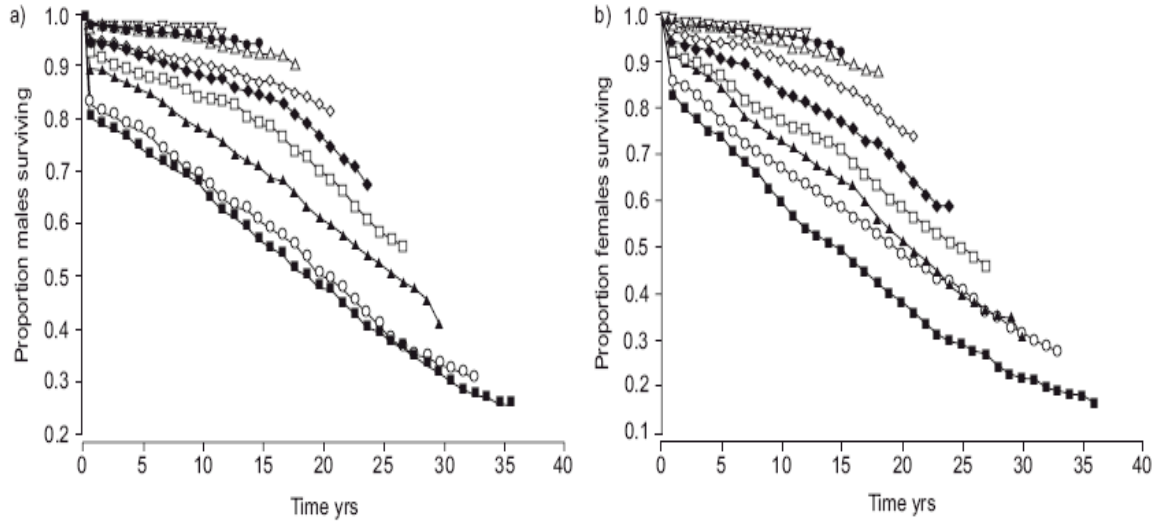


Figure 1. Proportion of a) males and b) females in the UK with CF, of each 3-year cohort, surviving until 2003.

■ 1968-70, ○ 1971-73, ▲ 1974-76, □ 1977-79, ♦ 1980-82, ◊ 1983-85, Δ 1986-88, ● 1989-91, ∇ 1992-94 (Dodge *et al.*, 2007)

1.2. Clinical aspects of cystic fibrosis

1.2.1. *Introduction*

As outlined above, CF is a multisystem disorder that arises from variants in the CFTR gene. (O'Sullivan and Freedman, 2009) Defects in ion and water transport across epithelia lead to a progressive disease that affects a number of different organs, including the upper and lower airways, pancreas, liver, and gastrointestinal and reproductive tracts. (Davies *et al.*, 2007)

1.2.2. *Newborn screening and diagnosis*

In 2007 screening for CF was introduced in the UK as part of the newborn bloodspot programme. (Downing and Pollitt, 2008) This programme involves initial screening of samples for the level of immunoreactive trypsinogen (IRT). Positive samples (around 0.5%) are then analysed for the most common CFTR variants and appropriate infants then referred for diagnostic sweat chloride testing. Importantly, the UK screening programme is designed to identify infants with CF, which is subsequently diagnosed formally by sweat testing, rather than to highlight heterozygote carriers. (Downing and Pollitt, 2008) It is inevitable however that some carrriers will be found by this method and also that some families will undergo the stress associated with an initial 'false positive' IRT result. (Kai *et al.*, 2009)

There is evidence from a North American randomised trial of nutritional benefits in children with CF diagnosed by newborn screening. (Farrell *et al.*, 1997) Pulmonary outcomes were influenced by confounding factors in this study, none the less there are genuine concerns regarding possible earlier acquisition of *Pseudomonas aeruginosa* in screened children. (Southern *et al.*, 2009) Prior to the introduction of newborn screening children were typically diagnosed when a sweat test was performed because of a chronic history of recurrent lower respiratory tract infections and growth faltering. Delay in diagnosis was common along with the inevitable associated morbidity for the child and emotional stress for the family involved. (Jackson *et al.*, 2010)

1.2.3. *Clinical aspects of cystic fibrosis lung disease*

It is estimated that lung disease is responsible for over 95% of morbidity and mortality in people with CF. (Doring *et al.*, 2007) Clinically CF lung disease is characterised by chronic endobronchial infection with specific microorganisms including *Staphylococcus aureus, Haemophilus influenzae* and *P. aeruginosa*, neutrophilic inflammation and retention of mucopurulent secretions. This results in progressive bronchiectasis, decline in lung function and ultimately respiratory failure in young adulthood (Figure 2). The mainstays of treatment include intensive physiotherapy and inhaled mucolytics to aid clearance of secretions and prophylactic or treatment antimicrobials to deal with infection. (Davies *et al.*, 2007) Lung transplantation is the only life-preserving intervention for end-stage CF lung disease. (Meachery *et al.*, 2008)

1.2.4. *Non-pulmonary manifestations of cystic fibrosis*

Brief details of the common non-pulmonary clinical manifestations of CF and appropriate interventions are summarised in Table 1.

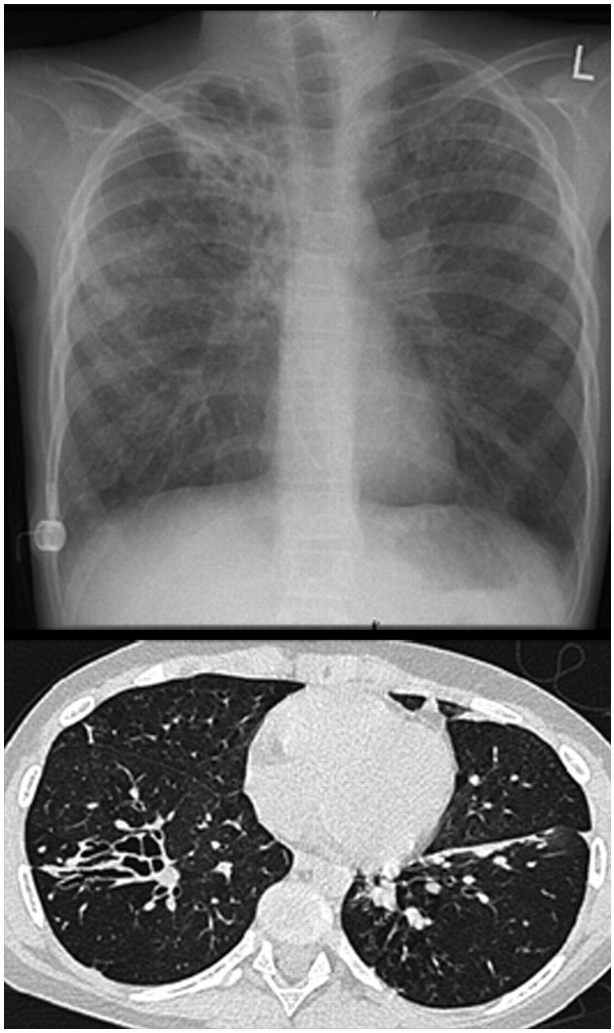


Figure 2. Severe bronchiectasis in end stage cystic fibrosis shown in chest radiograph and computed tomogram.

(Davies *et al.*, 2007)

Organ	Problem(s)	Intervention
Pancreas	Exocrine insufficiency and malabsorption	High-fat diet, supplementation of pancreatic enzymes and fat- soluble vitamins
	Pancreatitis	Largely symptomatic
	Endocrine insufficiency: CF-related diabetes	Insulin
Oesophagus	Gastro-oesophageal reflux	Pro-kinetics, acid suppression, fundoplication
Small intestine	Meconium ileus	Gastrografin enemas, surgery
	Distal Intestinal Obstruction Syndrome	Gastrografin, surgery
Large bowel	Rectal prolapse	Pancreatic enzymes, surgery
Liver	CF-related liver disease	Ursodeoxycholic acid, vitamin K supplements, transplantation
Upper airway	Nasal polyps	Topical steroids, surgery
	Sinusitis	Topical steroids or antibiotics, surgery
Bones	Osteopaenia	Exercise, calcium and vitamin D supplements
Sweat glands	Increased electrolyte loss	Electrolyte supplements
Reproductive tract	Bilateral absence of *vas deferens*	Assisted fertilisation techniques

Table 1. Non-pulmonary manifestations of cystic fibrosis and interventions

1.3. The cystic fibrosis transmembrane conductance regulator (CFTR)

The gene encoding CFTR was first cloned in 1989 and is situated on the long arm of chromosome 7. (Riordan et al., 1989, Kerem et al., 1989, Rommens et al., 1989) CFTR is a member of the ATP-binding cassette transporter superfamily. (Verkman and Galietta, 2009) The major function of CFTR is as a chloride channel that regulates ion and water balance across epithelia. (Sheppard and Welsh, 1999) CFTR is also involved in the regulation of a multitude of other physiological cellular processes including bicarbonate-chloride exchange, sodium transport via the epithelial sodium channel (ENaC), acidification of intracellular organelles and calcium-activated chloride channel (CaCC) function. (Quinton, 2008, Reisin et al., 1994, Schwiebert et al., 1995, Stutts et al., 1995, Mehta, 2005) The protein contains 1480 amino acids and includes two membrane-spanning domains, two nucleotide-binding domains and a unique regulatory domain. (Hwang and Sheppard, 2009)

Activation of CFTR relies on phosphorylation, particularly through protein kinase A, but most probably also involves a complex system of other kinases and phosphatases as well. (Ostedgaard et al., 2001, Hwang and Sheppard, 2009) Channel activity is governed by the two nucleotide-binding domains, which regulate channel gating. The carboxyl terminal of CFTR is anchored through a PDZ-type-binding interaction with the cytoskeleton and is kept in close approximation with a number of associated proteins. (Borthwick *et al.*, 2007) These proteins influence CFTR function, including conductance, regulation of other channels, signal transduction, and localization at the apical plasma membrane. (Rowe *et al.*, 2005) The hypothesized structure of CFTR is shown in Figure 3.

Over 1500 loss-of-function CFTR variants have been identified, although the number of true disease-causing variants is probably much lower. (Proesmans et al., 2008, Verkman and Galietta, 2009) CFTR variants are recessive and may be classified in to five classes depending on their effect on the CFTR protein. (Wilschanski et al., 1995) Table 2 describes the classes of variants I to V and Figure 4 depicts each class diagrammatically.

By far the most frequent disease-causing variant is p.Phe508del. It is traditionally considered to be a class II variant resulting from deletion of phenylalanine at position 508 and leading to aberrantly folded CFTR protein that is identified and degraded in the endoplasmic reticulum. (Cheung and Deber, 2008) This defective trafficking of the protein is thought to result in the absence of mature CFTR at the apical membrane, although this is still a subject of some debate. (LA Borthwick, personal communication August 2010) There is evidence from work in nasal brushings and mice that p.Phe508del should also be considered to be a class IV variant with some apically localised p.Phe508del CFTR present in homozygotes. (Penque et al., 2000) Importantly p.Phe508del CFTR exhibits a reduced open probabililty compared to wild type CFTR. (Pilewski and Frizzell, 1999) p.Phe508del accounts for 70% of all variant alleles with 10-20 less common variants accounting for another 15%. (Proesmans *et al.*, 2008)

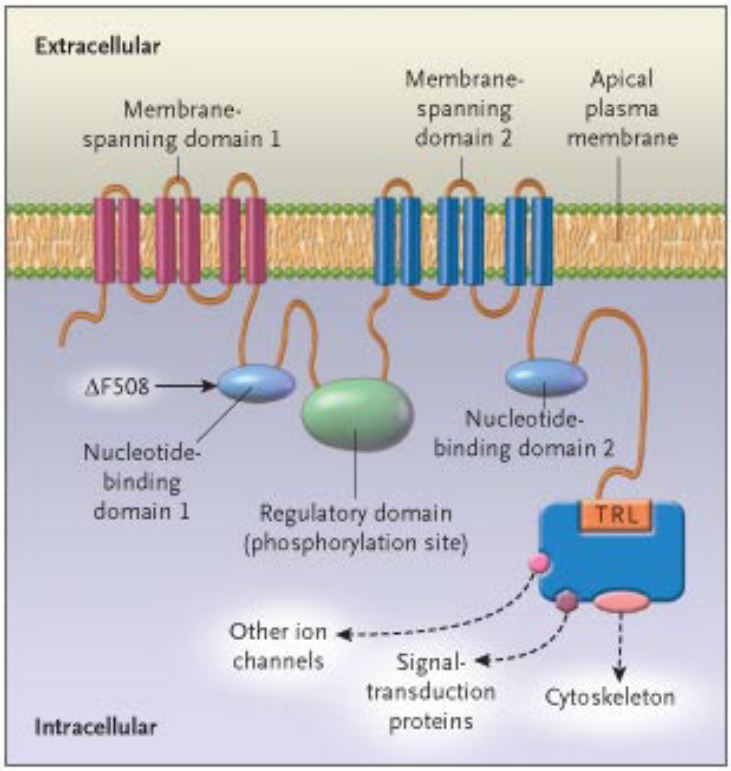


Figure 3. Hypothesised structure of the cystic fibrosis transmembrane conductance regulator (CFTR)

(Rowe *et al.*, 2005)

Class of variant	Effect	Example of common variant allele
I	No CFTR mRNA or protein formed, e.g. nonsense, frame shift or splice site mutation	p.Gly542X
II	Trafficking defect. CFTR protein forms but fails to traffic to cell membrane	p.Phe508del (also class IV)
III	Regulation defect. CFTR reaches the cell membrane but does not respond to cAMP stimulation	p.Gly551Asp
IV	Channel defect. CFTR functions as a defective Cl^- channel	p.Arg117His
V	Synthesis defect. Reduced or defective processing of normal CFTR	p.Ala455Glu

Table 2. Description of different classes of cystic fibrosis transmembrane conductance regulator (CFTR) variants

(Proesmans et al., 2008, Wilschanski et al., 1995)

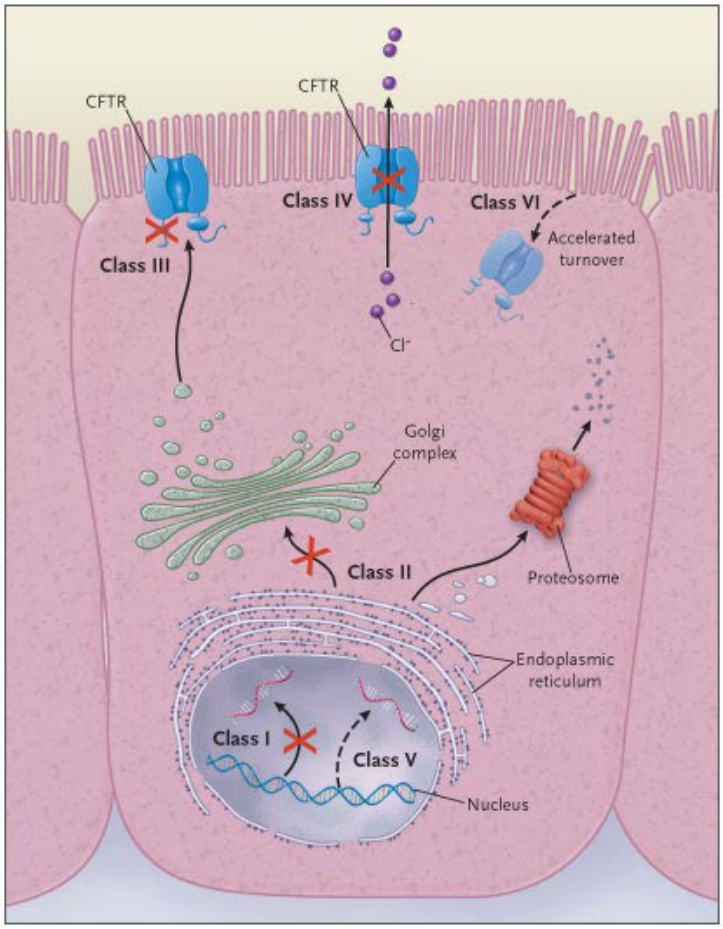


Figure 4. Schematic representation of the different classes of cystic fibrosis transmembrane conductance regulator (CFTR) variants

(Rowe *et al.*, 2005)

1.4. The science of cystic fibrosis lung disease

1.4.1. *Pathogenesis*

The lung histology of newborns with CF is essentially structurally normal with the exception of mild dilatation and plugging of submucosal gland ducts. (Konstan and Berger, 1997, Elizur et al., 2008) As outlined later in this thesis, there is some evidence of a pro-inflammatory milieu in the lungs of foetuses with CF however. (Hubeau *et al.*, 2001b) The respiratory tract is constantly exposed to numerous airborne pathogens. A wide array of innate host defences and clearance mechanisms are therefore critical to prevent infection. (Martin and Frevert, 2005, Chilvers and O'Callaghan, 2000) In CF these defences fail and the airways of young children with CF become inflamed with a predominantly neutrophilic infiltrate and infected with bacteria such as *S. aureus* and *H. influenzae* quite rapidly. (O'Sullivan and Freedman, 2009, Stafler et al., 2009) Airway neutophilia has been detected in bronchoalveolar lavage (BAL) fluid from clinically well infants with CF (Khan *et al.*, 1995) A longstanding debate has existed in CF research as to whether inflammation precedes infection, or *vice versa*, this is discussed in more detail later in the thesis. (Wine, 2010)

P. aeruginosa subsequently becomes the most prominent airway pathogen in older children and adults with CF. (Rosenfeld *et al.*, 2003) *P. aeruginosa* has a particular predilection for the CF airway and chronic infection appears to be frequently established with a number of potential explanations. These include the presence of hypoxic microenvironmental niches in adherent mucus plugs, increased bacterial binding to epithelial cells deficient in CFTR, reduced innate immune defences and complex adaptations in the bacteria themselves, including biofilm formation, quorum signalling and even immunomodulation. (Matsui et al., 2006, Worlitzsch et al., 2002, Imundo et al., 1995, Campodonico et al., 2008, Davies et al., 1998, Buchanan et al., 2009)

An ever-increasing vicious cycle then ensues of chronic endobronchial infection, neutrophilic inflammation and obstruction of airways and submucosal gland ducts by tenacious secretions consisting of mucus, inflammatory debris and bacteria. (Rubin, 2009, Elizur et al., 2008) The natural history of CF lung disease is typified by a gradual

drop in lung function punctuated by more marked drops associated with acute infective exacerbations. Progressive, severe bilateral bronchiectasis and ultimately respiratory failure and premature death occur. (O'Sullivan and Freedman, 2009) The only life-sustaining intervention for end-stage disease is lung transplantation. (Meachery *et al.*, 2008)

The precise connection between CFTR dysfunction and the chronic inflammation and infection present in CF lung disease has not been fully elucidated and has been the subject of a huge amount of research and indeed debate in the scientific literature. (Wine, 2010) The *low volume hypothesis* proposed by Boucher and colleagues from the University of North Carolina, USA is currently the most widely accepted explanation. (Boucher, 2007a) This theory involves excessive isotonic absorption in the airway reducing the periciliary liquid volume and concentrating mucus resulting in grossly defective mucociliary transport. Retained mucus then serves as a nidus for chronic bacterial infection. In addition to the results of several elegant *in vitro* experiments performed by Matsui (Figure 5) and colleagues to support this theory, the most striking clinical correlate is the recent proof that nebulisation of hypertonic saline, resulting in increased airway surface liquid volume, in people with CF improves mucus clearance and lung function and reduces pulmonary exacerbations (Figure 6). (Elkins et al., 2006, Donaldson et al., 2006, Matsui et al., 1998, Tarran et al., 2007, Boucher, 2007b)

At least four other hypotheses have been put forward to explain how the presence of defective CFTR leads to the clinical CF phenotype and it is possible that aspects of all the various hypotheses contribute to the pathogenesis of CF lung disease *in vivo*. (O'Sullivan and Freedman, 2009) The *high-salt hypothesis* argues that as a result of defective CFTR the concentration of sodium and chloride is raised in the airway surface liquid leading to dysfunction of innate antimicrobial peptides and subsequent chronic opportunistic pulmonary infection. (Goldman et al., 1997, Smith et al., 1996, Zabner et al., 1998)

In addition to effects on airway surface liquid and mucociliary transport there is also evidence that CFTR dysfunction *per se* leads to a pro-inflammatory milieu that results in airway inflammation prior to infection and exaggerated inflammatory responses once infection is established. (Major and Elborn, 2009) CFTR dysfunction may also cause a primary predisposition to infection. It is known that *P. aeruginosa* binds to functional

CFTR and normally initiates an appropriate, rapid and self-limiting innate immune response. This effect is lost in the presence of defective CFTR and furthermore there is increased binding of *S. aureus* and *P. aeruginosa* via lipid rafts to the apical membrane of epithelial cells. (Campodonico et al., 2008, Imundo et al., 1995) Accumulation of ceramide in airway epithelial cells has also been recently implicated in the pathogenesis of CF lung disease as discussed comprehensively in section 1.7. Paul Quinton, University of California, has also postulated that defective bicarbonate secretion plays a significant role in the pathogenesis of CF and leads to poorly solubilised mucins with the subsequent accumulation of mucus that is found in the affected organs of people with CF. (Quinton, 2008)

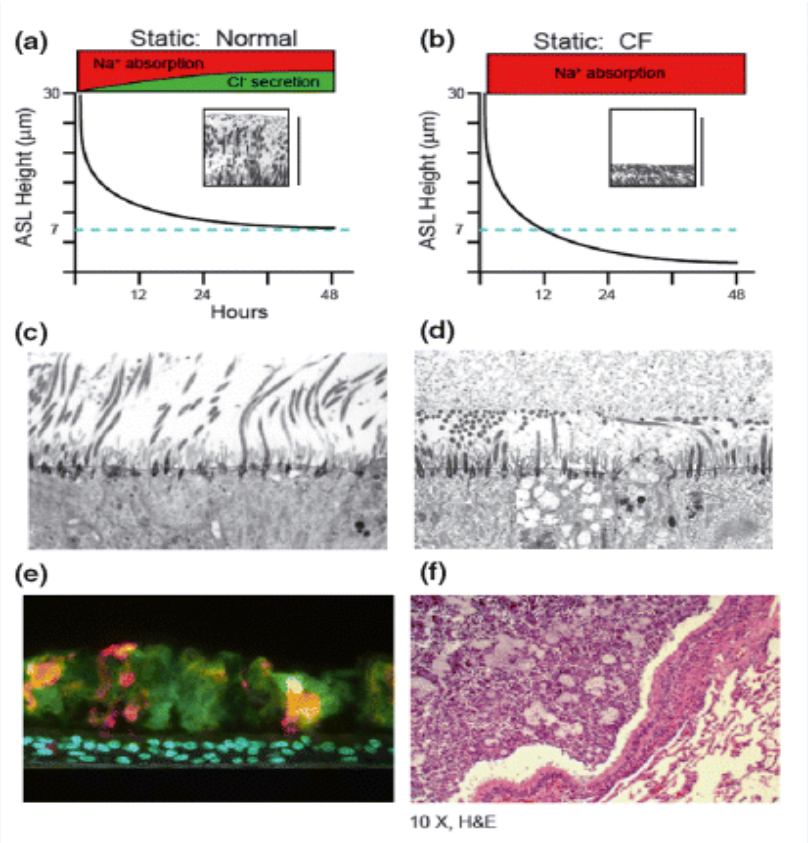


Figure 5. Evidence from well-differentiated human airway cultures supporting airway surface liquid (ASL) volume depletion in the pathogenesis of cystic fibrosis lung disease

(a) Measurement of ASL volume after addition of small volume of 'ASL' in normal airway epithelia devoid of mucus layer. ASL is absorbed until height is reached (~7 µm) that maintains beating of extended cilia (see EM insert). Bar above depicts relative rates of Na+ absorption versus Cl− secretion with ASL height. (b) Measurement of ASL volume (height) after addition of ASL to CF culture. Note, added ASL is absorbed more rapidly than in normal cultures and all ASL is absorbed, as defined by the absence of sufficient liquid to maintain ciliary extension (see EM insert). Bar above illustrates that Na+ absorption persists, and there is a failure to secrete Cl−, despite inappropriately low ASL height/volume. (c, d) Similar experiments, but with mucus layer left intact on normal (c) and CF (d) cultures. Note concentration of mucus and apposition/adhesion of mucus to 'bent over' cilia in CF. e) Immunohistochemistry of mucus layer adherent to CF culture after ASL volume depletion. A mucus plaque has formed on the epithelial surface (containing nuclei labelled with DAPI) composed of MUC5AC (red) and MUC5B (green). (f) Light micrograph of airway excised from young adult CF patient. Within the airway lumen is mucopurulent material containing multiple round macrocolony-like biofilms of *Pseudomonas aeruginosa*. (Boucher, 2007b)

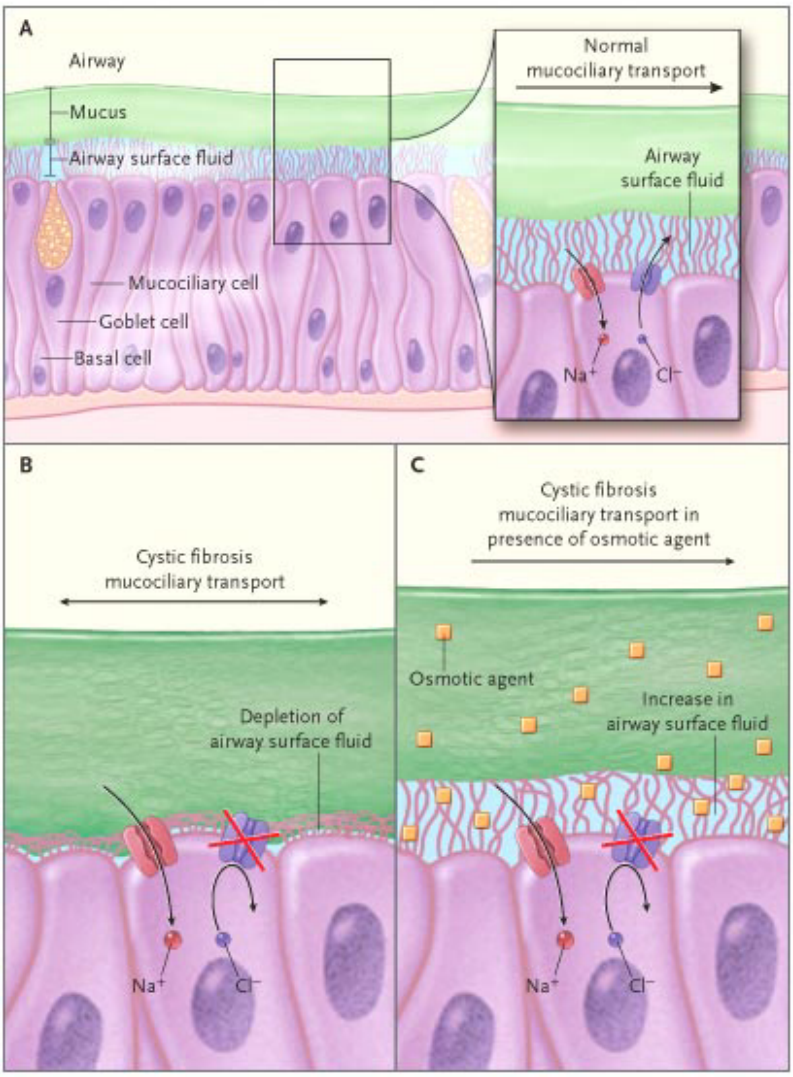


Figure 6. Mucociliary transport in the healthy lung and in cystic fibrosis and the therapeutic effect of osmotic agents.

A fluid layer that is maintained through a balance of chloride secretion and sodium absorption covers the surface of airway epithelial cells. The airway surface fluid supports a thin mucus layer produced by mucosal secretory glands. The mucus layer is transported by respiratory cilia from the lower airways to the airway opening (Panel A). In cystic fibrosis, defective chloride secretion and sodium hyperabsorption lead to the depletion of the layer of airway surface fluid, with consecutive breakdown of mucociliary transport (Panel B). Osmotic agents increase the volume of airway surface fluid through an increase in water influx, thereby restoring mucociliary function (Panel C). (Ratjen, 2006)

1.4.2. *The role of inflammation in cystic fibrosis lung disease*

As mentioned earlier in this thesis, lung histopathology from newborns with CF is essentially structurally normal with the exception of mild dilatation and plugging of submucosal gland ducts. (Konstan and Berger, 1997, Elizur et al., 2008) However, there is some evidence of an *in vivo* pro-inflammatory state in the lungs of foetuses with CF. This includes dysregulated activation of the nuclear factor-kappa B (NF-κB) pathway and infiltration of the airways by macrophages. (Verhaeghe et al., 2007, Hubeau et al., 2001b)

Direct sampling studies from young children with CF have identified raised baseline levels of inflammatory biomarkers and both increased and exaggerated inflammatory responses to bacterial infection. Khan and colleagues demonstrated raised neutrophil counts and levels of interleukin (IL)-8 and neutrophil elastase in BAL fluid from asymptomatic infants with CF diagnosed by newborn screening compared to controls. Moreover the BAL fluid from 7 of the 16 children with CF was negative on culture for bacteria and viruses but markers of inflammation were still raised. (Khan *et al.*, 1995) Similarly Balough *et al.* (1995) and Rosenfeld *et al.* (2001) found raised neutrophil counts in sterile BAL fluid from young children with CF but minimal respiratory symptoms. (Balough et al., 1995, Rosenfeld et al., 2001) In a larger study, Muhlebach and colleagues obtained BAL fluid from children with CF and controls both with and without positive cultures for bacterial lower respiratory tract infection. They found that children with CF in both the infected and non-infected groups had raised neutrophil counts and that the ratio of IL-8 and neutrophils to number of bacteria in those infected were raised. (Muhlebach *et al.*, 1999)

An important deficiency of these studies however is that in order to warrant a bronchoscopy all of these children had a history of recent lower respiratory tract symptoms. Conversely Armstrong *et al.* found no significant difference in BAL fluid neutrophil counts and inflammatory markers in truly infection naïve children with CF compared to controls. (Armstrong et al., 2005, Armstrong et al., 1995) It is perhaps significant though that around a third of the control group had either a positive growth

from BAL fluid or symptoms of lower respiratory tract infection within 48 hours of the bronchoscopy. (Rao and Grigg, 2006)

There is indirect evidence of a pro-inflammatory state in CF from work showing that cultured peripheral monocytes from adults with the disease produce increased amounts of tumour necrosis factor-alpha (TNF-α) compared to controls. (Pfeffer *et al.*, 1993) Macrophages cultured from a CF mouse model contain four times the TNF-α messenger ribonucleic acid (mRNA) found in macrophages from wild-type mice. (Thomas *et al.*, 2000) In addition, airways from infection naïve CF mice grafted on to animals with severe combined immunodeficiency produce raised amounts of IL-8 relative to controls and ultimately display luminal inflammation. (Tirouvanziam *et al.*, 2000)

In contrast however, recently published work performed using a new porcine model of CF suggests that there is no significant difference between CF and wild-type newborn piglets in baseline levels of BAL fluid IL-8 and inflammatory cell counts. (Stoltz *et al.*, 2010) The newborn CF piglets did have evidence of a defective capacity to eliminate bacteria however. (Stoltz *et al.*, 2010)

There is also evidence that in addition to a pro-inflammatory state that there may be defective down regulation of inflammation in CF lung disease. Work performed by Bonfield *et al.* has demonstrated that IL-10 production is decreased in bronchial epithelial cells from people with CF compared to controls. (Bonfield *et al.*, 1995)

In conclusion there is good evidence that an exaggerated inflammatory response occurs in the CF airway to infection. There is also inconclusive evidence that the airway may be primed in a pro-inflammatory state prior to infection.

1.4.3. *How is airway inflammation damaging in cystic fibrosis lung disease?*

Intense endobronchial and peribronchial neutrophilic inflammation are the hallmarks of the histopathology of established CF lung disease. (Hubeau *et al.*, 2001a) The principal chemoattractant for neutrophils in the CF airway is IL-8. (Downey *et al.*, 2009) Bronchial epithelial cells and immune cells such as macrophages and dendritic cells

produce IL-8 in the lungs. A wide range of other factors such as leukotriene B4, IL-1, IL-6, granulocyte-macrophage colony-stimulating factor (GMCSF), TNF-α and more recently IL-17 have also been identified as important in the modulation of the inflammatory response. (Downey et al., 2009, Brodlie et al., 2009c) This includes stimulating further production of IL-8 and recruitment of immune cells such as neutrophils and macrophages. (Elizur *et al.*, 2008) Neutrophils then trigger the release of additional pro-inflammatory mediators and chemoattractants resulting in a sustained inflammatory response and positive feedback loop. (Downey *et al.*, 2009)

Neutrophils contain a number of proteolytic enzymes that are stored in specific cytoplasmic granules and are critical in intracellular pathogen destruction and hence response to infection. (Haslett *et al.*, 1989) Large amounts of these proteases, such as the serine proteases neutrophil elastase, proteinase 3 and cathepsin G and matrix metalloproteases-8 and -9, are released in to the extracellular milieu on neutrophil degranulation or death and subsequently mediate tissue damage. (Linden et al., 2005, Korkmaz et al., 2008) Neutrophil elastase in particular digests a diverse range of substrates found in the lung including the structural proteins elastin and fibronectin and further activates pro-matrix metalloprotease-9. (Gaggar et al., 2007, Korkmaz et al., 2008) In the CF lung this large protease burden overwhelms intrinsic anti-protease activity and results in epithelial injury, airway remodelling and irreversible damage to structure leading to severe bronchiectasis. (Balfour-Lynn, 1999, Griese et al., 2008, Hilliard et al., 2007) There is also evidence from *ex vivo* studies that neutrophil elastase stimulates airway gland mucus secretion. (Schuster et al., 1992, Cardell et al., 1999)

On a clinical basis there is indirect evidence of the role of neutrophil elastase from work published by Sagel *et al.* (2002). They found that the concentration of neutrophil elastase in induced sputum from stable children with CF correlated negatively with their forced expiratory volume in one second (FEV_1). (Sagel *et al.*, 2002)

Pathogens are also eliminated by neutrophils via cytotoxic reactive oxygen species that are generated through a membrane-associated nicotinamide adenine dinucleotide phosphate (NADP)-oxidase system. (Roos *et al.*, 2003) Along with oxygen radicals from bacterial products and the environment this produces a cumulative oxidative stress in the CF lung that serves to further exacerbate airway injury. (Elizur *et al.*, 2008) Kettle and colleagues found evidence of oxidative stress in the airways of young

children with CF in terms of raised concentrations of myeloperoxidase, 3-chlorotyrosine, a biomarker for hypochlorous acid, and protein carbonyls in BAL fluid. (Kettle *et al.*, 2004)

Finally, on cell death neutrophils release large amounts of deoxyribonucleic acid (DNA) that constitutes a major polymeric component of the highly adhesive secretions characteristic of CF lung disease. (Rubin, 2007) The successful use of nebulised dornase alpha, which hydrolyses DNA in the airway, to improve mucociliary clearance in many people with CF provides practical evidence of the importance of increased concentrations of DNA in secretions in CF lung disease. (Jones and Wallis, 2003)

1.5. Interleukin-17

1.5.1. *Introduction*

IL-17 is the signature cytokine of the recently described T_H-17 subset of $CD4^+$ T cells. (Gaffen, 2009) The T_H-17 cell lineage represents the first major revision to the landmark T_H-1/T_H-2 paradigm proposed by Mossman and Coffman over 20 years ago and addresses a number of discrepancies that had become apparent in the model. (Mosmann *et al.*, 1986) IL-17 was first described and cloned in 1993. (Yao et al., 1995b, Rouvier et al., 1993) A family of cytokines has now been described that comprises of six unique homodimeric domains, namely IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F. The IL-17 family of cytokines are structurally very similar with homologous features such as disulphide linkage, C-terminal amino acid sequences and cysteine residues. (Linden *et al.*, 2005) The most widely studied and prevalent members of the family in human biology are IL-17A and the related T cell isoform IL-17F. From this point onwards in this thesis unless specifically stated IL-17 refers to the cytokine IL-17A.

T_H-17 cells are characterised in humans by the production of the cytokines IL-17A, IL-17F, IL-22, IL-21 and IL-26. (Gaffen, 2009) In mice the differentiation of naïve T cells to a T_H-17 phenotype is regulated by the transcription factors signal transducer and activator of transcription 3 (STAT3), retinoic acid receptor-related orphan receptor-γt (RORγt) and aryl hydrocarbon receptor. This process is driven by transforming growth factor-β (TGF-β), IL-1 and IL-6. (Gaffen, 2009, Ivanov et al., 2006)

Our understanding of the immunobiology of IL-17 in humans is currently evolving rapidly and, interestingly there appear to be significant differences to the situation in mice. (Laurence and O'Shea, 2007, de Jong et al., 2010) Yang *et al.* (2008) found that TGF-β and IL-21 uniquely promote the differentiation of naïve human $CD4^+$ T cells to T_H-17 cells that is accompanied by expression of the transcription factor RORC2, the human orthologue of RORγt. (Yang et al., 2008, Unutmaz, 2009) IL-1β and IL-6 induced IL-17A production from human central memory $CD4^+$ T cells but not from naïve $CD4^+$ T cells. IL-23 is required to expand and stabilise the T_H-17 population but is

not involved in the differentiation of naïve T cells. (Bettelli et al., 2007, Aggarwal et al., 2003) Figure 7 illustrates the differentiation of T_H cell subsets.

T_H-17 cells are not the only source of IL-17 identified however. IL-17 is also known to be produced by γδ T cells and natural killer T cells. (Michel et al., 2007, Roark et al., 2008) Apart from lymphocytes there is also an emerging literature showing that myeloid cells can also express IL-17. It has recently been shown that mast cells in rheumatoid arthritis synovium and alveolar macrophages in a mouse model of allergy express IL-17. (Hueber et al., 2010, Song et al., 2008) Moreover it has also been suggested in human alcoholic liver disease and mouse models of lipopolysaccharide-induced airway inflammation and kidney ischaemia-reperfusion injury that neutrophils are a potential source of IL-17. (Lemmers et al., 2009, Ferretti et al., 2003, Li et al., 2010)

The current overall understanding of IL-17 receptor (IL-17R) biology is weak. The IL-17R consists of five subunits, IL-17RA to IL-17RE. (Aggarwal and Gurney, 2002) IL-17RA is by far the largest and most well studied receptor. (Gaffen, 2009) IL-17RA is expressed by a wide range of cells relevant to lung immunobiology, including airway epithelial cells, fibroblasts, B- and T-lymphocytes, endothelial cells and myelomonocytic cells. (Linden et al., 2005, Yao et al., 1995a, McAllister et al., 2005) The majority of these cells express the receptor constitutively suggesting that under physiological conditions they are primed for an immediate response on exposure to IL-17. IL-17RA activation by IL-17A induces a highly pro-inflammatory programme of gene expression, including the NFκB and mitogen-activated protein kinase (MAPK) pathways, similar to that induced by receptors associated with innate immunity such as Toll-like and IL-1 receptors (Figure 8). (Gaffen, 2009) In the future an improved understanding of the receptor biology of IL-17 is likely to lead to novel therapeutic approaches with greater precision in terms of targeting the harmful aspects of inflammation while avoiding more general deleterious effects on the host immune system. (Gaffen, 2009)

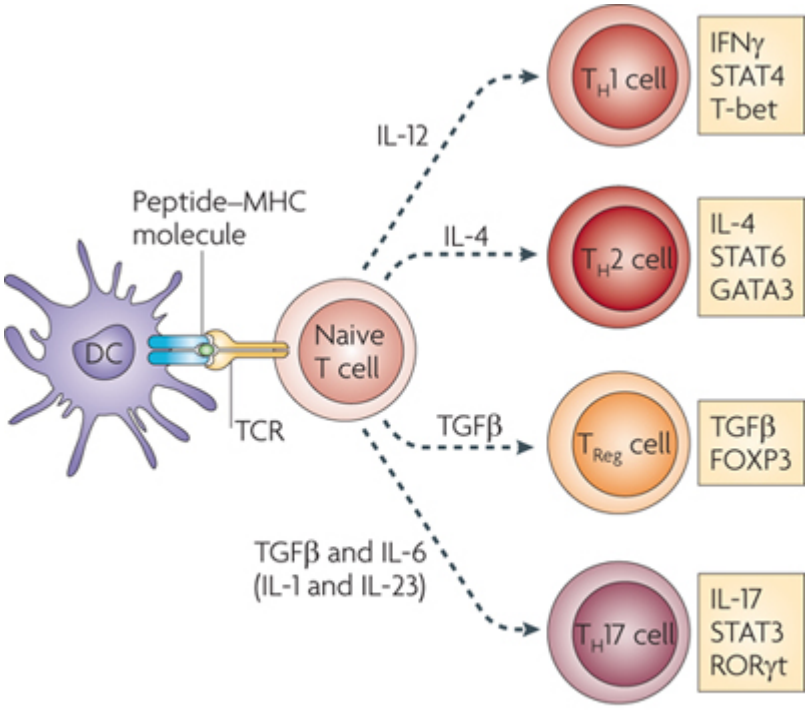


Figure 7. Differentiation of T_H cell subsets

Following activation by antigen-presenting cells such as dendritic cells (DCs), naive CD4+ T cells can be polarized into different effector T cell subsets — T helper 1 (TH1), TH2, TH17 and regulatory T (TReg) cells — depending on the local cytokine environment. The differentiation of each of these effector T cell subsets is controlled by distinct sets of transcription factors. In the presence of interleukin-6 (IL-6) and transforming growth factor-β (TGFβ), naive T cells can differentiate into TH17 cells, which are characterized by expression of the transcription factors retinoic acid receptor-related orphan receptor-γt (RORγt) and signal transducer and activator of transcription 3 (STAT3). Furthermore, IL-1 and IL-23 can promote and/or stabilize TH17 cell differentiation and expansion. FOXP3, forkhead box P3; GATA3, GATA-binding protein 3; IFNγ, interferon-γ; TCR, T cell receptor. (Zou and Restifo, 2010)

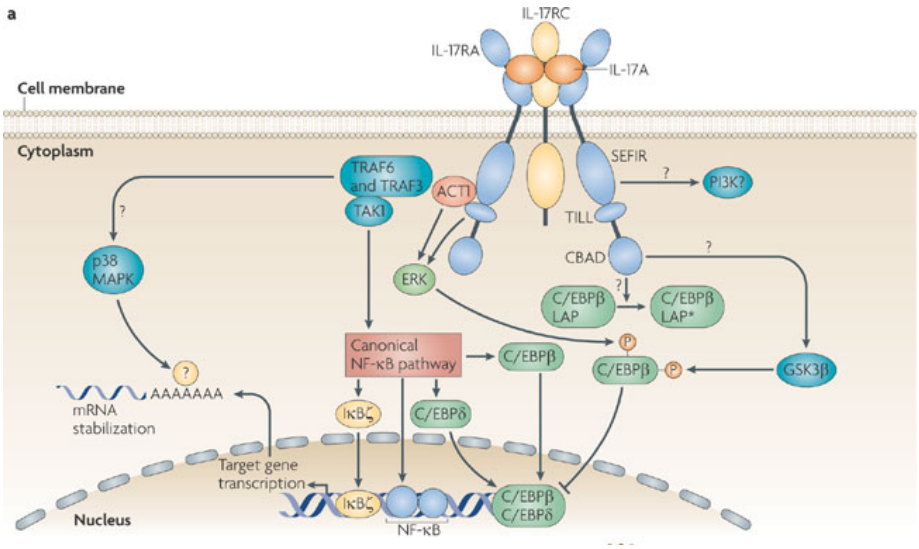


Figure 8. Interleukin-17 induced signalling pathways

Schematic depicting interleukin-17 receptor (IL-17R) signalling. The IL-17R complex is composed of IL-17RA and IL-17RC. IL-17RA engages the SEFIR domain-containing adaptor ACT1 to mediate various downstream events. Specifically, ACT1 is required for recruitment of TNFR-associated factor 6 (TRAF6) and possibly TRAF3, which are essential upstream activators of the canonical nuclear factor-B (NF-B) pathway. It is not clear whether TRAF6 is also required for the activation of the mitogen-activated protein kinase (MAPK) p38. ACT1, but not TRAF6, is required for IL-17A-induced stabilization of several target mRNAs, particularly those encoding chemokines and cytokines. A second functional domain on IL-17RA is located in the carboxy-terminal region and is not required for efficient activation of NF-B and MAPK pathways. (Gaffen, 2009)

1.5.2. *Interleukin-17 and neutrophils*

A substantial body of evidence supports a central role for IL-17A in host defence in the lungs. Principally IL-17A exerts an orchestrating effect on the accumulation and associated activity of neutrophils in the bronchoalveolar space. (Linden *et al.*, 2005) This effect is achieved indirectly via the local release of neutrophil-mobilising factors, including IL-8, IL-6, GM-CSF, IL-1β, TGF-β, TNF-α and prostaglandin E2, from cells resident in the lung such as bronchial epithelial cells, fibroblasts, macrophages and endothelial cells. (Aujla *et al.*, 2007) There is clear evidence that airway challenge with IL-17 causes substantial neutrophil accumulation in rats and mice *in vivo*. (Hoshino et al., 2000, Prause et al., 2004) The activated neutrophil products neutrophil elastase and myeloperoxidase are also similarly increased. (Hoshino *et al.*, 2000) Animal studies have also demonstrated that IL-17 signalling plays a central role in defence against Gram-negative bacterial infections in the lungs. Mice deficient for the IL-17 receptor have an increased mortality due to *Klebsiella pneumoniae* lower respiratory tract infection, decreased neutrophil mobilisation and poorer clearance of bacteria. (Ye *et al.*, 2001a)

As described earlier in this thesis, T_H-17 cells are thought to be an important source of IL-17. (Bettelli *et al.*, 2007) IL-17 therefore represents a strategic link between acquired and innate immunity and there is growing evidence that T lymphocytes play a role in the sustained mobilisation and activation of neutrophils through this mechanism. (Linden et al., 2005, Glader et al., 2010)

1.5.3. *Interleukin-17 and disease*

The IL-17 family of cytokines has been the focus of a great deal of research over recent years and has been implicated in the pathogenesis of a diverse range of inflammatory conditions. These include the neutrophilic lung diseases asthma, chronic bronchitis, chronic obstructive pulmonary disease and chronic lung allograft rejection along with non-respiratory conditions such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and autoimmune encephalomyelitis. (Bullens et al., 2006, Laan et al.,

2002, Curtis et al., 2007, Murphy et al., 2008b, Asquith and McInnes, 2007, McFarland and Martin, 2007, Bamias and Cominelli, 2007, McGeachy and Anderton, 2005)

1.5.4. *Interleukin-17 and cystic fibrosis*

As outlined above, IL-17 is linked to both neutrophilic inflammation and mucus excess, which are two cardinal features of CF lung disease, in addition to dysregulation of acquired immunity.(Chen et al., 2003, Bettelli et al., 2007, Aujla et al., 2007) It has been shown that people with CF who exhibit robust T cell responses to *P. aeruginosa* manifest more severe lung disease. (Winnie and Cowan, 1991) Other findings in the published literature pertinent to a role for IL-17 in CF lung disease include the reports that immortalised airway epithelial cells that do not express functional CFTR upregulate their innate immune responses following stimulation with IL-17. In particular nucleotide-binding oligomerisation domain 1 is increased. (Roussel and Rousseau, 2009) IL-17 also modulates bicarbonate secretion in normal airway epithelial cells suggesting that it may affect airway surface liquid physiology. (Kreindler *et al.*, 2009) In addition treatment with IL-17 increases expression of the mucin genes MUC5AC and MUC5B in bronchial epithelial cells *in vitro*. (Chen *et al.*, 2003)

Dendritic cells (DCs) are an important source of IL-23 in the lung. DCs form an immune sensing mesh that surrounds the airway and rapidly processes antigens. This is achieved by a process known as “snorkelling” where by DCs extend processes through intercellular spaces in the epithelium and sample the airway milieu. (Vermaelen et al., 2001, Holt, 2005) Dubin *et al.* (2007) hypothesise that the resultant high antigen load generates marked induction of IL-23 production by DCs in the airways of people with CF. (Dubin *et al.*, 2007) It follows that a cytokine milieu rich in IL-23 would favour expansion of T_H-17 cells and hence IL-17 production.

Published human studies of IL-17 in CF are rare but some support the importance of this axis. McAllister *et al.* (2005) analysed sputum from 8 young adults with CF, known to be colonised with *P. aeruginosa*, during hospitalisation for intravenous antibiotic therapy to treat an infective exacerbation. Sputum was collected pre-treatment and on days 10 and 20 of antibiotics. Levels of IL-17A, IL-17F and IL-23 along with a panel of IL-17-induced cytokines including IL-8 and GM-CSF were measured in the sputum

samples by enzyme-linked immunosorbent assay (ELISA). They found that IL-17A, IL-17F and IL-23 were raised in sputum during the exacerbation and were significantly reduced by day 20 of antibiotic treatment. A similar pattern was observed in the panel of IL-17-induced cytokines, most notably IL-8. (McAllister *et al.*, 2005) Raised levels of IL-17 have also been reported in BAL fluid from children with CF during infective exacerbations by the same research group. (Dubin et al., 2007, Aujla et al., 2008) IL-17 has also been found to be raised in the serum of clinically stable adults with CF. (Dufresne *et al.*, 2009)

In summary, there is strong evidence from animal and cellular studies that IL-17 is linked to neutrophilic airway inflammation and IL-17 has also been demonstrated *in vitro* to increase mucin gene expression. Neutrophilic inflammation and mucus excess are two of the cardinal features of the CF airway making IL-17 a highly plausible mediator in the pathogenesis of CF lung disease. To date actual evidence of the probable role of IL-17 in CF lung disease is limited to a sputum study involving a small number of patients. It follows that further research in this area is likely to yield important results in terms of enhancing our understanding of the pathogenesis of CF lung disease and elucidating potential therapeutic targets in the cascade of neutrophilic inflammation that is responsible for progressive bronchiectasis.

1.6. Experimental models of cystic fibrosis lung disease

1.6.1. *Introduction*

As outlined earlier in this chapter, over 95% of morbidity and mortality in CF is associated with lung disease. (Doring *et al.*, 2007) Developments in clinical care have yielded ever-increasing survival for people with CF over recent decades. (Dodge *et al.*, 2007) However, 20 years after the identification of the CFTR gene, the exact pathogenesis of CF lung disease remains poorly understood. (Rogers et al., 2008a, Riordan et al., 1989) Valid experimental models are therefore required to further determine the pathogenesis of CF lung disease. (Wine, 2010)

A number of exciting novel, small-molecular compounds targeted at specific CFTR mutations have been described in recent years. For example, VX-770 is a CFTR potentiator that has shown promising early results in a phase IIa clinical trial in people with at least one p.Gly551Asp class III variant. (O'Sullivan and Freedman, 2009, Van Goor et al., 2009) PTC124 was initially identified using a cell-based assay and induces ribosomes to read through premature stop codons. (Welch *et al.*, 2007) This compound has potential benefit in approximately 10% of people with CF who have in-frame nonsense variants, such as p.Trp1282X or p.Gly542X, and PTC124 has been shown to restore CFTR protein and function in a mouse model with the p.Gly542X mutation. (Du et al., 2008, O'Sullivan and Freedman, 2009) A subsequent phase II clinical trial in people with nonsense variants showed an increase in nasal potential difference in some but not all. (Kerem *et al.*, 2008) These findings highlight the huge importance of accurate experimental models to enable the development and evaluation of novel potentially therapeutic strategies. (Auld *et al.*, 2009)

1.6.2. *Animal models*

Since the cloning of the CFTR gene it has been possible to develop animal models of CF. (Riordan *et al.*, 1989) A large amount of work has been performed in this area, principally focussed on mice, that has generated over 2500 publications to date. (Scholte *et al.*, 2004) There has also been exciting progress recently in the development of CF pig and ferret models. (Rogers et al., 2008b, Sun et al., 2008, Stoltz et al., 2010) The obvious strength of animal models is that they allow *in vivo* study designs of disease pathogenesis or the evaluation of novel therapeutic strategies, which are not technically or ethically possible in humans. Once the appropriate genetic manipulation has been achieved, however, the utility of an animal model is dependent on how closely the phenotype of the animal matches that observed in human disease. This is further complicated by the relatively weak correlation between CFTR genotype and phenotype of lung disease seen clinically in people with CF. (Rowntree and Harris, 2003, Bronsveld et al., 2001) When considering this area, it is important to be aware of the timely establishment of organisations such as the National Centre for the Replacement, Refinement and Reduction of Animals in Research in the United Kingdom to facilitate the careful use of animals in medical research. (NC3Rs, 2010)

1.6.3. *Mouse models*

The first murine models were produced by interruption of the CFTR gene and produced a knockout effect resulting in no detectable mouse CFTR mRNA production. (Snouwaert et al., 1992, Ratcliff et al., 1993, Hasty et al., 1995, Rozmahel et al., 1996) Mice with residual function that produce low levels of mouse CFTR mRNA were subsequently produced by an insertional strategy. (Dorin et al., 1992, O'Neal et al., 1993) Although fundamental to subsequent research it became apparent that the mutations in these early models did not accurately simulate the effects of relevant clinical mutations such as the dysfunctional CFTR produced with the p.Phe508del mutation. (Grubb and Boucher, 1999)

In view of this recombinant mice were therefore generated with specific clinically relevant mutations. The first example of which were p.Phe508del CFTR mice

developed to replicate the most frequent human CF mutation. (Colledge et al., 1995, Zeiher et al., 1995, van Doorninck et al., 1995) In addition mice carrying the human CF mutations p.Gly480Cys, p.Gly551Asp and p.Gly542X have been produced. (Delaney et al., 1996, Dickinson et al., 2002, Du et al., 2002)

Once the appropriate genetic manipulation has been achieved clearly a vital consideration are the phenotypic properties of the various strains of CFTR mutant mice. In general the mice exhibit a characteristic CF bioelectric phenotype. (Grubb and Boucher, 1999) Intestinal disease, typified by intestinal obstruction, dilated crypts filled with mucus and goblet cell hyperplasia, is the most prominent feature in CF mice. This intestinal phenotype closely mimics that seen in humans and is widely accepted as a valid model. (Guilbault *et al.*, 2007) Pancreatic insufficiency is a problematic feature of classical CF in humans but is not convincingly replicated in most mouse models. Pancreatic disease appears to be less severe in CF mice due to lower levels of CFTR expression in the murine pancreas and the presence of an alternative secretory pathway. (Gray *et al.*, 1995) In male mice CFTR dysfunction leads to mucoid obstruction of the *vas deferens* rather than complete absence as is seen in human CF. The net effect however is similarly severely reduced fertility. (Scholte *et al.*, 2004)

CF lung disease in humans is characterised by chronic infection with bacteria such as *S. aureus, H. influenzae* and *P. aeruginosa*, neutrophilic inflammation and retention of mucopurulent secretions. (Davidson et al., 1995, Downey et al., 2009) Unfortunately there are significant differences between the human CF lung phenotype and that seen in CF mice. Despite promising bioelectric features of the airway in some CF mice, establishing chronic infection with *P. aeruginosa* and subsequent inflammation and fibrotic lung damage has proved difficult. Scholte *et al.* in a review of animal models for CF concluded that: *"the development of an ideal mouse model of CF lung disease, to enable the dissection of pathogenesis, or testing of novel therapeutics, is yet to be achieved."* (Scholte *et al.*, 2004) Possible explanations for this observation include inter-species differences in lung physiology, innate immunity, airway epithelial cell composition, alternative chloride channels and less widespread submucosal glands. (Pack et al., 1980, Grubb et al., 1994, Borthwick et al., 1999, Maxwell et al., 2003)

In an alternative approach, Mall and colleagues, developed a mouse with airway-specific overexpression of the β epithelial sodium channel subunit. (Mall *et al.*, 2004)

Interestingly the resultant increase in sodium absorption *in vivo* generated a spontaneous CF-like lung disease in the mice including reduced airway surface liquid, increased mucus concentration, defective mucus transport, neutrophilic inflammation and poor bacterial clearance. This model provides an important mechanistic link between altered ion transport, dysregulation of airway surface liquid and CF-like lung disease. However, unlike human CF lung disease CFTR function is preserved and the mice also develop pulmonary emphysema. (Mall *et al.*, 2008) The model is therefore currently of limited value in translation to human CF.

1.6.4. *Other animal models*

The limitations outlined above of murine models have led to attempts in recent years to develop other animal models. Larger animals such as pigs, sheep or ferrets have the advantage that their lungs resemble human lungs more closely in terms of anatomy and in particular submucosal glands are extensively distributed throughout the respiratory tract. (Rogers *et al.*, 2008a)

The preliminary results of a major programme of work based at the University of Iowa to develop pigs and ferrets with CFTR mutations have recently been published and presented at international meetings. (Rogers et al., 2008b, Sun et al., 2008, Rogers et al., 2008c) Piglets with disrupted CFTR have been observed to develop an extreme gastrointestinal phenotype including meconium ileus, necessitating ileostomy, exocrine pancreatic destruction and focal biliary cirrhosis. (Rogers et al., 2008c, Meyerholz et al., 2010) Gastrointestinal morbidity has also included gastric ulceration and abdominal pathology has been responsible for a significant early mortality rate.

The initial results with regard to the recapitulation of CF lung disease have recently been published. (Stoltz *et al.*, 2010) As mentioned earlier, newborn CF piglets did not show increased evidence of inflammation (BAL fluid IL-8 and neutrophil counts) but were less often sterile and eliminated bacteria less effectively. This suggests an intrinsic host defense defect against bacteria is the initial pathogenic event in the lungs of CF piglets. Within only months of birth the piglets developed spontaneously several features of human CF lung disease including mucus accumulation, neutrophilic inflammation and endobronchial infection. (Stoltz *et al.*, 2010) Figure 9 illustrates some of the pathological features found in the CF pigs. (Stoltz *et al.*, 2010) The pig model

remains at an experimental stage and certainly does not represent a mainstream research tool that is available to other researchers currently. A major challenge for this model to realise its full potential will be to increase the length of survival of the pigs. This has been limited to 6 months due to severe morbidity, requiring euthanasia, associated with meconium ileus, gastric ulceration and respiratory infections. (Wine, 2010)

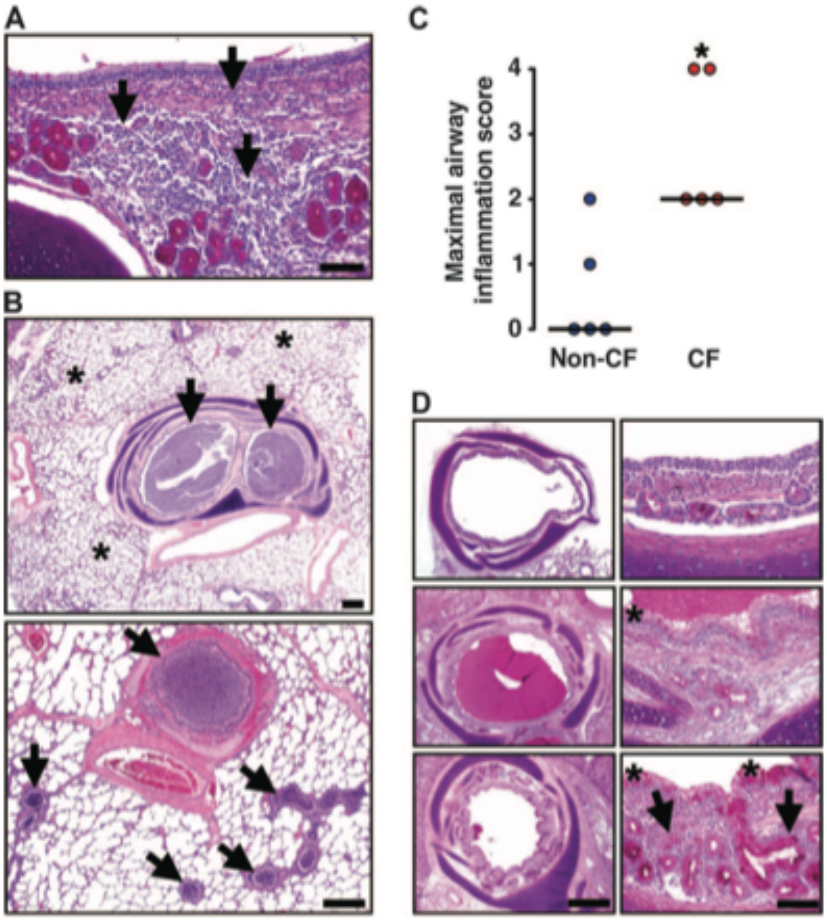


Figure 9. Lung disease in cystic fibrosis pigs

(A) Infiltration of the airway wall by lymphocytes and plasma cells (arrows). PAS stain. Scale bar, 80 μm. (B) Obstruction (arrows) of bronchi and small bronchioles was a striking feature in otherwise unaffected lung (asterisks). H&E stain. Scale bars, 0.75 mm. (C) Airways from non-CF and CF pigs were scored for the most severe degree of leukocytic infiltration de- tected, *P < 0.05, Mann-Whitney test. Horizontal line indicates the median. (D) Airways (left panels) (scale bar, 0.7 mm) ranged from relatively unaffected (top) to severe disease (bottom; note that the luminal mucocellular plug was removed at necropsy) with airway wall thickening. Surface epithelium (right panels) (scale bar, 70 μm) ranged from near normal (top) to mucinous and hyperplastic change (asterisks) in moderate to severe disease (middle and bottom panels). Note that hypertrophy or hyperplasia of submucosal glands (arrows, bottom right) was uncommon and generally restricted to the most severe and chronically affected airways. (Stoltz *et al.*, 2010)

1.6.5. *Cellular models*

In addition to structural and barrier functions airway epithelial cells are increasingly recognised to operate as 'effector' cells. Airway epithelial cells produce a wide range of inflammatory and immunomodulatory cytokines and growth factors that contribute to the pathogenesis of respiratory disease. (Ward et al., 2009, Smyth, 2009, Crystal et al., 2008, Wang et al., 2009, Brodlie et al., 2009a)

For a number of decades it has been possible to culture human airway epithelial cells *in vitro*. (Gruenert et al., 1995, Lechner et al., 1981) Cells may be cultured most simply under submerged conditions on plastic in petri dishes or flasks, however, primary cells revert to a poorly differentiated phenotype within a limited number of passages. (Gruenert et al., 1995, Araya et al., 2007) More advanced culture techniques, such as within collagen gels, as three-dimensional spheroids or most commonly on semi-permeable membranes at an air-liquid interface, allow more accurate reproduction of the native airway epithelium. (de Jong et al., 1993, Ulrich and Doring, 2004, Bals et al., 2004, Choe et al., 2006)

Air-liquid interface cultures have been pivotal in several major advances in our current understanding of the pathogenesis of CF lung disease, including depletion of the periciliary liquid layer. (Matsui *et al.*, 1998) Semi-permeable membranes may also be used to co-culture different cell types and investigate their interactions, for instance airway epithelial cells and fibroblasts. (Zhang *et al.*, 1999) Airway epithelial cells may also be used in electrophysiology experiments in whole-cell patch clamping or cultured monolayers in assays of iodide efflux or Ussing chambers. (Rakonczay et al., 2008, Jurkuvenaite et al., 2009, Brodlie et al., 2010f)

1.6.6. *Immortalised cell lines*

Immortalised airway epithelial cell lines, originating from human neoplasms or produced *in vitro* by physical or chemical mutagenesis or introduction of viral oncogenes, are used extensively in CF research and have contributed significantly.

(Gruenert et al., 1995, Lundberg et al., 2002, Fulcher et al., 2009, Pedemonte et al., 2005) Immortalised cell lines have been particularly useful for the investigation of relationships between CFTR genotypes, metabolic and biochemical characteristics and disease phenotypes. (Gruenert *et al.*, 2004) Immortalised cells are also essential in the early stages of high-throughput screening strategies to identify novel therapeutic compounds. (Pedemonte *et al.*, 2005) Advantages of immortalised cell lines include their widespread availability, especially when compared to the scarcity of primary CF tissue and cells, homogeneity in terms of biochemical, electrophysiological and growth characteristics and the presence of matched isogenic control lines. (Gruenert *et al.*, 2004) Cell lines that are commonly used in CF research have been comprehensively reviewed. (Gruenert *et al.*, 2004)

However, the process of immortalisation may generate karyotypic instability and have major effects on cellular differentiation, morphology or function compared to the situation *in vivo*. (Gruenert et al., 2004, Fulcher et al., 2009, Karp et al., 2002) The condition of the primary culture prior to immortalisation is crucial in determining the validity and utility of the cell line. Careful selection must be made of the most appropriate immortalised cell line guided by the biological end points that are to be evaluated. Although the dependence of these end points on the cellular context cannot always be regulated. (Gruenert *et al.*, 2004) Karyotypic instability may also lead to the emergence of subpopulations that do not retain the phenotypic characteristics of interest. (Gruenert *et al.*, 2004)

1.6.7. *Primary tissue or cellular* ex vivo *models*

CF is a uniquely human disease and therefore primary tissue or cells cultured directly from people with CF represent a highly valuable experimental resource. The *ex vivo* culture of primary airway epithelial cells from people with CF, that have not been immortalised, is likely to reproduce the behaviour of cells *in vivo* more accurately than immortalised cell lines. Furthermore, primary airway epithelial cell cultures have been instrumental in several important developments in our understanding of the pathophysiology of CF lung disease. (Fulcher M. L., 2005, Matsui et al., 1998, Blouquit et al., 2006, Joseph et al., 2005, Widdicombe, 1990)

Primary airway epithelial cells are particularly important in the new era of targeted therapies for the molecular defects associated with specific CFTR variants. (O'Sullivan and Freedman, 2009) The problems outlined earlier with the murine models of CF lung disease clearly limit their utility in drug discovery. (Scholte *et al.*, 2004) Although immortalised cell lines are extremely valuable in the early stages of high-throughput screening, primary cells are vital to confirm and validate any initial findings in a immortalised cell line prior to more advanced stages of analysis. (Pedemonte et al., 2005, Van Goor et al., 2006, Ma et al., 2002, O'Sullivan and Freedman, 2009)

In CF there is a diverse spectrum of disease and often a weak correlation between CFTR genotype and clinical lung phenotype. (Rowntree and Harris, 2003) Primary airway epithelial cells from individual donors are likely to reflect this inherent biological heterogeneity in terms of their function. (Becker *et al.*, 2004) Indeed, for some experiments the homogeneity of an immortalised cell line along with isogenic controls may be preferable. Primary cells possess a finite reproductive capacity before they senesce and this has provided impetus to establish novel immortalised cell lines derived from primary cells. (Fulcher et al., 2009, Ben-Porath and Weinberg, 2005)

Several potential sources of primary airway epithelial cells from people with CF are described below, including explanted lungs, bronchial and nasal brushings. Appropriate ethical approval and strict adherence with human tissue legislation are clearly essential along with the informed consent of the participants and any research sampling must not compromise the care or health of patients. (Trouet, 2004, Anon, 2006, Trouet et al., 2004, Dodge, 2004, Mallory, 2006, Bush and Davies, 2006)

1.6.8. *Explanted lungs*

The only life-sustaining intervention for end-stage CF lung disease is transplantation. (Meachery *et al.*, 2008) Explanted lungs removed at the time of transplantation from people with CF represent a potential source of large numbers of primary bronchial epithelial cells. The procurement of appropriate lung tissue is logistically demanding however, lung transplantation is unpredictable in nature, informed consent is required in advance from patients and the cooperation of the multidisciplinary transplant team is

essential. (Brodlie et al., 2010f) It is also important to note that cells and tissue obtained from explanted lungs maybe only representative of advanced disease.

It is imperative that the multidrug-resistant microorganisms, which frequently colonise the airways of people with end-stage CF lung disease, are eradicated from cultures at an early stage to achieve success. (Gruenert et al., 2004, Randell et al., 2001) Chapter 5 of this thesis reports the establishment of a successful technique and programme to culture primary bronchial epithelial cells from explanted lungs in Newcastle upon Tyne.

Intact sheets of bronchial epithelium may also be resected from lungs shortly after explantation and placed in perfused mini-Ussing chambers. This allows the investigation of electrophysiological responses, for example with small molecule CFTR correctors or potentiators, in the context of an intact epithelium complete with submucosal glands. (Derichs, 2009, Jaffar et al., 1999)

1.6.9. *Bronchial brushings*

Bronchial epithelial cells maybe cultured in smaller numbers from brushings of the lower airway. Once the appropriate ethical approval is in place an opportunistic approach is likely to be required on behalf of the clinical researcher to obtain informed consent and bronchial brushings from people with CF when they undergo elective procedures such as gastrostomy or totally implantable vascular access device insertion. Eradication of infection remains essential for the successful *ex vivo* culture of cells.

Bronchial brushings may be obtained blindly from patients who have been intubated by passing a cytology brush down the endotracheal tube. (Doherty *et al.*, 2003) Alternatively, brushings may be performed under direct vision via a flexible bronchoscope in patients, including children, who are intubated or with a laryngeal mask airway *in situ*, or in those who are undergoing bronchoscopy for a clinical indication. (McNamara et al., 2008, Forrest et al., 2005, Lane et al., 2005)

1.6.10. *Nasal brushings*

The nose is an alternative source of airway epithelial cells that is easily accessible. Nasal brushings may be performed in a relatively non-invasive manner in a clinic setting. In adults and older children the technique does not require any sedation or anaesthesia and therefore allows repeated isolations from the same individual, for instance with a particular CFTR genotype of interest. This also provides the potential for longitudinal studies of aetiology and treatment strategies, the evaluation of the effects of primary CFTR defects and subsequent host-pathogen interactions on phenotype. Nasal brushings may also be performed on groups such as children who are less likely to undergo bronchoscopy due to practical or ethical reasons. (Mosler et al., 2008, McDougall et al., 2008) There are several published methods for the culture of nasal epithelial cells from people with CF and feasibility of the technique has even been demonstrated in infants. (Bridges et al., 1991, Mosler et al., 2008)

McDougall *et al.* compared the release of proinflammatory mediators and surface expression of receptors by undifferentiated monolayers of nasal and bronchial epithelial cells from the same individuals under resting conditions and in response to cytokine stimulation. (McDougall *et al.*, 2008) They found differences in absolute mediator levels but similar responses to stimulation and comparable cell surface receptor expression suggesting that nasal epithelial cells may represent an accessible surrogate for lower airway epithelial cells to study inflammation. (McDougall *et al.*, 2008)

1.6.11. *Experimental model summary*

Experimental models are critically important for use in research to advance both our knowledge of the pathogenesis of CF lung disease and to discover and evaluate novel therapeutic compounds and strategies. This is especially relevant in light of the recent exciting advances in the development of small-molecule compounds directed at specific CFTR mutations. A large amount of work over the last two decades has unfortunately failed to yield a good CFTR-deficient murine model of CF lung disease. The development of larger animal models, for example, the porcine model shows promise but remains in its infancy and is yet to be fully validated. Immortalised airway epithelial cell lines have contributed significantly to CF research but have inherent limitations,

including karyotypic instability and poor replication of behaviour *in vivo*. Primary lung tissue and airway epithelial cells harvested from nasal or bronchial brushings or explanted lungs from people with CF represent a valuable resource for the *ex vivo* study of CF lung disease. A schematic diagram of approaches to the use of different experimental models in drug discovery for CF lung disease is shown in Figure 10.

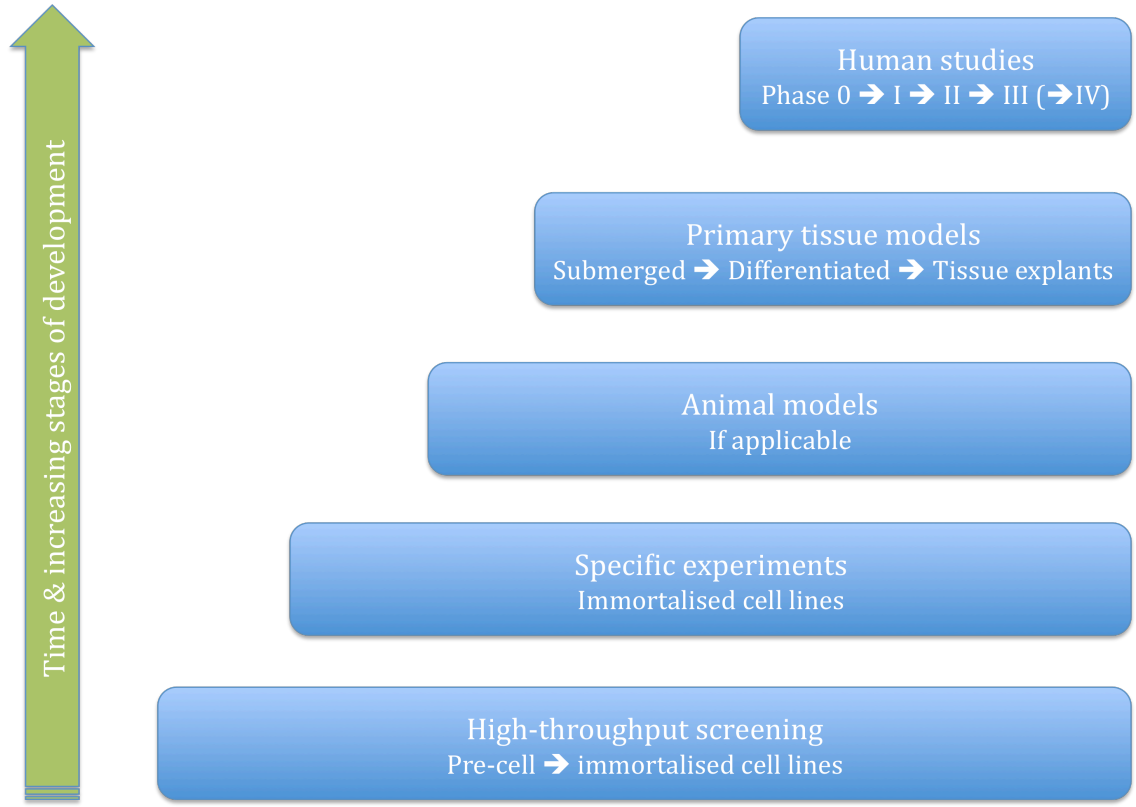


Figure 10. Schematic diagram illustrating the use of different experimental models in drug discovery for CF lung disease

1.7. Ceramide and cystic fibrosis lung disease

1.7.1. *Introduction and biochemistry*

Ceramide is a ubiquitous sphingolipid that is found in the membrane of cells. The original *'fluid mosaic model'* of cell membrane structure has subsequently been revised to include the concept of membrane microdomains including ceramide-enriched lipid rafts. (Singer and Nicolson, 1972, Simons and Ikonen, 1997, Gulbins et al., 2004) Lipid rafts act as anchoring points in cell membranes for the cytoskeleton and as receptors for the start of signalling cascades and the uptake of microorganisms. (Grassme et al., 2003, Uhlig and Gulbins, 2008, Manes et al., 2003) In addition to their function in cell membranes, sphingolipids are also recognised to regulate a number of key physiological intracellular processes *via* roles as pleiotropic second-messengers. (Hannun and Obeid, 2002) This includes apoptosis, senescence, innate and acquired immunity, vascular permeability and smooth muscle tone. (Uhlig and Gulbins, 2008, Novgorodov and Gudz, 2009)

Sphingolipids are amphiphatic and comprise of a hydrophobic ceramide moiety and a hydrophilic headgroup. (Becker *et al.*, 2010c) Sphingolipds are principally derived from sphingosine. Sphingosine-1-phosphate (S1P) is formed by phosphorylation of sphingosine and its acylation results in ceramide. Ceramide is coupled with phosphocholine to produce sphingomyelin. The reverse of this processs, the production of ceramide from sphingomyelin by sphingomyelinase, is of greater significance in disease. Ceramide itself may be degraded by acid ceramidase to sphingosine and fatty acid. (Uhlig and Gulbins, 2008) Figure 11 provides a summary of sphingolipid metabolism. Figure 12 illustrates the *de novo* synthesis pathway and the salvage pathway that ceramide is principally generated by. (Novgorodov and Gudz, 2009)

Sphingolipids exert profound effects on cell fate. S1P mediates survival and proliferation where as ceramide promotes differentiation, apoptosis and cell-cycle arrest. (Uhlig and Gulbins, 2008) Hence the term *'the sphingolipid rheostat'* has been coined to describe the critical role of sphingolipids in cell fate, particularly in response

to different stressors. (Pyne and Pyne, 2000) Accordingly sphingolipid levels are tightly regulated in cells. (Novgorodov and Gudz, 2009)

There is wide-ranging evidence that ceramide in particular is a key sphingolipid messenger in the regulation of cellular responses to stress, including apoptosis and senescence. (Pettus *et al.*, 2002) Ceramide consists of a family of approximately 50 distinct species that are defined by molecular structure including acylation, desaturation and hydroxylation. (Novgorodov and Gudz, 2009)

The study of sphingolipids in biological systems is highly complex and techinically demanding. Ceramide is hydrophobic and therefore the identification of specific intracellular binding partners has proved difficult. (Uhlig and Gulbins, 2008) Another major challenge is difficulties with the lack of an efficient method to quantify levels of sphingolipids in biological samples. Two ceramide antibodies are now commercially available, a mouse IgM monoclonal antibody (Sigma-Aldrich) and an antiserum enriched for IgM polyclonal antibody (Glycobiotech). Cowart *et al.* compared the specificity of these two antibodies and found the polyclonal antibody to be more specific for ceramide than the monoclonal antibody, which also detects dihydroceramide, phosphatidylcholine and sphingomyelin. (Cowart *et al.*, 2002)

As mentioned above, ceramide truly consists of a large family of distinct molecular species. Importantly, there is evidence that individual ceramide species have specific biological functions and vary in their relative concentrations, for example C16 and C24 ceramide in apoptosis. (Kroesen *et al.*, 2003) In order to identify and accurately quantify individual ceramide species analytical chemistry techniques are required. For example high performance liquid chromatography-mass spectrometry (HPLC-MS) may be used to analyse homogenates of tissues. (Pettus *et al.*, 2003) Clearly such an approach does not allow architectural localisation of ceramide species in tissues however. (Brodlie et al., 2010d)

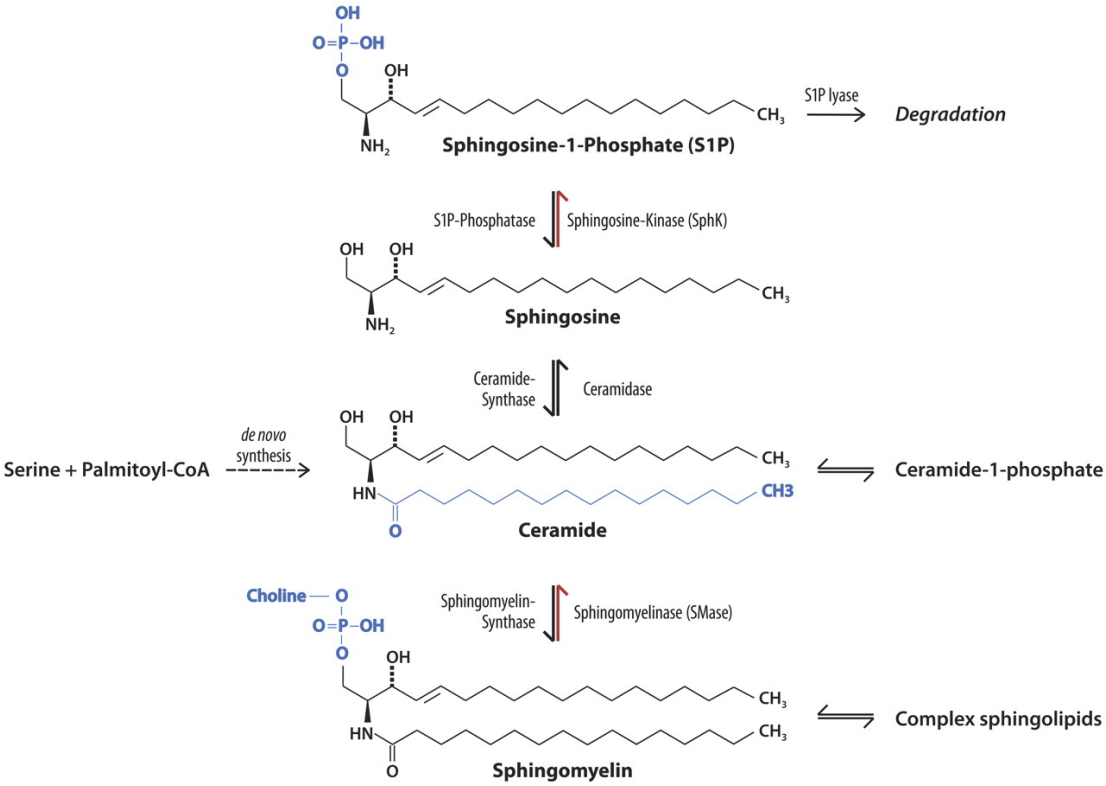


Figure 11. Overview of sphingolipid metabolism.

Blue colours indicate chemical moieties transferred by the enzymatic reactions. Red arrows indicate heavily regulated enzymes that are thought to play a major role in pulmonary disease (Uhlig and Gulbins, 2008)

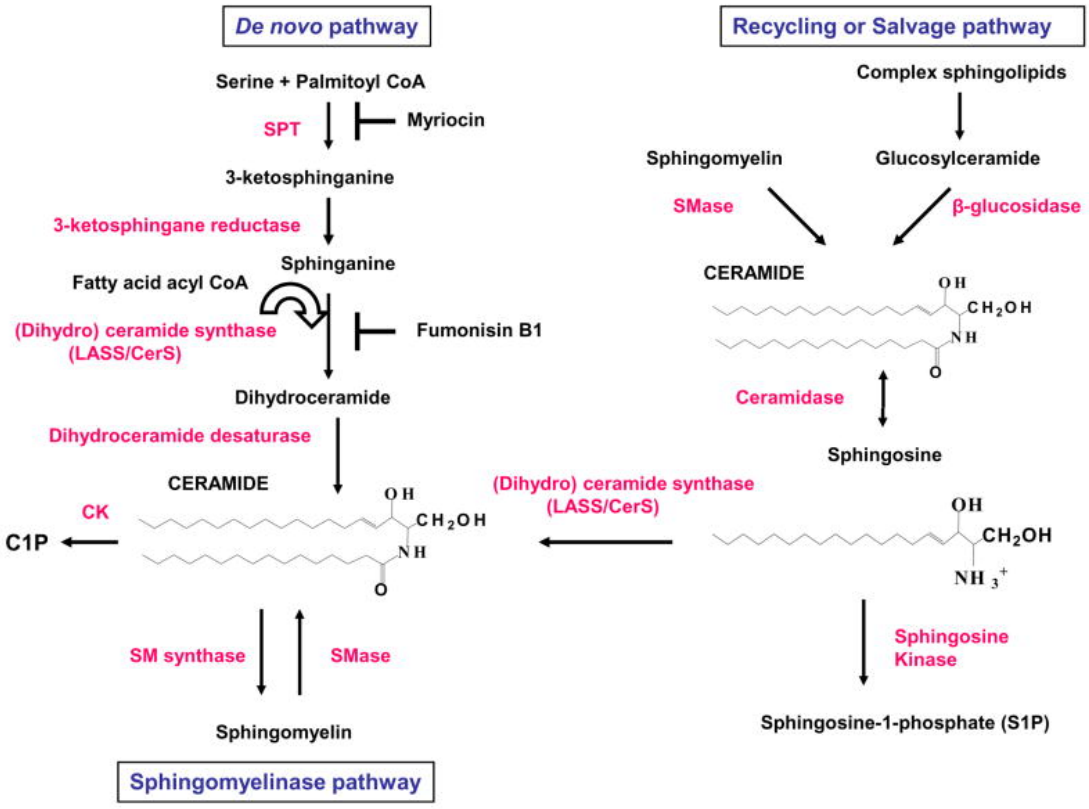


Figure 12. Biosynthesis of ceramide including the *de novo* and salvage pathways

(Novgorodov and Gudz, 2009)

1.7.2. *Sphingolipids and pulmonary disease*

Sphingolipids are increasingly recognised to play a role in the pathogenesis of several lung conditions. The metabolic conditions Niemann-Pick disease (NPD) type A and B result from deficiency of sphingomyelinase and subsequent lysosomal accumulation of sphingomyelin. NPD is associated with pulmonary infection and interstitial lung disease. (Schuchman, 2007) In the context of acute lung injury and pulmonary oedema acid sphingomyelinase activity has been shown to be increased and may represent a potentially useful therapeutic target. (Niessen et al., 2008, Claus et al., 2005, Lindner et al., 2005) Asthma is characterised by chronic inflammation involving eosinophils, mast cells, neutrophils and lymphocytes, airway smooth muscle contraction and hyperresponsiveness. (Brodlie and McKean, 2009, Holgate, 2008) Sphingolipids are involved in the regulation of all of the aforementioned processes. (Ammit *et al.*, 2001) Ceramide has also been identified as an important mediator in the development of emphysema. In this context ceramide is thought to act by inducing oxidative stress and apoptosis of alveolar endothelial and epithelial cells. (Petrache et al., 2005, Petrache et al., 2008)

Ceramide-enriched lipid rafts are intimately involved in airway epithelial cells defences against infection by microorganisms. This includes hosting key signalling events in the immune-surveillance of pathogens. (Manes *et al.*, 2003) In addition a number of pathogens have evolved strategies to enter airway epithelial cells *via* lipid rafts. (Manes *et al.*, 2003) The invasion of epithelial cells by rhinovirus involves ceramide-enriched lipid rafts. (Grassme *et al.*, 2005) The infection of epithelial cells with *P. aeruginosa* also critically involves ceramide-enriched membrane domains. (Grassme *et al.*, 2003) Furthermore it has been shown that CFTR acts as a receptor for the internalization of *P. aeruginosa*. (Pier et al., 1996, Pier et al., 1997)

1.7.3. *Ceramide and cystic fibrosis lung disease*

Chronic infection with *P. aeruginosa* and intense neutrophilic inflammation are two hallmarks of CF lung disease. (Davidson et al., 1995, Downey et al., 2009) In lung endothelial cells CFTR function is also required for stress-induced apoptosis by maintaining ceramide activation. (Noe *et al.*, 2009) Ceramide is therefore a plausible mediator in the pathogenesis of CF lung disease.

Two specific strains of CFTR-deficient mice have recently been demonstrated to accumulate ceramide in airway epithelial cells. (Teichgraber *et al.*, 2008) The accumulation of ceramide triggered chronic neutrophilic pulmonary inflammation, death of airway epithelial cells and extracellular deposition of DNA in the airways, thereby increasing susceptibility to infection with *P. aeruginosa*. (Teichgraber *et al.*, 2008) Importantly, treatment with the tricyclic antidepressant amitriptyline, which is an acid sphingomyelinase inhibitor, normalised pulmonary ceramide, inflammation and susceptibility to *P. aeruginosa* infection. (Becker et al., 2010b, Teichgraber et al., 2008) Ceramide accumulation was also shown in nasal epithelial cells and demonstrated at a qualitative level in three sections of lower airway from people with CF. (Teichgraber *et al.*, 2008)

The precise role of epithelial ceramide accumulation in the pathogenesis of CF lung disease is yet to be fully elucidated. The work performed by Teichgräber *et al.* (2008) suggests that ceramide accumulation occured in a constitutive, age-dependent fashion in the airway epithelial cells of CFTR-deficient animals and promoted pro-inflammatory cytokine release, apoptosis and DNA deposition in the airway. (Teichgraber *et al.*, 2008) A proposed mechanism for ceramide accumulation is that defective CFTR leads to inappropriate alkalinisation of intracellular vesicles, in particular pre-lysosomes and lysosomes, resulting in an increase in acid sphingomyelase and reduction in acid ceramidase activity and subsequent ceramide accumulation (Figure 13). (Teichgraber *et al.*, 2008) However, the role of CFTR in the acidification of lysosomes has been hotly debated recently with apparently contradictory results published by different investigators. (Haggie and Verkman, 2009a, Haggie and Verkman, 2009b, Di et al., 2006, Teichgraber et al., 2008, Poschet et al., 2002, Noe et al., 2009)

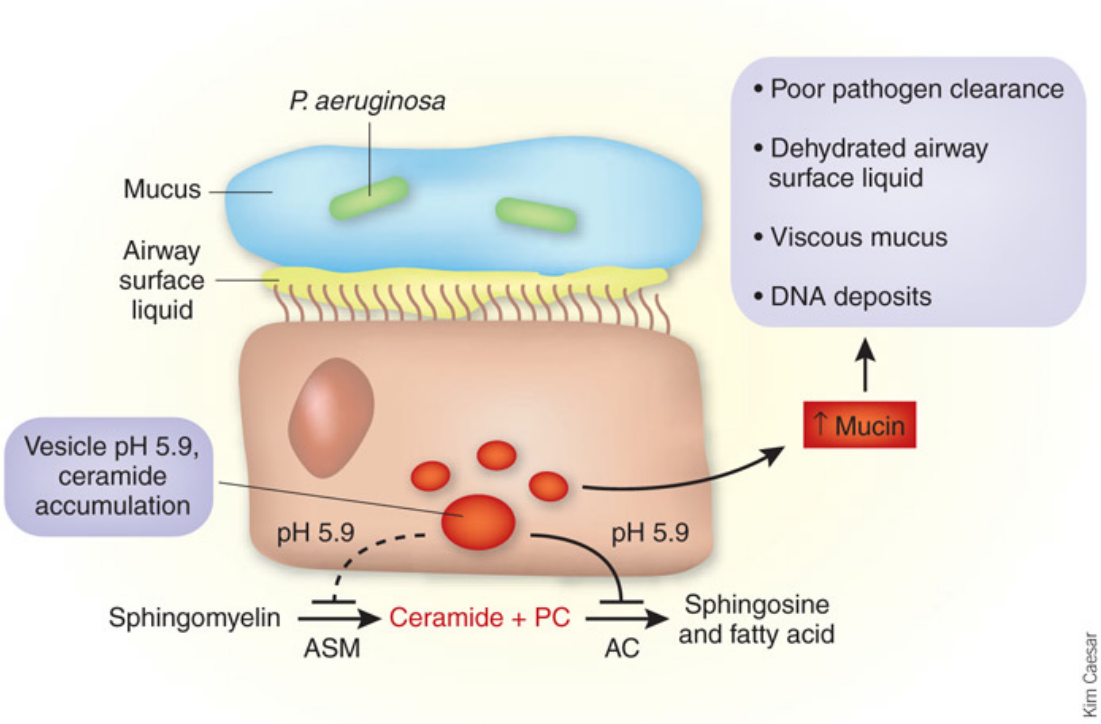


Figure 13. Schematic diagram of proposed mechanism of ceramide accumulation in the pathogenesis of cystic fibrosis lung disease

Synthesis of ceramide from sphingomyelin by acid sphingomyelinase (ASM) and degradation by acid ceramidase (AC) is optimal at an acidic pH, such as that of normal intracellular vesicles (pH 4.5). Lack of functional CFTR increases vesicle pH to 5.9, partially inhibiting ASM and highly inhibiting AC, resulting in ceramide accumulation in the vesicle and age-dependent pulmonary inflammation. Other properties of the cystic fibrosis–affected lung, including lack of the CFTR receptor needed for clearing Pseudomonas aeruginosa, enhanced mucin secretion, a dehydrated airway surface liquid, viscous mucus and DNA deposits released from ceramide-engorged apoptotic cells, also contribute to P. aeruginosa airway colonization. Eventually, this microbe establishes a chronic infection wherein bacterial cells reside in low-oxygen mucus plugs and become highly resistant to clearance or killing by host defenses. (Pier, 2008)

The findings of current research into ceramide and CF pathophysiology are divergent however, and possibly specific to the model systems studied. Furthermore, there is a lack of human data in the literature, particularly in relation to the lower airways, which are the site of the vast majority of morbidity and mortality in people with CF. (Guilbault et al., 2008b, Guilbault et al., 2008a, Teichgraber et al., 2008)

Low plasma levels of ceramide have been reported by Guilbault *et al.* (2008a and 2008b) in people with CF compared to healthy volunteers. The same researchers also found reduced levels of ceramide in the plasma, lungs, pancreas and ileum of CFTR-deficient mice that could be corrected by treatment with fenretinide. (Guilbault et al., 2008b, Guilbault et al., 2008a) Yu *et al.* found no significant difference in basal ceramide levels in IB3-1 immortalised CF bronchial epithelial cells and lung homogenate from CFTR knock out mice compared to wild type cells and mice. (Yu *et al.*, 2009) Physiological levels of ceramide appear to be essential for the homeostasis of cells and inflammatory responses and therefore it is plausible that too little ceramide may be equally as deleterious to epithelial physiology as a situation where ceramide accumulation occurs. (Guilbault *et al.*, 2008a)

1.8. Airway remodelling in cystic fibrosis lung disease

CF lung disease is characterised by airflow obstruction, neutrophilic inflammation and chronic endobronchial infection. (Davies *et al.*, 2007) Children with CF are born with airways of essentially normal structure however. (Konstan and Berger, 1997) Airway remodelling, in the form of architectural changes in the airway wall, is well recognised in people with asthma and chronic obstructive pulmonary disease (COPD). (Jeffery, 2001, Davies, 2009)

One specific feature of airway remodelling seen in adults and children with asthma is apparent thickening of the sub-epithelial reticular basement membrane (RBM) due to collagen matrix deposition (Figure 14). (Ward et al., 2002, Payne et al., 2003) It has also been postulated that airway remodelling may be implicated in the pathogenesis of CF lung disease and RBM thickening has been reported in endobronchial biopsies from children with CF. (Hilliard *et al.*, 2007) Clinical evidence of structural airway remodelling at an early stage in CF lung disease includes the observation that airway function is demonstrably abnormal in infants newly diagnosed clinically with CF. (Ranganathan *et al.*, 2001) Furthermore, their lung function does not improve despite intensive treatment, and the airway obstruction would appear to be 'fixed'. (Ranganathan *et al.*, 2004)

However, in an earlier study Durieu *et al.* examined RBM thickness in a mixture of endobronchial and lobectomy specimens from a total of 9 children and adults with a spectrum of severity of CF lung disease. (Durieu *et al.*, 1998) They assessed the basal lamina thickness of the basement membrane qualitatively using immunostaining for murine laminin in fresh frozen sections and transmission electron microscopy. In contrast to the findings of Hilliard *et al.*, they found the RBM to be thinned in the CF specimens compared to 3 healthy, non-smoking volunteers. (Hilliard et al., 2007, Durieu et al., 1998) A dense, fibrous, acellular sub-epithelial deposit was observed however. (Durieu *et al.*, 1998)

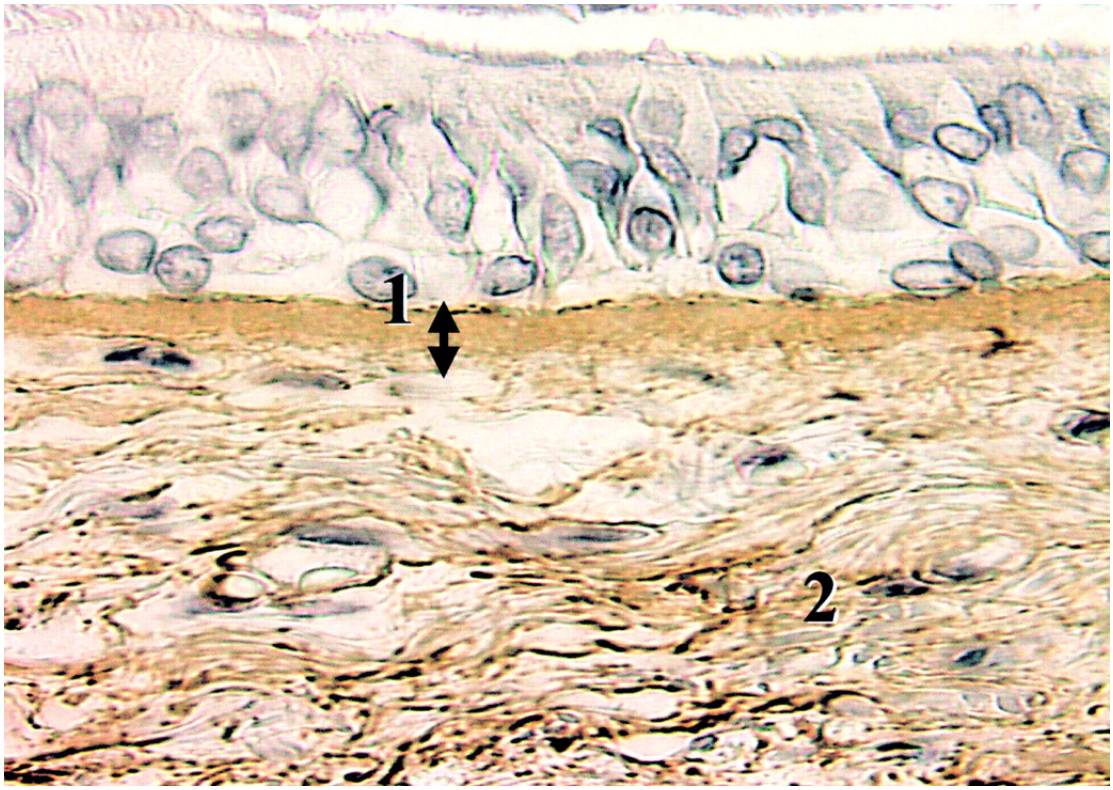


Figure 14. Example of airway remodelling in an endobronchial biopsy from a person with asthma

(1) subepithelial reticular basement membrane thickening as denoted by collagen subtype I staining and (2) diffuse staining in the lamina propria. (Ward *et al.*, 2001)

Wojnarowski *et al.* found the RBM to be thickened in association with a metaplastic epithelium in endobronchial biopsies taken from children during an acute exacerbation of CF. (Wojnarowski *et al.*, 1999) In this study the biopsies were fixed in paraformaldehyde and embedded in paraffin prior to haematoxylin and eosin staining of 2μm sections. Biopsies from clinically stable children with CF were found to contain an intact respiratory epithelium with normal RBM appearances. (Wojnarowski *et al.*, 1999) Changes in airway dimension have been described in explanted CF lungs but RBM thickness has not been specifically investigated in end-stage disease to the best of my knowledge. (Tiddens *et al.*, 2000)

Eosinophilic inflammation has been implicated in the pathogenesis of airway remodelling in asthma *via* the maintenance and progression of aberrant airway tissue injury and repair. (Holgate and Polosa, 2008) The biological activity of eosinophils is largely mediated by the release of stored granules containing potent cytotoxic proteins such as eosinophilic cationic protein, eosinophil peroxidase and major basic protein. (Stone *et al.*, 2010) In addition, eosinophils produce oxygen radicals, lipid mediators and a wide range of pro-inflmmatory cytokines and chemokines. (Hamid and Tulic, 2009) A number of cytokines produced by eosinophils are associated with remodelling and fibrosis, most notably TGF-β but also IL-6, IL-11, IL-13, IL-17 and IL-25. (Hamid and Tulic, 2009, Minshall et al., 1997) Interestingly, Hilliard *et al.* found a positive correlation between RBM thickness and total TGF-β_1 concentration in bronchoalveolar lavage fluid in chidren with CF. (Hilliard *et al.*, 2007)

In summary, there are contradictory reports in the literature regarding RBM thickness in CF lung disease and no descriptions of RBM thickness in end-stage disease.

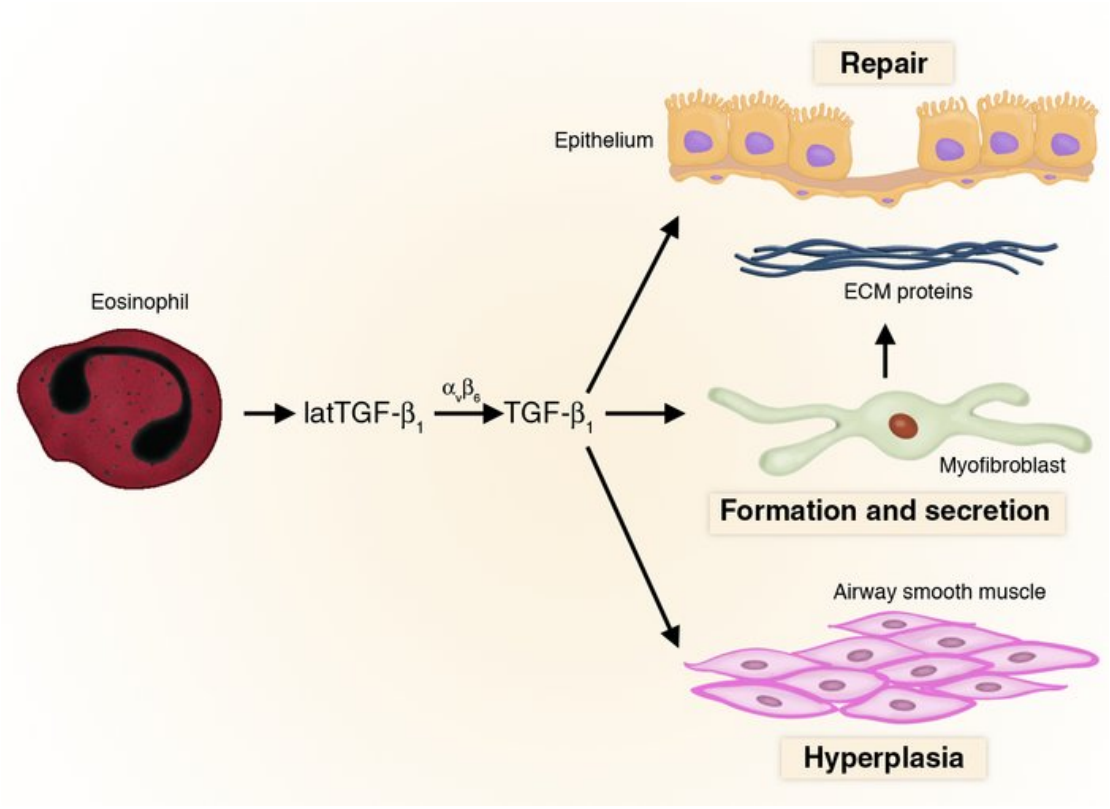


Figure 15. Schematic diagram of eosinophilic inflammation and airway remodelling in asthma

Eosinophils in the asthmatic lung release latent TGF-β1. Latent TGF-β1 (latTGF-β1) is activated by αvβ6 expressed on airway epithelial cells. TGF-β1 mediates airway remodeling by inducing the formation of myofibroblasts that secrete extracellular matrix proteins, and the growth factor also stimulates airway smooth muscle hyperplasia. (Williams, 2004)

2. Chapter 2. Hypotheses

The work described in this thesis investigated the following hypotheses

1. It is technically possible to culture PBECs from explanted CF lungs and to establish a programme to do this at the Freeman Hospital, Newcastle upon Tyne.
2. Expression of interleukin-17 is raised in the lower airway of people with advanced CF lung disease.
3. Interleukin-17 may be localised to cells other than lymphocytes in the lower airway of people with CF
4. Stimulation with interleukin-17 would increase the production of pro-neutrophilic mediators by *ex vivo* cultures of PBECs from people with advanced CF lung disease.
5. Ceramide is raised in the lower airway epithelium of people with advanced CF lung disease and is correlated with markers of neutrophilic inflammation and *P. aeruginosa* infection.
6. RBM thickness is increased in advanced CF lung disease and that this is linked to mucosal eosinophils.

3. Chapter 3. Aims

In accordance with the hypotheses outlined in Chapter 2 the aims of the work described in this thesis were to:

1. Develop and optimise a method to culture PBECs from explanted CF lungs
2. Establish a programme to culture PBECs from lungs removed at the time of transplantation from people with CF at the Freeman Hospital in Newcastle upon Tyne
3. Investigate the protein expression of interleukin-17 in the lower airway of people with advanced CF lung disease and compare to the non-suppurative condition pulmonary hypertension.
4. Describe the cellular localisation of interleukin-17 in the lower airway of people with advanced CF lung disease.
5. Investigate the effects of stimulation with interleukin-17 on the production of the pro-neutrophilic mediators interleukin-8, interleukin-6 and granulocyte monocyte colony-stimulating factor by *ex vivo* primary bronchial epithelial cell cultures from people with advanced CF lung disease.
6. Investigate levels of ceramide in the lower airway of people with advanced CF lung disease. More specifically to:
    a. Quantify immunoreactive ceramide localised to the lower airway epithelium in advanced CF lung disease compared to pulmonary hypertension, emphysema and, previously healthy, unused lung donors.
    b. Quantify the specific ceramide species C16:0, C18:0, C20:0 and C22:0 in whole lung tissue from people with advanced CF lung disease compared to pulmonary hypertension by the independent technique of high performance liquid chromatography-mass spectrometry.
7. Correlate the amount of immunoreactive ceramide in the lower airway epithelium in advanced CF lung disease, pulmonary hypertension, emphysema and unused lung donors with levels of neutrophilic inflammaion in the lower airway mucosa, as measured by the number of cells staining positive for neutrophil elastase and myeloperoxidase per mm of basement membrane.

8. Investigate the relationship between the amount of immunoreactive ceramide in the lower airway epithelium in advanced CF lung disease, pulmonary hypertension and emphysema, and colonisation with *P. aeruginosa*.
9. Quantify RBM thickness in the lower airways of people with advanced CF lung disease requiring transplantation
10. Investigate any relationship between RBM thickness and number of mucosal eosinophils
11. Investigate any relationship between RBM thickness and age at time of lung transplantation

4. Chapter 4. Materials and methods

4.1. Ethics and consent

Approval was obtained for this study from the Newcastle and North Tyneside 2 Research Ethics Committee, reference number 07/Q0906/47, on an application by Drs Malcolm Brodlie, Christopher Ward, Michael McKean and James Lordan. The study was also approved by the Research and Development Department of the Newcastle upon Tyne Hospitals Foundation NHS Trust, reference number 3910.

I obtained informed consent from all participants at the time of acceptance on to the active lung transplantation list at the Freeman Hospital, Newcastle upon Tyne.

4.2. Culture of primary bronchial epithelial cells from the lungs of people with cystic fibrosis removed at the time of transplantation

4.2.1. *Procurement of explanted lungs*

As a matter of routine lungs removed at the time of transplantation are examined macroscopically and histologically by a Consultant Pathologist in order to confirm the nature of the underlying disease and to identify any additional pathology present that may have been unidentified prior to transplantation, for example a malignancy. (Abrahams *et al.*, 2004) I am grateful to Dr Fiona Black, Consultant Pathologist, Newcastle upon Tyne Hospitals NHS Foundation Trust, for permission to remove the tissue samples for this study prior to processing of the lungs for routine pathology.

Explanted lungs were stored at 4°C once samples of frank airway secretions had been obtained for microbiology testing. Tissue was processed as soon as possible following explantation, typically in under 60 minutes. This work has therefore necessitated a permanent on-call commitment on my part in the event of a lung transplant occurring. The support of Dr Laura MacKay, MRC Clinical Research Training Fellow, Newcastle

University, the cardiopulmonary transplant coordinators, cardiothoracic surgeons and theatre staff at the Freeman Hospital has also been invaluable.

4.2.2. *Airway lavage of explanted lungs*

A small-volume airway lavage of 15mL of phosphate-buffered saline (PBS) was performed on each CF lung (total volume of 30mL/patient). This was achieved by installation of saline in to the main or first generation bronchus *via* a 20mL syringe with a 5cm quill attached that was then aspirated back in to the syringe. The median lavage return was 22mL/patient (73%) using this technique. The resulting lavage fluid was then promptly centrifuged at 2000 revolutions per minute (rpm) and the supernatant aliquoted and frozen at -20°C prior to analysis. The lower phase, containing cellular and mucoid material was also frozen and stored.

4.2.3. *Resection of main bronchus and initial processing*

All culture work was performed using strict aseptic technique in a class II laminar flow hood. Pieces of main bronchus, around 0.5cm by 2cm in dimension, were resected and cut free from surrounding connective, lymphoid, alveolar and vascular tissue. Around four pieces of bronchus were removed from each lung. The pieces of bronchus were then rinsed twice in 25mL volumes of sterile phosphate buffered saline (Sigma-Aldrich, Dorset, UK) at 4°C. The rinsed tissue was then submerged in 20mls of washing solution A at 4°C (Table 3). After 30 minutes the solution was agitated and the pieces of bronchus were rinsed twice in 25mL volumes of Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Paisley, UK) at 4°C before being submerged in 20mls of washing solution B (Table 4). The tissue was then maintained at 4°C whilst being agitated on a rocker-shaker set at 50Hz for 48 to 72 hours.

Component	Concentration	Supplier
DMEM	Neat	Invitrogen
Dnase	10μg/mL	Sigma-Aldrich
Dithiothreitol	500μg/mL	Sigma-Aldrich
Designated antimicrobials	See Table 5	

Table 3. Constituents of washing solution A

Component	Concentration	Supplier
DMEM	Neat	Invitrogen
Dnase	1μg/mL	Sigma-Aldrich
Protease	0.1%	Sigma-Aldrich
Designated antimicrobials	See Table 5	

Table 4. Constituents of washing solution B

Initially a fixed, 'best-guess', combination of antimicrobials (amphotericin B, ceftazidime, vancomycin and tobramycin) was used based on discussions with Professor John Perry, Microbiology Department, Freeman Hospital. This approach was subsequently changed however to a tailored, 'patient-specific', approach that was based on expert microbiology advice from Professor John Perry and Audrey Nicholson taking in to account where possible the sensitivity results for organisms recently isolated from the sputum of specific patients. The antimicrobial strategy is discussed fully in section 5.6 and this was the principal difference from the method previously published by Dr Scott Randell, University of North Carolina at Chapel Hill, USA. (Randell et al., 2001)

The various antimicrobials used along with the working concentrations are detailed in Table 9. The concentration of each antimicrobial used was decided based on data published by Randell *et al.* (2001) regarding the relative cytotoxicity of different agents in primary bronchial epithelial cell (PBEC) cultures and discussion with Professor Perry concerning the concentration required for the desired antimicrobial effect. (Randell *et al.*, 2001)

Antimicrobial	Concentration
Ceftazidime	128μg/mL
Tobramycin	16μg/mL
Vancomycin	10μg/mL
Colistin	5μg/mL
Meropenem	100μg/mL
Co-trimoxazole	12μg/mL
Ticarcillin and clavulanate	16μg/mL
Amphotericin B	4μg/mL
Voriconazole	10μg/mL

Table 5. Working concentrations of antimicrobials used in media

4.2.4. *Harvesting of primary bronchial epithelial cells*

Following 48 to 72 hours in washing solution B the PBECs were harvested. This was achieved by firstly adding 5mls of Roswell Park Memorial Institute-1640 (RPMI) (Invitrogen) containing 10% fetal calf serum (FCS) (Invitrogen) to the solution in order to neutralise any remaining protease activity. The pieces of bronchus were then transferred to a sterile petri dish. A scalpel blade was used to lightly scrape the lumenal side of the bronchi and thus remove the epithelial cells. The scraped cells were then suspended in 10mls of RPMI containing 10% FCS and centrifuged at 1000 rpm for 5 minutes.

The supernatant was discarded and the cells were resuspended in 5mls of Bronchial Epithelial Growth Medium (BEGM) (Lonza, Basel, Switzerland) warmed to 37°C and supplemented with the single quots detailed in Table 6, 1% streptomycin (Sigma-Aldrich), 1% penicillin (Sigma-Aldrich) and designated antimicrobials as described in Table 5. The resulting cell suspension was then seeded in to a 25cm^2 tissue culture flask (Corning, New York, USA) pre-coated with type I collagen (Purecol, Nutacon, Leimuiden, Netherlands) and placed in a carbon dioxide (5%) enriched incubator at 37°C.

Component	Volume added to 500mL*
Bovine pituitary extract	2mL
Insulin	500µL
Hydrocortisone	500µL
Retinoic acid	500µL
Transferrin	500µL
Epinephrine	500µL
Human epidermal growth factor	500µL
Tri-iodothyronine	500µL
Gentamicin/amphotericin	500µL

*concentrations not published by the manufacturer

(available as product number CC-4175 from Lonza)

Table 6. Components (single quots) added to bronchial epithelial basal medium to create bronchial epithelial growth medium

The PBEC cultures were then carefully observed to ensure that the cells were growing satisfactorily and for any evidence of infection. In the latter event the flask was immediately removed from the incubator and the contents sent for routine bacterial and fungal cultures to identify the infecting organism and relevant antimicrobial sensitivities (performed by Professor John Perry and Audrey Nicholson). The BEGM was replaced every 48 hours. The designated antimicrobials were normally removed from the BEGM after around 96 hours of successful cell culture free of overt infection.

Once flasks were around 80% confluent the PBECs were passaged using trypsin/ethylene diamine tetraacetic acid (EDTA) (Sigma-Aldrich) by the following method. The culture medium was removed from the flask and replaced with 2mls of trypsin/EDTA pre-warmed to 37°C. Adherent PBECs were then gently removed by a shaking action. The level of trypsinisation was assessed dynamically by light microscopy. Once it was gauged that a sufficient number of cells had been lifted from the base of the flask the trypsin was neutralised by the addition of an equal volume of RPMI containing 10% FCS. The resultant suspension was then removed and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was either resuspended in BEGM and seeded in to flasks or 24-well plates (Corning) for

further submerged culture, on to Transwell® inserts (Corning) for air-liquid interface (ALI) culture or cryopreserved.

4.2.5. *Cryopreservation*

Cell pellets were generated using the trypsinisation method described above. The pellets were resuspended in 1ml of 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) in FCS and transferred to sterile cryotubes (Fisher Scientific, Loughborough, UK). Tubes were placed in an isopropranolol cell freezer (Fisher Scientific) at room temperature that was then stored at -80°C for 24 hours. At this point the tubes were transferred to a liquid nitrogen cell freezer for long-term storage. It is important to note that no cryopreserved cells were used for the experiements described in this thesis. All experiments were performed using cells freshly isolated from expanted cystic fibrosis lungs.

4.2.6. *Fixation of submerged cultures for tinctorial staining and immunohistochemistry*

First-passage PBECs were grown on 8-chamber slides (Fisher Scientific) coated with type I collagen as outlined above. Once confluent the cells were fixed with 4% paraformaldehyde (Mallinckrodt Baker, Deventer, Netherlands) for 20 minutes.

4.2.7. *Reconstitution of cryopreserved cells*

Cryotubes containing 1ml cell suspensions in 10% DMSO in FCS were removed from a liquid nitrogen cell freezer and rapidly rewarmed in a 37°C water bath. Once defrosted the suspension was centrifuged at 1000rpm for 5 minutes. The supernatant was discarded and the cells were immediately resuspended in 2mls of BEGM pre-warmed to 37°C and seeded in a 25cm^2 tissue culture flask and grown as described previously.

4.2.8. *Electrophysiology studies*

First passage PBECs from patient 9, (see Table 9), were grown on type I collagen (Nutacon) coated glass coverslips. The PBECs were transferred to a tissue chamber and mounted on the stage of an inverted microscope. Whole-cell currents were recorded with an EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany). Currents were elicited using a step voltage protocol over the range ± 100 mV and analysed as described previously. (Winpenny *et al.*, 1995)

The pipette solution for experiments involving ionomycin contained (mM): 120.0 CsC1, 2.0 $MgCl_2$, 0.2 EGTA, 1.0 ATP and 10.0 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), (pH 7.2 with CsOH). Pipette solution for experiments using forskolin had EGTA adjusted to 5 mM and the addition of 2.12 mM $CaCl_2$ (calculated free Ca^{2+} concentration 100 nM). The standard bath solution contained (mM): 145 NaCl, 4.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10.0 HEPES, and 5.0 glucose (pH 7.4). Cl^- selectivity of whole cell current was confirmed by measuring the change in reversal potential (*Erev*) following the replacement of 100mM NaCl in the standard bath solution with 100mM sodium aspartate. Stock solutions of 100mM forskolin (Tocris, Bristol, UK) and 10mM ionomycin (Calbiochem, San Diego, USA) were prepared in DMSO.

4.2.9. *Air-liquid interface culture of primary bronchial epithelial cells*

A solution containing $1*10^5$ cells/mL suspended in BEGM was generated by the trypsinisation method described above from PBECs grown in submerged culture at either passage level 0 or 1. Transwell® inserts were pre-coated with type IV collagen (Nutacon) and seeded with 0.5mL of this suspension and placed in a carbon dioxide (5%) enriched incubator at 37°C. The lower basolateral chamber was filled with 1ml of pre-warmed BEGM. Once at 80-90% confluency, typically after 48 to 72 hours, the apical medium was removed and the PBECs were exposed to air at the apical membrane. At this stage the medium in the basolateral chamber was changed to ALI medium (Table 7) and was replaced every 48 to 72 hours.

Once mucus was visible by light microscopy at the apical surface it was washed every 72 hours in order to remove excess mucus and to harvest it for analysis. This was

achieved by gently applying 500μL of PBS warmed to 37°C. The PBS was left *in situ* for 5 minutes after which time it was carefully removed by pipette and stored at -20°C immediately prior to analysis.

Constituent	Volume or concentration
Bronchial epithelial basal medium (Lonza)	250mL
DMEM high glucose (Invitrogen)	250mL
1% Penicillin/streptomycin (Sigma-Aldrich)	5mL
Human epidermal growth factor	0.5ng/mL
Bovine pituitary extract	2mL*
Insulin	500μL*
Hydrocortisone	500μL*
Retinoic acid	500μL*
Transferrin	500μL*
Epinephrine	500μL*
Tri-iodothyronine	500μL*
Calcium chloride	1mM

*concentrations not published by the manufacturer
(available as product number CC-4175 from Lonza)

Table 7. Air-liquid interface medium

4.2.10. *Fixation of air-liquid interface cultures for tinctorial staining*

ALI cultures were fixed by addition of 4% paraformaldehyde (Mallinckrodt Baker) for 20 minutes.

4.2.11. *Scanning electron microscopy of air-liquid interface cultures*

Scanning electron microscopy (SEM) of ALI cultures was performed by Electron Microscopy Research Services, Newcastle University. In brief, cultures were fixed by treatment with gluteraldehyde followed by osmium tetroxide. This was followed by dehydration through graded alcohols and embedding in resin. SEM was performed by Electron Microscopy Research Services staff using a Cambridge Stereoscan 240 electron microscope with digital image capture.

4.2.12. *Slot blot enzyme-linked immunosorbent assay for MUC5B*

A slot blot technique was used to detect the airway gel-forming mucin MUC5B in apical washings from ALI cultures. (Taylor et al., 2004, Smirnova et al., 2003) Nitrocellulose membrane sheets (Whatman, Kent, UK) of 0.2μm pore size were mounted on the Minifold II 72-well slot blot apparatus (Schleicher and Schuell, Dassel, Germany) and supported on blotting paper wetted with deionised water. Standards of MUC5B, purified from human saliva (Professor JP Pearson, Newcastle University), and 100μL aliquots of ALI washings were added to individual wells in triplicate and the block attached to a vacuum source. Once the samples were absorbed on to the membrane it was removed and blocked overnight at 4°C, to minimise non-specific binding in PBS containing 2% bovine serum albumin (BSA). After blocking the membrane was incubated with the primary antibody, MUC5B thick effusion polyclonal anti-serum (Queen's Medical Centre, Nottingham, UK), at 1:1000 dilution in PBS containing 1% BSA for 2 hours at room temperature. The membrane was then washed twice in 0.5% Tween20 in PBS followed by 3 changes of PBS. The secondary antibody, anti-rabbit IgG horseradish peroxidase conjugated (Sigma-Aldrich) diluted at 1:10000 in 1% BSA in PBS, was then added for 1.5 hours at room temperature. The membrane was then washed as described above and developed using 0.05% 3, 3'diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) with 0.3% H_2O_2 in PBS for 5 minutes. Once sufficiently developed the membrane was washed in tap water and left to dry overnight. The membrane was then read at 595nm using a Shimadzu scanning densitometer (Columbia, USA). Negative controls were performed to determine non-specific binding of the primary antibody by incubating it with 1% BSA in PBS alone.

4.2.13. *Measurement of trans-epithelial resistance of air-liquid interface cultures of primary bronchial epithelial cells*

Trans-epithelial resistance (TER) measurements were performed using an epithelial volt-ohmmeter (World Precision Instruments, Stevenage, UK) as per the manufacturer's instructions. In order to measure the TER 0.5mL of pre-warmed BEGM was applied to the apical surface and allowed to equilibriate for 20 minutes prior to performance of the measurements. The resistance of a 'blank' membrane included in each plate that was treated identically, but not seeded with cells, was subtracted from each measurement.

4.2.14. *Stimulation of primary bronchial epithelial cells with interleukin-17*

First or second passage PBECs were grown to 70-80% confluence in 24-well plates coated with type I collagen using BEGM. Cells were rested for 24 hours with serum-free resting medium (Table 8) prior to stimulation with 500μL of resting medium containing 1, 10 or 100ng/mL of recombinant human IL-17 (R&D Systems).

Constituent	Volume added to 500mL of bronchial epithelial basal medium (Lonza)
ITS liquid media supplement: 1mg/mL insulin from bovine pancreas, 0.55mg/mL human transferrin and 0.5μg/mL sodium selenite (Sigma-Aldrich)	5mL
1% Penicillin/streptomycin (Sigma-Aldrich)	5mL
Gentamicin/amphotericin single quot (Lonza)	500μL*

*concentrations not published by the manufacturer
(available as product number CC-4175 from Lonza)

Table 8. Constituents of resting medium

4.2.15. *Stimulation of primary bronchial epithelial cells with flagellin*

First or second passage PBECs were grown to 70-80% confluence in 24-well plates coated with type I collagen using BEGM. Cells were rested for 24 hours with serum-free resting medium (Table 8) prior to stimulation with 500μL of resting medium containing 1, 10 or 100 ng/mL of flagellin (Alexis biochemicals, Exeter, UK).

4.2.16. *Measurement of interleukin-17 in airway lavage fluid from explanted cystic fibrosis lungs*

Airway lavage fluid supernatants were defrosted on ice and then promptly assayed for IL-17 concentration using an ultrasensitive ELISA kit (MesoScale Discovery, Maryland, USA) with a lower limit of detection of 0.2pg/mL as per manufacturer's instructions. The plates were read using the MSD Discovery Workbench analyser and software package. (MesoScale Discovery).

4.2.17. *Measurement of interleukin-23 in airway lavage fluid from explanted cystic fibrosis lungs*

Airway lavage fluid supernatants were defrosted on ice and then promptly assayed for IL-23 concentration using a Quantikine® ELISA kit (R&D Systems) with a lower limit of detection of 6.8pg/mL as per manufacturer's instructions. Each sample and standard was assayed in triplicate and a mean taken. A standard curve was then constructed and the IL-23 concentration was quantified for each sample.

4.2.18. *Measurement of interleukin-8, interleukin-6 and granulocyte macrophage colony-stimulating factor in culture supernatants*

The contents of each well was removed by pipette after 24 hours, centrifuged at 1000rpm for 2 minutes and the supernatant immediately frozen at -80°C prior to analysis. A multiplex enzyme-linked immunosorbent assay (ELISA) was then

performed as per manufacturer's instructions (Meso Scale Discovery). The plates were read using the MSD Discovery Workbench analyser and software package. (MesoScale Discovery).

4.3. Histology

4.3.1. *Preparation of paraffin embedded blocks of airway*

A 2cm by 0.5cm piece of lung parenchyma was resected using tweezers and a fresh scalpel. The intention being to produce sections of intermediate-large sized airways, in the region of 1-5mm diameter and lined by columnar ciliated respiratory epithelium. The tissue was then fixed in 10% buffered formalin (Genta Medical, York, UK) for 12 hours.

Fixed tissue was then placed in cassettes (Simport, Beloeil, Canada) and dehydrated through graded alcohols. This was performed by immersion for 20 minutes in: 70% industrial methylated spirit (IMS), 70% IMS, 80% IMS, 80% IMS, 95% IMS, 95% IMS, 74OP IMS, 74OP IMS and 74OP IMS respectively. Finally the tissue was submerged in xylene (VWR) twice for 20 minutes and then embedded in paraffin wax for 1 hour at 60ºC.

4.3.2. *Preparation of paraffin sections*

Sections of 5μm thickness were cut using a microtome from paraffin blocks of distal airway. The sections were dewaxed by soaking in xylene for 5 minutes and then rehydrated through graded alcohols, 1 minute each in 95% IMS, 99% IMS and 99% IMS again.

4.4. Tinctorial stains

4.4.1. *Haematoxylin and eosin staining*

Haematoxylin and eosin staining was used to examine the basic histology of the sections. (Kiernan, 2008) Sections were cut, dewaxed and rehydrated as described above. In the case of 8-chamber slides the rehydration steps were unnecessary. The sections were then washed in water and stained in freshly filtered Harris Haematoxylin (Fisher Scientfic) for 1 minute. The staining process was assessed dynamically under a light microscope. Next the sections were washed in running tap water for 2-3 minutes before being differentiated in 0.1% acid alcohol and washed in running tap water until the nuclei were appropriately blue. The sections were then counterstained with Eosin Y (Fisher Scientific) for 2 minutes. Finally the sections were washed well in tap water and dehydrated back through graded alcohols and xylene. The sections were then mounted with DPX (VWR, Lutterworth, UK).

4.4.2. *Periodic acid-Schiff staining*

Periodic acid-Schiff (PAS) staining was used to stain for the mucin glycoproteins in mucus. (Kiernan, 2008) Sections were cut, dewaxed and rehydrated as described above. In the case of 8-chamber slides the rehydration steps were unnecessary. Firstly the sections were soaked in 1% periodic acid for 5 minutes and then washed under running tap water for 2 minutes. Next they were soaked in Schiff's solution (Sigma-Aldrich), diluted 1 in 4 with distilled water, for 6 minutes and then washed under running tap water for 5 minutes. The sections were then counter-stained with Harris Haematoxylin for 30 seconds and washed until blue. Finally the sections were dehydrated and mounted with DPX as described previously.

4.4.3. *Alcian blue/periodic acid-Schiff staining*

The combination of alcian blue and PAS staining was used to stain acidic and neutral mucins in airway sections. (Kiernan, 2008) Sections were cut, dewaxed and rehydrated as described above. Firstly sections were incubated with 0.1% diastase (VWR) for 30 minutes at 37°C followed by alcian blue pH 2.5 (Sigma-Aldrich) for 20 minutes and then washed thoroughly with tap water. Periodic acid-Schiff staining was then performed as outlined above.

4.4.4. *Sirius red staining*

Sirius red was used to stain for eosinophils. (Meyerholz et al., 2009) Sections were cut, dewaxed and rehydrated as described above. Nuclei were firstly stained with Harris Haematoxylin as outlined above. Sections were then stained with Sirius red (Sigma-Aldrich) for 1 hour and then thoroughly washed in tap water for 10 minutes. Finally the sections were dehydrated and mounted with DPX as described previously.

4.5. Immunohistochemistry

4.5.1. *Immunohistochemistry for pan-cytokeratin panel*

Confluent monolayers of PBECs were fixed on 8-chamber slides as described in section 4.2.6 and stained for an epithelial panel of pan-cytokeratin markers as previously described. (Forrest *et al.*, 2005) The cells were initially treated with 5% normal lamb serum (Dako, Ely, UK) in PBS for 1 hour to minimise non-specific antibody binding. The primary mouse monoclonal anti-human cytokeratin clone LP34 (Dako) was then applied at a dilution of 1:50 in normal lamb serum for 2 hours at room temperature. The slides were washed with PBS and then treated with an appropriate secondary system as per manufacturers instructions (EnVision/HRP, Dako). Matched istotype negative controls were used.

4.5.2. *Immunohistochemistry for interleukin-17*

Paraffin sections were cut, dewaxed and rehydrated as described above. Endogenous peroxidase activity was blocked by soaking sections in methanolic hydrogen peroxide. The sections were then washed for 10 minutes in running tap water. Antigen retrieval was performed in citrate buffer at pH 6 for 5 minutes in a microwave set at 30% power. The sections were then washed 3 times in tris-buffered saline (TBS) and blocked with 20% normal horse serum (Vector Laboratories, Peterborough, UK) for 30 minutes. The primary antibody (goat polyclonal affinity purified anti-human IL-17, R&D Systems, Abingdon, UK) was diluted 1 in 40 in 3% bovine serum albumin (BSA) and applied for 1 hour at room temperature. The sections were then washed twice with TBS and treated with biotinylated horse anti-goat secondary antibody (Vector Laboratories) for 30 minutes. The sections were washed twice with TBS and treated with the ABC Vectastain Elite kit and DAB (Vector Laboratories) as per the manufacturer's instructions. The sections were finally counter-stained with Carazzi's stain for 1 minute and then mounted. Isotype negative controls were performed using normal goat immunoglobulins (R&D Systems).

4.5.3. *Immunohistochemistry for retinoic acid receptor-related orphan receptor gamma*

Paraffin sections were cut, dewaxed and rehydrated as described above. Endogenous peroxidase activity was blocked by soaking sections in methanolic hydrogen peroxide. The sections were then washed for 10 minutes in running tap water. The sections were steamed in citrate buffer pH 6 at 100°C for 20 minutes and then left to cool in the buffer at room temperature for a further 20 minutes. The sections were then washed 3 times in tris-buffered saline (TBS) and blocked with 20% normal horse serum (Vector Laboratories, Peterborough, UK) for 30 minutes. The primary antibody (retinoic acid receptor-related orphan receptor gamma rabbit polyclonal, AbCam, Cambridge, UK) was diluted 1 in 40 in 3% bovine serum albumin (BSA) and applied for 1 hour at room temperature. The sections were then washed twice with TBS and treated with biotinylated horse anti-rabbit secondary antibody (Vector Laboratories) for 30 minutes. The sections were washed twice with TBS and treated with the ABC Vectastain Elite kit

and DAB (Vector Laboratories) as per the manufacturer's instructions. The sections were finally counter-stained with Carazzi's stain for 1 minute and then mounted. Isotype negative controls were performed using normal rabbit immunoglobulins (R&D Systems).

4.5.4. *Immunohistochemistry for ceramide (Glycobiotech antibody)*

Paraffin sections were cut, dewaxed and rehydrated as described above. The antiserum enriched for IgM anti-ceramide polyclonal antibody (Glycobiotech, Kükels, Germany) is commercially available and has been utilised by other researchers and demonstrated by lipid overlay assays to be specific for ceramide. (Cowart et al., 2002, Teichgraber et al., 2008, Vielhaber et al., 2001) Antigen retrieval was performed in citrate buffer at pH 6.0 in a microwave on high power for 3 minutes. The primary antibody was used at 1/150 dilution in 3% BSA. Sections were incubated for 48 hours at 4ºC with the primary antibody before treatment with the EnVision peroxidase-based secondary system (Dako). Appropriate negative controls; no primary antibody added and mouse IgM (Dako) istotype negative control were performed.

4.5.5. *Immunohistochemistry for ceramide (Sigma antibody)*

Paraffin sections were cut, dewaxed and rehydrated as described above. The monoclonal anti-ceramide mouse IgM antibody (Sigma-Aldrich) was used at 1/50 dilution in 3% BSA. Antigen retrieval was performed in citrate buffer at pH 6.0 in a microwave on high power for 3 minutes. Sections were incubated for 48 hours at 4ºC with the primary antibody before treatment with the EnVision peroxidase-based secondary system (Dako). Appropriate negative controls; no primary antibody added and mouse IgM (Dako) istotype negative control were performed. It is important to note that this antibody has been shown in lipid overlay assays to be less specific for ceramide than the polyclonal antibody in that it also detects dihydroceramide, phophatidylcholine and sphingomyelin. (Cowart *et al.*, 2002)

4.5.6. *Immunohistochemistry for neutrophil elastase*

Paraffin sections were cut, dewaxed and rehydrated as described above. Sections were then incubated at room temperature for 45 minutes with mouse anti-neutrophil elastase monoclonal antibody (Dako) diluted 1/300 in 3% bovine serum albumin. No antigen retrieval was required. The EnVision secondary system (Dako) was used. Mouse immunoglobulin G_1 (R&D Systems) isotype negative controls were performed.

4.5.7. *Immunohistochemistry for myeloperoxidase*

Paraffin sections were cut, dewaxed and rehydrated as described above. Antigen retrieval was performed for 10 minutes in citrate buffer at pH 6.0 in a microwave on high power. Sections were incubated overnight at 4°C with rabbit anti-myeloperoxidase polyclonal antibody (Novocastra, Newcastle upon Tyne, United Kingdom) diluted 1/1000 in 3% bovine serum albumin. The Immpress peroxidase-based secondary system was used (Vector Labs, Peterborough, United Kingdom). Appropriate rabbit immunoglobulin (Dako) negative controls were performed.

4.6. Quantification and analysis of staining

4.6.1. *Quantification and analysis of interleukin-17 staining*

Positive staining was measured in the airway epithelium in 5 randomly selected, non-overlapping x40 objective high power fields for each patient. I was blinded to the diagnosis of each patient. Image analysis software was used (Image Pro Plus 4.0, MediaCybernetics, Bethesda, USA) to quantify the staining in terms of mean percentage area of epithelium staining positive and number of positive inflammatory cells per mm of basement membrane in the lamina propria and epithelial compartments as previously described. (Ward *et al.*, 2005) Neutrophils were identified by appropriate dimensions

and characteristic morphological features such as multilobular nuclei and granular cytoplasm. (Bain, 2002)

4.6.2. *Quantification of ceramide staining*

Positive staining was measured in the airway epithelium in 5 randomly selected, non-overlapping x40 objective high power fields for each patient. Image analysis software was used (Image Pro Plus 4.0) to quantify the staining in terms of percentage area of epithelium positive for ceramide as previously described. (Ward *et al.*, 2005) I was blinded to the diagnosis of each patient. A further analysis using a Bland-Altman plot was performed to assess the intra-observer repeatability of these measurements that is presented in section 7.5.5. (Bland and Altman, 1986)

4.6.3. *Quantification of neutrophil elastase and myeloperoxidase staining*

The number of cells staining positive in the epithelium and lamina propria were counted in 5 randomly selected, non-overlapping x20 objective high power fields for each patient using image analysis software (Image Pro Plus 4.0). The length of basement membrane in each field was measured to calculate the number of positive cells/millimetre basement membrane. I was blinded to the diagnosis of each patient.

4.6.4. *Measurement of reticular basement membrane thickness*

Measurements were performed on 5μm sections stained with haematoxylin and eosin taken from formalin-fixed, paraffin-embeeded blocks of intermediate-large airway as described in sections 4.3.1 and 4.3.2. RBM thickness was quantified by measuring the distance between two demarcating lines in each high-power section using Image ProPlus software (MediaCybernetics) as shown in Figure 87.

4.7. High performance liquid chromatography-mass spectrometry for ceramide

4.7.1. *Clinical sample preparation and lipid extraction*

Biopsies were resected from freshly explanted lungs and immediately snap frozen in an isopentane slurry cooled with liquid nitrogen prior to storage at -80°C. At the time of analysis around 100mg of tissue was defrosted to room temperature, weighed and immediately homogenised. Lipids were then extracted from the homogenate using a previously described chloroform/methanol protocol. (Folch et al., 1957, Guilbault et al., 2008b)

4.7.2. *High performance liquid chromatography-mass spectrometry*

A Waters (Milford, MA, USA) nanoACQUITY HPLC pump, with autosampler and a LTQ-FT hybrid linear ion trap-FTMS (Thermo Electron, Bremen, Germany) mass spectrometer were interfaced using an ADVANCE (Michrom Bioresources, Auburn, CA, USA) MS ion source. The calibration standards used were C16:0 (N-Palmitoyl-D-Sphingosine, N-Hexadecanoyl-D-sphingosine, Palmitoyl ceramide, catalogue number C2777-8), C18:0 (N-Stearoyl-D-sphingosine, Stearoyl ceramide, catalogue number C2777-83), C20:0 (N-Eicosanoylsphingosine, catalogue number C2777-85) and C22:0 (N-[(3E)-2-hydroxy-1-(hydroxymethyl)-3-heptadecenyl]-docosanamide, catalogue number C2777-87) ceramides (US Biological, Swampscott, Ma, USA). Each standard was reconstituted using an appropriate volume of chloroform:methanol (2:1) to generate 20mM stock solutions for HPLC-MS analysis. Further dilutions were made using acetonitrile.

The lower (chloroform) phase of each patient sample was carefully transferred to a clean tube and then evaporated to dryness in a centrifugal evaporator. Samples were re-suspended in 1mL of chloroform:methanol (2:1) and then diluted 1:10 with acetonitrile prior to HPLC-MS analysis.

All samples and standards were analyzed in triplicate on a Waters (Milford, MA, USA) C18 symmetry column (0.3 by 100mm) maintained at 45°C and a flow rate of

10μL/min. A non-aqueous reversed phase HPLC (NARP) gradient was used. Buffer A comprised acetonitrile:formic acid (99.8:0.2) while buffer B was 20% isopropanol in acetonitrile. Samples were eluted using a gradient of 0 to 15% buffer B over 20 minutes.

The mass spectrometer method consisted of two scan events, a FTMS scan at 12,500 resolution in the range 475 – 675m/z (to minimize sample matrix noise), followed by one data-dependant ion trap MS/MS. The ADVANCE spray source was used without sheath gas and the spraying voltage was 1.5kV.

Calibration curves were constructed from triplicate readings of a four ceramide mixture at 5, 0.5, 0.05 and 0.005pmoles injected on-column, (Table 15 and Figure 69 to Figure 72 respectively). The initial plan was to monitor the MS/MS product ion at 264.3m/z as a means for selective quantitation. However it soon became apparent that the ceramide spectra were dominated by $[M+Na]^+$ adducts as the major ion species precluding observation of the desired product ion at a sufficiently high sensitivity. To circumvent this problem the mass accuracy of the FTMS was used for ion selection and quantitation.

Qualbrowser software (Thermo Electron, Bremen, Germany) was used to sum the ion currents for both observed $[M+H]^+$ and $[M+Na]^+$ singly –charged ion species of each ceramide. For selectivity the mass tolerance for each ion was set to within 0.02m/z. The peak areas of the detected ion signals were recorded and calibration plots constructed using this data for each of the four ceramide species. The calibration plots were then used to obtain quantitative results for C16:0, C18:0, C20:0 and C22:0 ceramides from triplicate measurements on the 10 patient samples.

4.7.3. *Protein assay*

The Bio-Rad Protein Micro Assay (Bio-Rad, Hemel Hempstead, UK), based on the Bradford protein assay technqiue, was used to measure protein levels spectroscopically in the upper phase of the HPLC-MS analysates. (Bradford, 1976) Standards ranging from 0.8 to 20μg/mL were prepared from a stock of 400μg/mL made by dissolving BSA in deionised water. Samples were diluted 1:2 with deionised water prior to analysis. In a clean dry test tube 800μL of standard or diluted sample was mixed with

200μL of reagent and incubated for 30 minutes at room temperature. The optical density at 595nm was then read in fresh cuvettes using a spectrometer (Unicam 8625 UV/VIS spectrometer, Cambridge, UK). The optical density of a 'blank' cuvette filled with 1mL of deionised water was subtracted from each reading. Each sample and standard was assayed in triplicate and a mean taken. A standard curve was then constructed and the protein concentration was quantified for each sample.

4.7.4. *Phosphate assay*

The PiBlue Phosphate Assay Kit (BioAssay Systems, Hayward, USA) was used to quantify levels of phosphate in the upper pahse of the HPLC-MS analysates. Standards ranging from 4 to 40μM phosphate were prepared from a stock 40μM solution as per the manufacturer's instructions. Using a clean dry 96-well plate 50μL of standard or sample was added to each well followed by 100μL and incubated for 30 minutes at room temperature. The optical density at 620nm was then read in fresh cuvettes using a spectrometer (Unicam 8625 UV/VIS spectrometer, Cambridge, UK). Each sample and standard was assayed in triplicate and a mean taken. A standard curve was then constructed and the phosphate concentration was quantified for each sample.

4.8. Statistics

Statistical analyses were performed using Minitab 15 (Minitab Incorporated, Pennsylvania, USA). Graphs were prepared using Prism 5 for Mac (GraphPad software, California, USA).

Non-parametric statistical tests were used to test the null hypothesis using the most conservative approach possible. A P value <0.05 was considered to be significant. Where multiple comparisons were performed correction was performed using the Holm-Bonferroni method. (Holm, 1979) The Holm–Bonferroni method is a closed test procedure that allows the performance of more than one hypothesis test simultaneously by adjusting the relevant P value accordingly. (Marcus et al., 1976) The specific statistical test used is stated in the figure caption directly below the graph in the thesis.

5. Chapter 5. Development of a method to culture primary bronchial epithelial cells from the lungs of people with cystic fibrosis removed at the time of transplantation

Elements of the work described in this chapter have been previously reported in the form of presentations at medical and scientific meetings and in a peer-reviewed paper. (Brodlie et al., 2008a, Brodlie et al., 2008b, Brodlie et al., 2009f, Brodlie et al., 2010f)

5.1. Abstract

Background: Lung disease is responsible for more than 95% of morbidity and mortality in cystic fibrosis. The exact pathogenesis of cystic fibrosis lung disease remains poorly understood. Experimental models are therefore vital for use in research. Animal models and immortalised cell lines both have inherent limitations. Explanted lungs removed from people with CF at the time of transplantation represent a potentially valuable but technically and logistically challenging source of primary cystic fibrosis bronchial epithelial cells.

Methods: Pieces of segmental bronchus from explanted lungs were treated with patient-specific antimicrobials prior to isolation of bronchial epithelial cells. Cultured cells were characterised by their morphology under light microscopy, cytokeratin and haematoxylin-eosin staining, electrophysiological profile and cytokine production.

Results: Primary bronchial epithelial cells were successfully cultured from 23 of 34 patients attempted. The cells exhibited typical epithelial morphology, staining for cytokeratin, lack of responsiveness to forskolin treatment, produced interleukin-8, interleukin-6 and granulocyte macrophage colony-stimulating factor and remained viable after storage in liquid nitrogen. Eleven unsuccessful cultures failed due to early infection with bacteria known to colonise the airways pre-transplant.

Conclusions: Primary bronchial epithelial cell culture is possible from explanted cystic fibrosis lungs and provides an important cellular model to elucidate the pathogenic mechanisms in cystic fibrosis lung disease and to investigate potential therapeutic targets.

5.2. Introduction

Promising developments in clinical care have yielded an increase in survival for people with CF over recent decades, however, the exact pathogenesis of CF lung disease remains poorly understood. (Dodge *et al.*, 2007) It follows that valid experimental models are required to further elucidate the pathogenesis of CF lung disease and to evaluate novel, potentially therapeutic compounds. (Doring *et al.*, 2007)

Shortly after the cloning of the CFTR gene in 1989 it has been possible to develop animal models of CF. (Riordan *et al.*, 1989) Work has principally focussed on murine models and has generated over 2500 publications to date. (Scholte *et al.*, 2004) Mice with complete interruption of the CFTR gene, residual function and with specific, clinically relevant mutations have been developed. (Davidson and Rolfe, 2001, Guilbault et al., 2007) However, despite promising bioelectric features and replication of gastrointestinal pathology it has proved impossible to reproduce the human CF lung phenotype. Indeed, Scholte *et al.* (2004) in a review of this topic concluded that:

> *"the development of an ideal mouse model of CF lung disease, to enable the dissection of pathogenesis, or testing of novel therapeutics is yet to be achieved."*

Possible explanations for this include inter-species differences in submucosal gland distribution, innate immunity, epithelial cell composition and chloride channels in the lung. (Pack et al., 1980, Grubb et al., 1994, Borthwick et al., 1999, Maxwell et al., 2003)

A major programme of work is under way to develop pigs and ferrets with mutant CFTR that may produce animals with a closer lung phenotype to that seen in CF but it will be a substantial number of years before they represent a practical tool for research. (Sun et al., 2008, Rogers et al., 2008c)

Immortalised cell lines, derived from relevant tissues in people with CF, non-CF individuals and other mammals, have also been used extensively in research and have contributed to our current understanding of the disease. (Lundberg *et al.*, 2002) There are a number of important limitations, however, that must be remembered if the results

of experiments involving immortalised cells are to be extrapolated to the situation *in vivo*.

Firstly, a cell line is always derived from a fixed clone of a phenotypically diverse mixed primary cell culture. The phenotype of cells is also likely to change with multiple passages *in vitro* and furthermore karyotypic instability is a feature of immortalised cells that may lead to the emergence of diverse subpopulations that do not retain the phenotypic characteristics of interest. (Gruenert *et al.*, 2004)

In summary, both animal models and immortalised cell lines have contributed significantly to CF research but there are inherent limitations to both systems. (Carvalho-Oliveira et al., 2007, Gruenert et al., 2004, Guilbault et al., 2007) In particular, animal models have failed to reproduce the lung disease characterised by neutrophilic inflammation, chronic bacterial infection and mucus hypersecretion that is responsible for 95% of mortality and morbidity in CF. Immortalised cell lines are accessible and convenient but it is widely acknowledged that high passage cells are likely to significantly differ from those found *in vivo* and that they display karyotypic instability.

Primary tissue and cells from people with CF therefore represent an important and rare experimental resource. The *ex vivo* culture of PBECs from people with CF is likely to produce a superior cellular model to that produced by immortalised cell lines with characteristics that replicate more accurately those found *in vivo*. Furthermore, well-differentiated PBEC cultures maintained at ALI are an excellent model of airway epithelial function and have been integral to several important developments in our current knowledge of the pathophysiology of CF lung disease. (Matsui et al., 1998, Fulcher et al., 2005)

Lung transplantation is the only life-sustaining option for end-stage CF lung disease. (Meachery et al., 2008, Corris, 2008) The organs explanted from people with CF during lung transplantation represent a potential source of CF PBECs. The culture of PBECs from explanted CF lungs poses scientific and logistical challenges however. The major practical obstacles to the successful culture of cells include firstly, actual procurement of appropriate lung tissue and secondly, infection of cell cultures by multi-resistant

organisms present in the copious mucopurulent secretions that are prevalent in end-stage CF lungs. (Randell *et al.*, 2001)

To the best of my knowledge, prior to the commencement of this work PBECs were not cultured routinely from explanted CF lungs outside of a handful of centres in North America, most notably at the University of North Carolina, Chapel Hill. (Karp et al., 2002, Yamaya et al., 1992, Randell et al., 2001) The Freeman Hospital in Newcastle upon Tyne is the largest lung transplantation centre in the United Kingdom. Lungs removed at the time of transplantation from people with CF in Newcastle therefore represent a previously under used and potentially highly valuable resource for CF research.

5.3. Aims

The aims of the work described in this chapter were to:

- Develop and optimise a method to culture PBECs from explanted CF lungs
- Establish a programme to culture PBECs from lungs removed at the time of transplantation from people with CF at the Freeman Hospital in Newcastle upon Tyne

5.4. Hypothesis

I hypothesised that it would be technically possible to culture PBECs from explanted CF lungs and to establish a programme to do this at the Freeman Hospital, Newcastle upon Tyne.

5.5. Results

5.5.1. *Development of a method to culture primary bronchial epithelial cells from explanted cystic fibrosis lungs*

The detailed method that I used to culture PBECs is described in Chapter 4. The technique published by Randell *et al.* (2001) was used as an initial template. This was subsequently optimised through personal experience, helpful discussions with Dr Scott Randell, University of North Carolina and Professor Christopher O'Callaghan, University of Leicester, input from my supervisors in Newcastle and further discussions with delegates and other invited speakers at the 'Workshop on Epithelial Cells from Lung: Production, Cultivation and Characterization' held by EuroCareCF in Lisbon, Portugal in July 2008.

I developed the novel approach of using patient-specific combinations of antimicrobials. These were based on up to date knowledge of the organisms cultured from the sputum of patients in their clinical management pre-transplant. (Corris, 2008, Meachery et al., 2008) The expert advice of Professor John Perry and Audrey Nicholson, Department of Medical Microbiology, Freeman Hospital, was invaluable in selecting an appropriate anti-microbial combination for each specific patient.

A key consideration when using primary tissue in research is that the clinical care of patients is not compromised. The method that I developed allows clinical specimens to be collected from explanted lungs for microbiology and involves only modest dissection thereby enabling a full examination of the lungs to be performed by a pathologist.

5.5.2. *Establishment of a programme to culture primary bronchial epithelial cells from explanted cystic fibrosis lungs at the Freeman Hospital, Newcastle upon Tyne*

In addition to the scientific challenges of culturing PBECs from end-stage CF lungs, the establishment of a programme to utilise explanted lungs relied on excellent working relationships with the entire multidisciplinary transplant team. This includes obtaining the appropriate ethical approval to perform such work and clearly the informed consent of the transplant recipients themselves. In my experience it is ideal if individuals are approached for consent at the time of visiting the transplant centre for assessment and not during the emergency admission immediately prior to a potential transplant occurring. Indeed, the Local Research Ethics Committee advised this timing of approach for consent when they considered our ethical application for this work.

Furthermore, the helpful co-operation of cardiothoracic surgeons, transplant coordinators and theatre staff is essential in order to ensure that the research team is informed. Due to the unpredictable nature of transplantation in my experience a permanent 'on-call' commitment is important in facilitating and ensuring the appropriate timely procurement of tissue in the event of a transplant occurring. An onward stream of interested researchers is likely to be essential to sustain this programme of tissue procurement in the future.

5.5.3. *Outcome of primary bronchial epithelial cell cultures from explanted cystic fibrosis lungs*

I actively procured lungs between October 2007 and March 2010. During this period 36 people with CF were transplanted at the Freeman Hospital. I attempted to culture PBECs from the lungs of 34 of the 36 patients. Figure 16 shows a typical explanted CF lung.

Cultures were successful from 23 (68%) of the 34 patients attempted. When unsuccessful this was universally due to early bacterial overgrowth once the initial anti-microbial cocktail had been removed from the culture medium. Routine microbiological

culture of infected supernatants was performed by Professor John Perry and Audrey Nicholson, which revealed that the bacteria responsible in each case were the same organisms that had colonised the lungs of the patients pre-transplantation. Table 9 outlines the clinical details, the organisms isolated from sputum pre-transplant, antimicrobial combination used and the outcome of cell culture for each patient. Figure 17 compares the success rate of cultures with the initial 'best guess' approach (50%) to that following adoption of a tailored patient-specific anti-microbial strategy (75%).

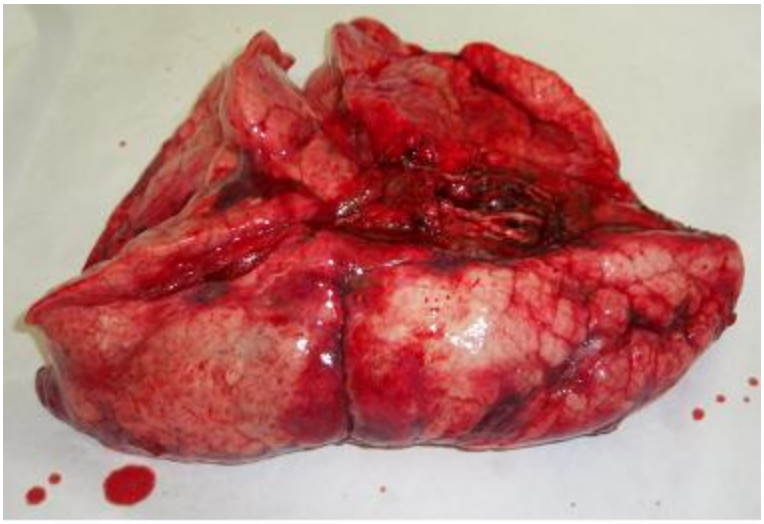


Figure 16. Typical example of an explanted cystic fibrosis lung.

Dimensions 230mm superior-inferior, 155mm anterior-posterior and 60mm medial-lateral

Table 9. Brief clinical details of patients, microbiology and outcome of primary bronchial epithelial cell cultures

No.	Age*	CFTR variant[$]	FEV_1[^]	Sputum microbiology	Antimicrobials used in cell culture	Outcome of cultures
1	28	p.Phe508del p.Phe508del	15	*Pseudomonas aeruginosa, Aspergillus fumigatus*	Ceft, Tobr, Vanc, Amph	Successful
2	57	p.Arg117His p.Arg553X	23	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph	Successful
3	32	Unknown	25	*P. aeruginosa, Burkholderia vietnamiensis*	Ceft, Tobr, Vanc, Amph	Not successful, *B. vietnamiensis* overgrowth
4	25	p.Phe508del Unknown	22	*P. aeruginosa, Staphylococcus aureus*	Ceft, Tobr, Vanc, Amph	Not successful, apparent infection, negative on culture
5	23	Unknown	21	*Scedosporium* sp., *Alcaligines* sp., *S. Aureus*	Ceft, Tobr, Vanc, Amph, Mero, Vori	Successful
6	39	p.Phe508del p.Phe508del	14	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph	Successful
7	29	p.Phe508del p.Gly551Asp	23	*P. aeruginosa,* Methicillin-Resistant *S. aureus*	Ceft, Tobr, Vanc, Amph, Mero	Not successful, *P. aeruginosa* overgrowth
8	27	p.Phe508del p.Phe508del	22	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero	Not successful, *P. aeruginosa* overgrowth
9	43	p.Phe508del p.Arg560Thr	15	*Stenotrophomonas maltophilia, Acinetobacter* sp.	Ceft, Tobr, Vanc, Amph, Colo, CoTrim, Tim	Successful
10	28	p.Phe508del p.Asp443fs	17	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo	Successful
11	29	p.Phe508del p.Phe508del	20	*P. aeruginosa, A. fumigatus*	Ceft, Tobr, Vanc, Amph, Mero, Colo	Successful
12	30	p.Phe508del p.Phe508del	30	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim	Successful

13	40	Unknown	24	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim Vori	Not successful, *P. aeruginosa* overgrowth
14	23	p.Phe508del p.Gly551Asp	30	*P. aeruginosa, A. fumigatus*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Vori	Successful
15	46	Unknown	30	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful
16	29	p.Phe508del p.Gln685fs	17	*B. multivorans, A. fumigatus, Geosmithia argillacae*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim Vori	Not successful, *B. multivorans* overgrowth
17	22	p.Phe508del p.Phe508del	23	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful
18	20	p.Phe508del p.Phe508del	21	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim	Successful
19	29	p.Phe508del c.317+10kbC>T	29	*P. aeruginosa, S. maltophilia*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Not successful, *P. aeruginosa* overgrowth
20	28	Unknown	20	*P. aeruginosa,* Methicillin-Resistant *S. aureus*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful
21	48	p.Phe508del Unknown	29	*P. aeruginosa, S. aureus*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful
22	33	Unknown	23	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful
23	22	p.Phe508del p.Phe508del	25	*P. aeruginosa, A. fumigatus*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Not successful, *P. aeruginosa* overgrowth
24	40	Unknown	36	*P. aeruginosa, S. maltophilia, Achromobacter xylosoxidans*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful

25	24	p.Phe508del p.Phe508del	26	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful
26	21	Unknown	25	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful
27	45	p.Phe508del p.Phe508del	21	*P. aeruginosa, A. fumigatus*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Not successful, *P. aeruginosa* overgrowth
28	33	Unknown	17	*P. aeruginosa, Mycobacterium gordonae*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful
29	33	Unknown	18	*P.aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful
30	26	Unknown	25	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Not successful, *P. aeruginosa* overgrowth
31	22	Unknown	20	*B. multivorans*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Not successful, *B. multivorans* overgrowth
32	29	p.Phe508del p.Phe508del	19	*P. aeruginosa, S. maltophilia, Achromobacter* sp., *M. avium, A. fumigatus, G. argillacea*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful
33	23	Unknown	27	*P. aeruginosa, P. putida, A. fumigatus, A. xylosoxidans, Paecilomyces* sp., *M. avium* complex	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful
34	27	Phe508del Gly551Asp	22	*P. aeruginosa*	Ceft, Tobr, Vanc, Amph, Mero, Colo, CoTrim, Tim	Successful

Abbreviations: *Age in years at time of transplant, $cystic fibrosis transmembrane conductance regulator genotype (where known), ^forced expiratory volume in 1 second (% predicted), Ceft: ceftazidime, Tobr: tobramycin, Vanc: vancomycin, Amph: amphotericin, Mero: meropenem, Vori: voriconazole, Colo: colomycin, CoTrim: co-trimoxazole, Tim: timentin

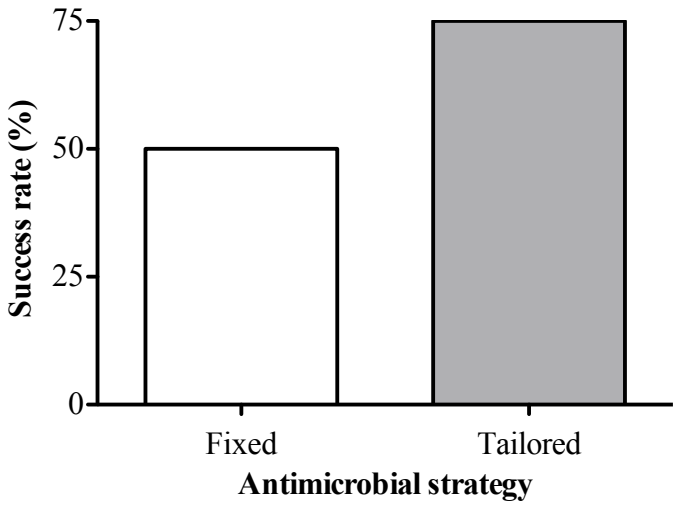


Figure 17. Culture success rates with fixed and tailored antimicrobial strategies

5.5.4. *Characterisation of primary bronchial epithelial cells*

5.5.5. *Morphology*

Brightfield light microscopy on repeated occasions revealed characteristic epithelial 'cobblestone' morphology and growth characteristics as shown in Figure 18. (Borthwick et al., 2009, Ward et al., 2005)

5.5.6. *Haematoxylin and eosin staining*

First passage PBECs were grown under submerged conditions to confluence on collagen coated eight-chamber slides and stained with haematoxylin and eosin as described in Chapter 4. The PBECs displayed typical 'cobblestone' epithelial morphology (Figure 19).

5.5.7. *Cytokeratin immunohistochemistry*

First passage PBECs were grown under submerged conditions to confluence on collagen coated eight-chamber slides and stained with an epithelial, pan-cytokeratin marker, as described in section 4.5.1. The PBECs stained positively for cytokeratin in a similar pattern to that observed previously in PBECs cultured from bronchial brushings performed on lung transplant recipients (Figure 20). (Forrest *et al.*, 2005)

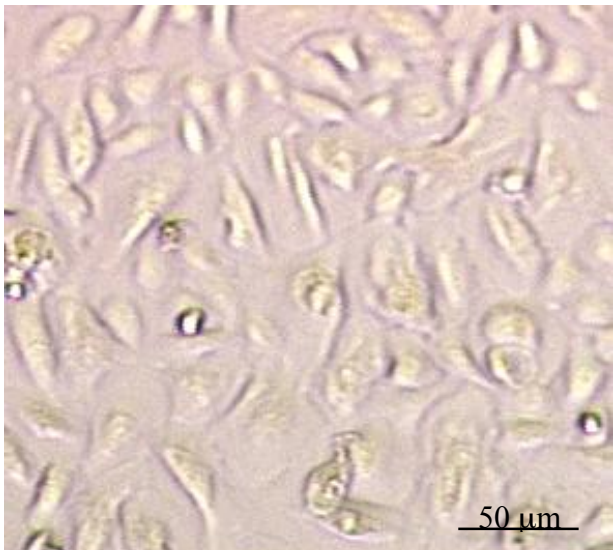


Figure 18. Brightfield light micrograph of primary bronchial epithelial cells cultured under submerged conditions from an explanted cystic fibrosis lung

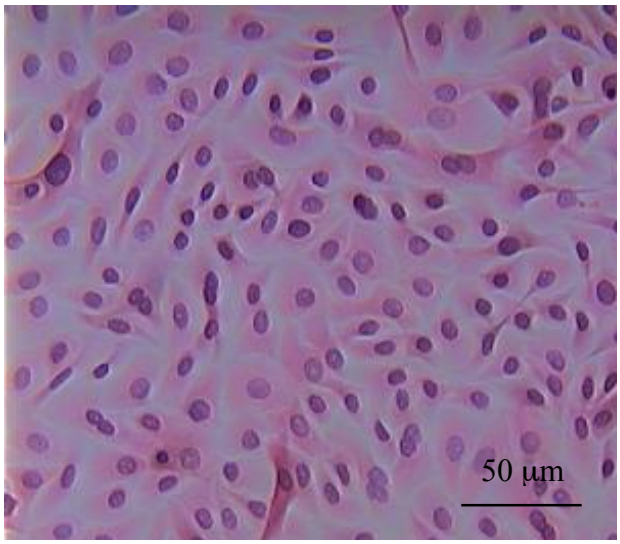


Figure 19. Haematoxylin and eosin staining of primary bronchial epithelial cells cultured under submerged conditions from an explanted cystic fibrosis lung

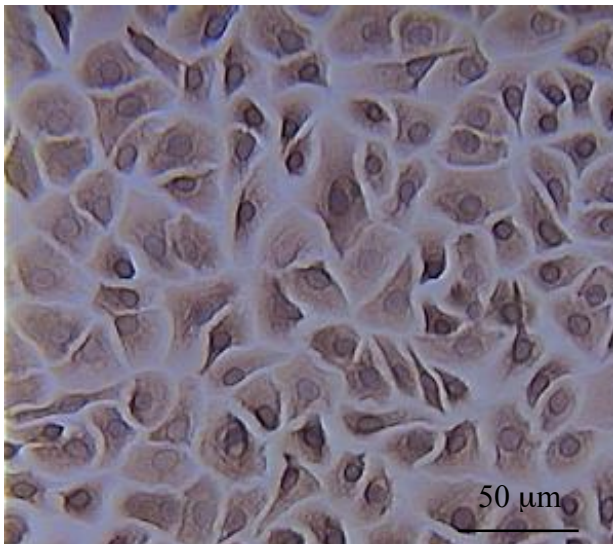


Figure 20. Pan-cytokeratin staining of primary bronchial epithelial cells cultured under submerged conditions from an explanted cystic fibrosis lung

5.5.8. *Electrophysiology*

A characteristic feature of CF airway epithelial cells is the lack of a cAMP/protein kinase A activated Cl^- conductance, due to dysfunctional mutant CFTR channels. (Moran and Zegarra-Moran, 2008) However, CF epithelial cells do retain a Cl^- conductance that can be activated by an elevation of intracellular calcium. (Mall et al., 2003, Hartzell et al., 2005) In order to investigate whether my cultured PBECs retained this signature CF phenotype electrophysiological experiments were performed on cells grown as monolayers on glass coverslips. Whole cell Cl^- currents were measured using the patch clamp technique.

Exposure of PBECs to 5μM forskolin (a cAMP-elevating agent), for a minimum of 4 minutes, failed to elevate whole cell current above basal levels (0/9), (Figure 21A). In contrast, treatment with 1μM ionomycin (a calcium elevating agent) initiated a rapid increase in whole cell current within 60 seconds of addition (7/12 cells). In 4 of these experiments, this current increase was transient in nature, however, in the remaining cells tested, this increase was sustained and the current was shown to be outwardly rectifying and possess the time dependent kinetics characteristic of a calcium-activated chloride current (CaCC) (Figure 21B). (Hartzell et al., 2005, Tarran et al., 2002) Furthermore, substitution of 100mM of bath Cl- with aspartate caused a shift in Erev of +22.5 ± 3.6mV (n = 3), indicating a predominantly Cl- selective current (under these conditions, a +29.3mV shift would indicate a perfectly Cl- selective current). These results therefore confirm that my cultured PBECs from explanted lungs retain the characteristic electrophysiological profile expected of CF cells.

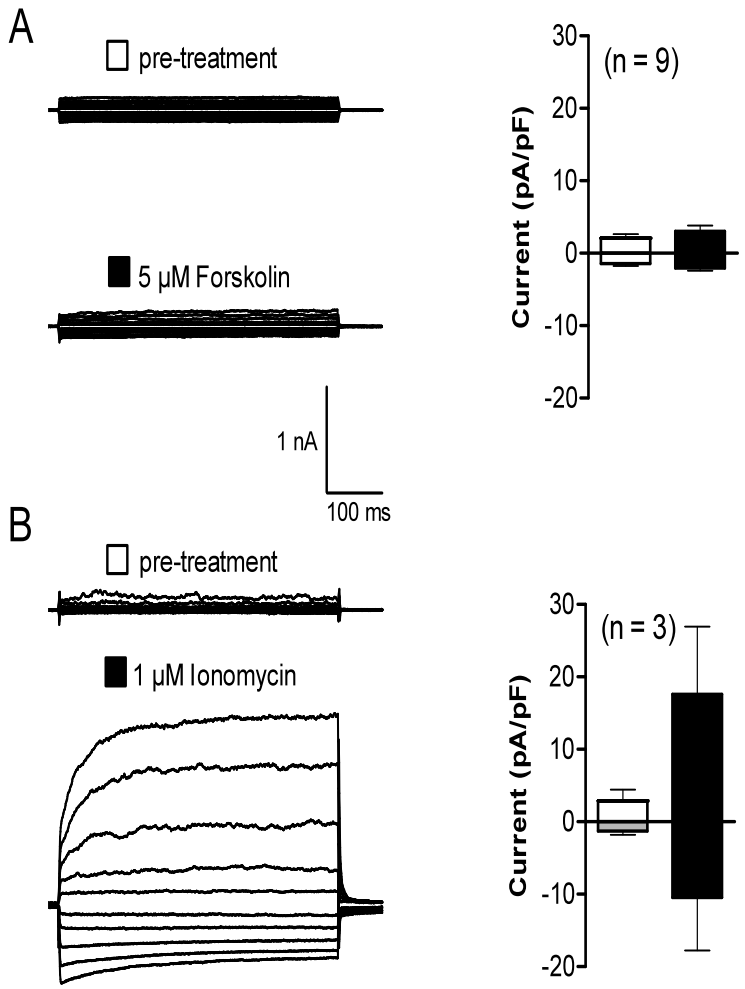


Figure 21. Representative current traces and summary data for cells treated with Forskolin (A) and Ionomycin (B).

For summary data, current was measured at *Erev* +60 mV (upright bars) and -60 mV (inverted bars) and normalised to cell capacitance.

5.5.9. *Cytokine production*

5.5.10. *Basal (unstimulated) cytokine production*

I investigated the production of the cytokines IL-8, IL-6 and GMCSF by submerged PBEC cultures by performing a multiplex ELISA on culture supernatants. Constitutive production of these cytokines is known to occur and would be expected to happen in viable airway epithelial cells. (Becker et al., 2004, Murphy et al., 2008a) As described fully in section 4.2, PBECS were rested for 24 hours prior to the medium being replaced with fresh resting medium for a further 24 hours and then harvested for analysis. Typical standard curves for the ELISA in my hands are shown in Figure 46 to Figure 48 respectively.

Figure 22 to Figure 24 display basal levels (unstimulated) of IL-8, IL-6 and GMCSF production by PBECs from people with advanced CF lung disease (see Table 9). As has been described previously by other researchers, there is variability in the baseline levels of production of these cytokines by PBECs from different individuals. (Becker et al., 2004, Ribeiro et al., 2009)

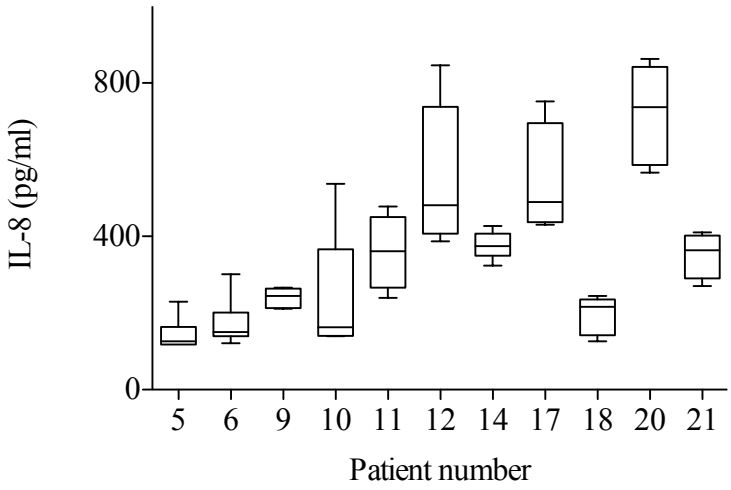


Figure 22. Basal production of interleukin-8 by primary bronchial epithelial cells from people with advanced cystic fibrosis lung disease

Horizontal lines indicate the median basal production of interleukin-8, results from four replicate experiments. The boxes indicate the 25th and 75th percentiles and whiskers minimum and maximum respectively. See Table 9 for clinical details relating to each patient number.

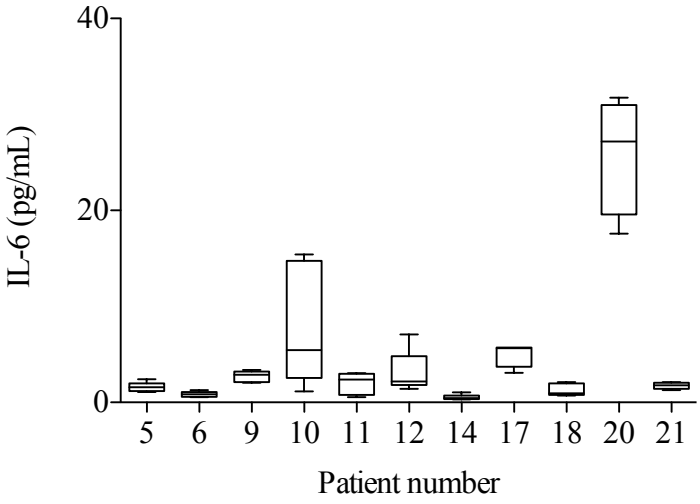


Figure 23. Basal production of interleukin-6 by primary bronchial epithelial cells from people with advanced cystic fibrosis lung disease

Horizontal lines indicate the median basal production of interleukin-6, results from four replicate experiments. The boxes indicate the 25th and 75th percentiles and whiskers minimum and maximum respectively. See Table 9 for details relating to each patient number.

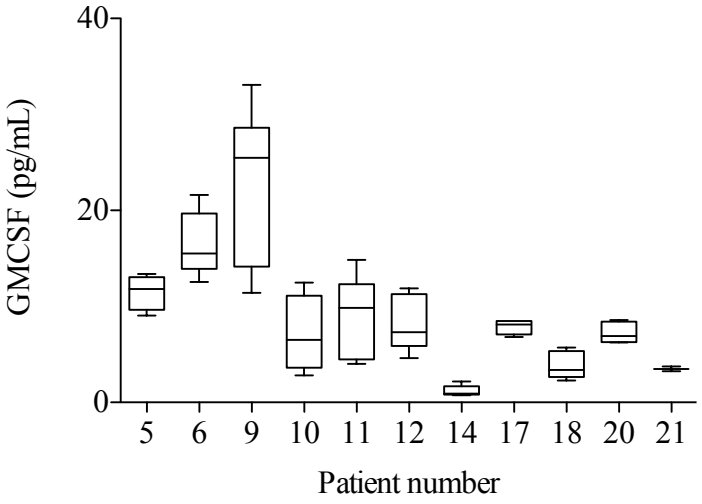


Figure 24. Basal production of granulocyte macrophage colony-stimulating factor by primary bronchial epithelial cells from people with advanced cystic fibrosis lung disease

Horizontal lines indicate the median basal production of granulocyte macrophage colony-stimulating factor, results from four replicate experiments. The boxes indicate the 25th and 75th percentiles and whiskers minimum and maximum respectively. See Table 9 for details relating to each patient number.

5.5.11. *Cytokine production following stimulation with flagellin*

Following on from the experiments described in section 5.5.10 investigating the basal production of the cytokines IL-8, IL-6 and GMCSF by PBECS from people with advanced CF lung disease I investigated the effect of stimulation with flagellin on the production of these cytokines. Flagellin is the major component of bacterial flagella and is found abundantly in flagellated bacteria, for example *Salmonella typhimurium, Escherichia coli* and *P. aeruginosa*. (Feldman *et al.*, 1998) Importantly flagellin is expressed by motile forms of *P. aeruginosa,* which in the context of CF lung disease is most relevant in the early stages of infection and colonisation. (Raoust et al., 2009, Prince, 2006) Flagellin is a key target of epithelial innate immunity and toll-like receptor (TLR) 5 is a specific receptor. (Vijay-Kumar and Gewirtz, 2009) Flagellin therefore represents a potent pro-inflammatory stimulus to epithelial cells that triggers a number of signalling cascades, including the NFκB and MAPK pathways, and leads to the production of IL-8, IL-6 and GMCSF, amongst other mediators. (Pena et al., 2009, Lorenz et al., 2010, Sha et al., 2004) Appropriate responses would therefore be expected to occur in viable airway epithelial cells following challenge with flagellin.

As described fully in section 4.2.15, PBECS were rested for 24 hours prior to the medium being replaced with fresh resting medium containing 1, 10 or 100ng/mL flagellin. After 24 hours the supernatant was harvested and analysed by multiplex ELISA. The flagellin used for these experiments was purified from *S. typhimurium* (Alexis Biochemicals).

Figure 25 to Figure 27 display levels of IL-8, IL-6 and GMCSF produced by PBECs from people with advanced CF lung disease following stimulation with 1, 10 or 100ng/mL. Typical standard curves for the ELISA in my hands are shown in Figure 46 to Figure 48 respectively. Increases in IL-8 production were statistically significant following stimulation with 1, 10 and 100ng/mL of flagellin (Figure 25). Although there was also an increase in production of IL-6 and GMCSF in some experiments, see Figure 26 and Figure 27, this was not consistent and was not statistically significant.

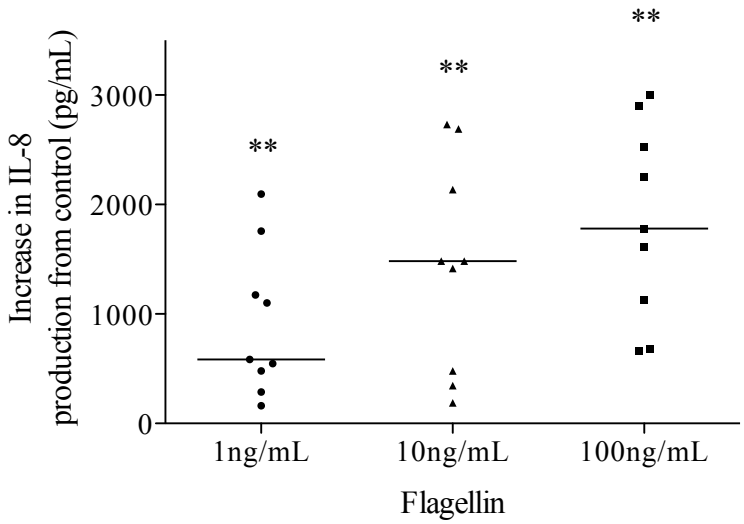


Figure 25. Increase in interleukin-8 production by primary bronchial epithelial cells from people with advanced cystic fibrosis lung disease following stimulation with flagellin

Each symbol represents an individual donor (n=9) and is the mean of three replicate experiments.
Horizontal lines indicate the median.
Median basal interleukin-8 production 242pg/mL.
***P*=0.009
Wilcoxon signed-rank test for each concentration of flagellin compared to basal production. (Wilcoxon, 1945) Holm-Bonferonni correction applied. (Holm, 1979)

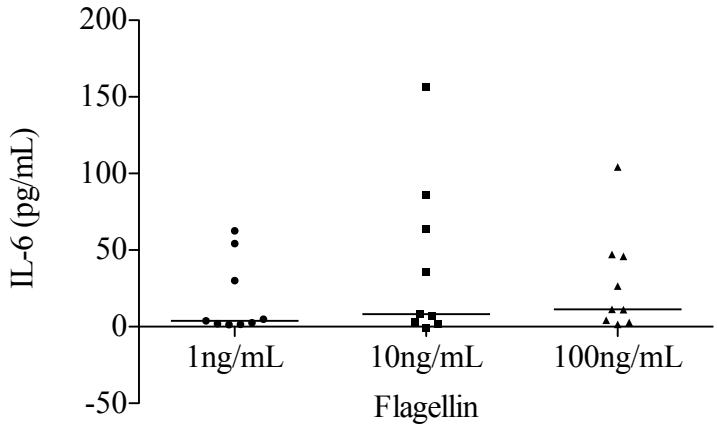


Figure 26. Increase in interleukin-6 production by primary bronchial epithelial cells from people with advanced cystic fibrosis lung disease following stimulation with flagellin

Each symbol represents an individual donor (n=9) and is the mean of three replicate experiments.
Horizontal lines indicate the median.
Median basal interleukin-6 production 2pg/mL.
Differences not significant by Wilcoxon signed-rank test for each concentration of flagellin compared to basal production. (Wilcoxon, 1945) Holm-Bonferonni correction applied. (Holm, 1979)

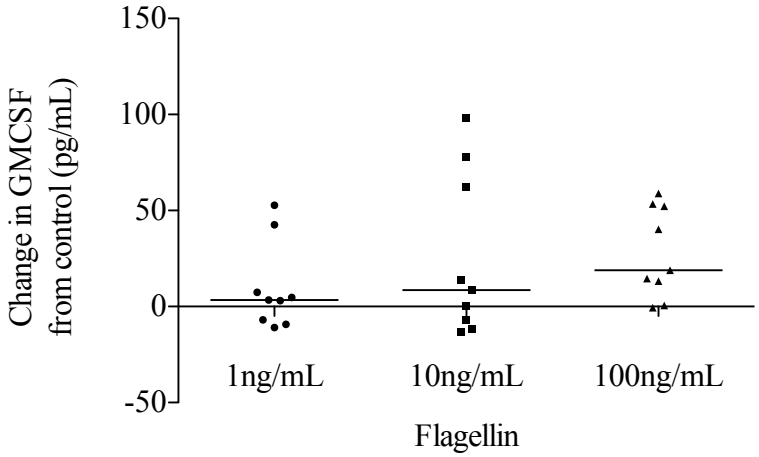


Figure 27. Change in granulocyte macrophage colony-stimulating factor production by primary bronchial epithelial cells from people with advanced cystic fibrosis lung disease following stimulation with flagellin

Each symbol represents an individual donor (n=9) and is the mean of three replicate experiments.
Horizontal lines indicate the median.
Median basal granulocyte macrophage colony-stimulating factor production 8.1pg/mL
Differences not significant by Wilcoxon signed-rank test for each concentration of flagellin compared to basal production. (Wilcoxon, 1945) Holm-Bonferonni correction applied. (Holm, 1979)

5.5.12. *Cryopreservation*

Cells were reconstituted after a prolonged period of cryopreservation using the method described in section 4.2.5 on over 20 occasions with a success rate of 80% for subsequent submerged culture.

5.5.13. *Air-liquid interface culture*

Airway epithelial cells may be cultured most simply *in vitro* under submerged conditions, as was the case in the work described in this chapter so far. However, primary airway epithelial cells revert to a poorly differentiated phenotype within a limited number of passages. (Gruenert et al., 1995, Araya et al., 2007) More advanced culture techniques, such as within collagen gels, as three-dimensional spheroids or most commonly on semi-permeable membranes at ALI, allow recapitulation of a more accurate representation of the native airway epithelium. (de Jong *et al.*, 1993) ALI cultures facilitate prolonged culture of cells *in vitro*, muco-ciliary differentiation and formation of a polarised epithelium characterised by tight junction formation and a raised trans-epithelial resistance.

The successful culture of primary airway epithelial cells at ALI is widely acknowledged to be technically challenging and to require specialist expertise. (Personal communications with research groups in Liverpool, Sheffield, Edinburgh, Belfast, Leeds and Leicester) To date only a small amount of ALI culture work has been performed in our research group in Newcastle. I therefore performed some preliminary experiments in an attempt to establish ALI cultures with PBECs from people with CF.

The method described in Chapter 4 that I used to establish the ALI cultures was largely based on personal discussions with Dr Scott Randell, University of North Carolina, and Professor Chris O'Callaghan, University of Leicester and the method published by Fulcher *et al.* (2005). In essence the technique involved the submerged culture of first passage cells to confluence on semi-permeable filters coated with type IV collagen using standard supplemented bronchial epithelial growth medium. Followed by

exposure of the apical membrane to air and switching of the basolateral media to a different, 'ALI medium'.

A trans-epithelial resistance in excess of 500Ω/cm^2, indicative of a polarised epithelium, was routinely achieved at ALI. (Zabner et al., 2003, Fulcher et al., 2005) An example of trans-epithelial resistance measurements for PBECs cultured from a person with CF is shown in Figure 28. The morphology of the apical surface of PBECs cultured at ALI is shown in Figure 29 with areas of mucus accumulation indicated. Washings from the apical surface of the cultures were positive for the gel-forming airway mucin MUC5B detected by slot-blot ELISA (Figure 30). Periodic acid-Schiff staining of formalin-fixed ALI cultures was also positive (Figure 31). These results confirm the presence of different cell types, including well-differentiated mucus-secreting cells. Scanning electron micrographs of PBECs from a person with CF cultured at an air-liquid interface, illustrating morphology at different time points and tight junction formation are shown in Figure 32.

In summary, it has been possible to generate polarised cultures with a consistently raised trans-epithelial resistance, which secrete mucus and form tight inter-cellular junctions and maintain them *in vitro* for over 6 weeks. True ciliogenesis did not occur and the cells appeared to be squamous rather than columnar in morphology after prolonged culture.

Alternative approaches to establishing well-differentiated airway epithelial cell cultures that have been used by other researchers include 3-dimensional models involving collagen gels and co-culture with fibroblasts alone or along with other inflammatory cells such as eosinophils. (Choe et al., 2003, Ulrich and Doring, 2004, Bals et al., 2004, Parker et al., 2010, Zhang et al., 1999) This suggests that 'cross-talk' between epithelial and other cell types is likely to be important in maintaining epithelial homeostasis and function. (Xu et al., 2002, Wang et al., 2009)

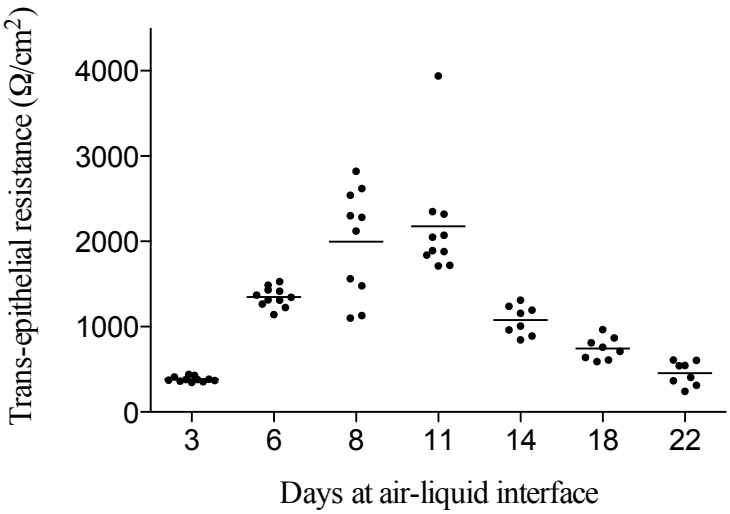


Figure 28. Trans-epithelial resistance measurements for primary bronchial epithelial cells cultured at an air-liquid interface for 22 days from an explanted cystic fibrosis lung

Horizontal bars represent the median resistance.

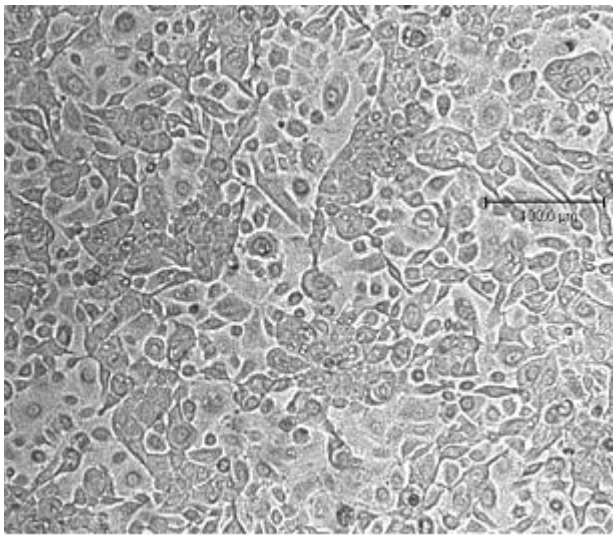


Figure 29. Light micrograph of primary bronchial epithelial cells cultured at an air-liquid interface from an explanted cystic fibrosis lung

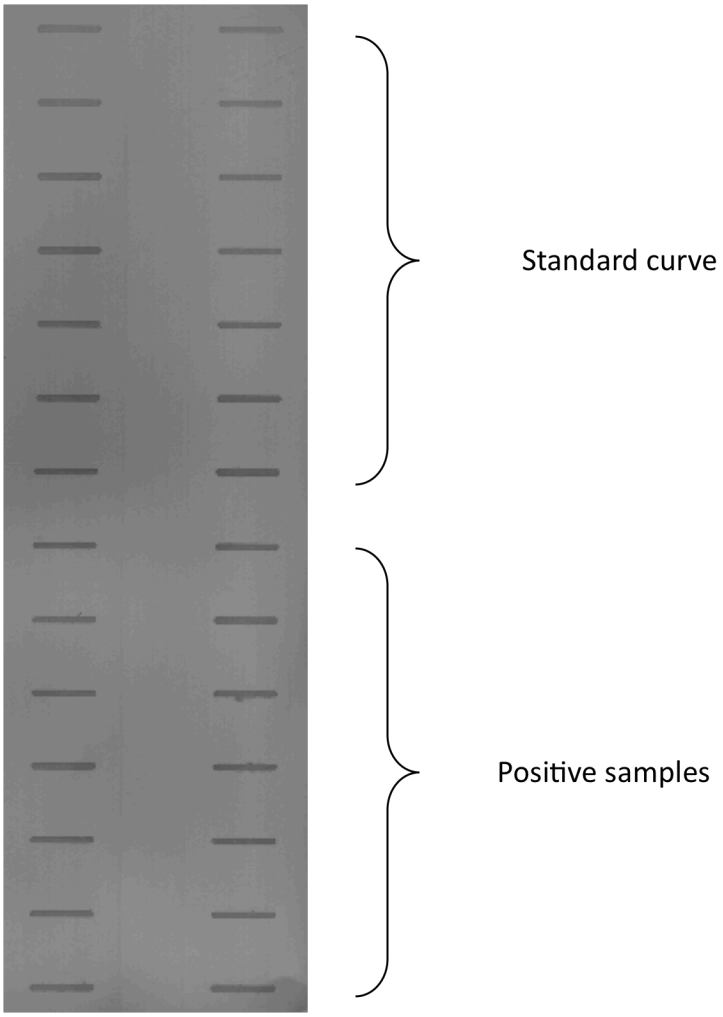


Figure 30. Example of positive slot-blot enzyme-linked immunosorbent assay for the gel-forming airway mucin MUC5B from washings from the apical surface of primary bronchial epithelial cells cultured at an air-liquid interface from explanted cystic fibrosis lungs

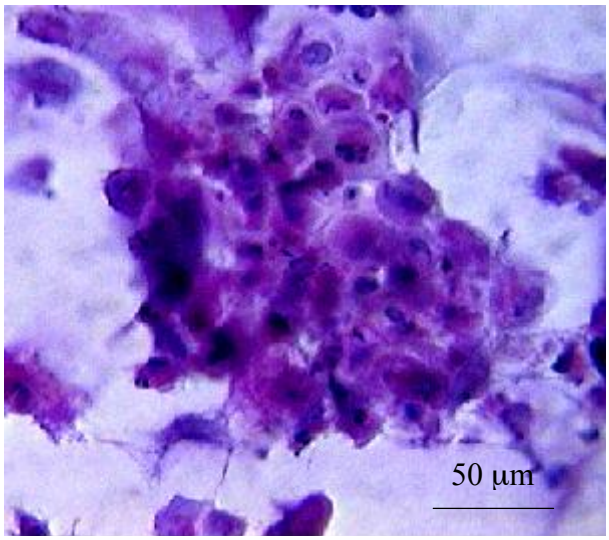
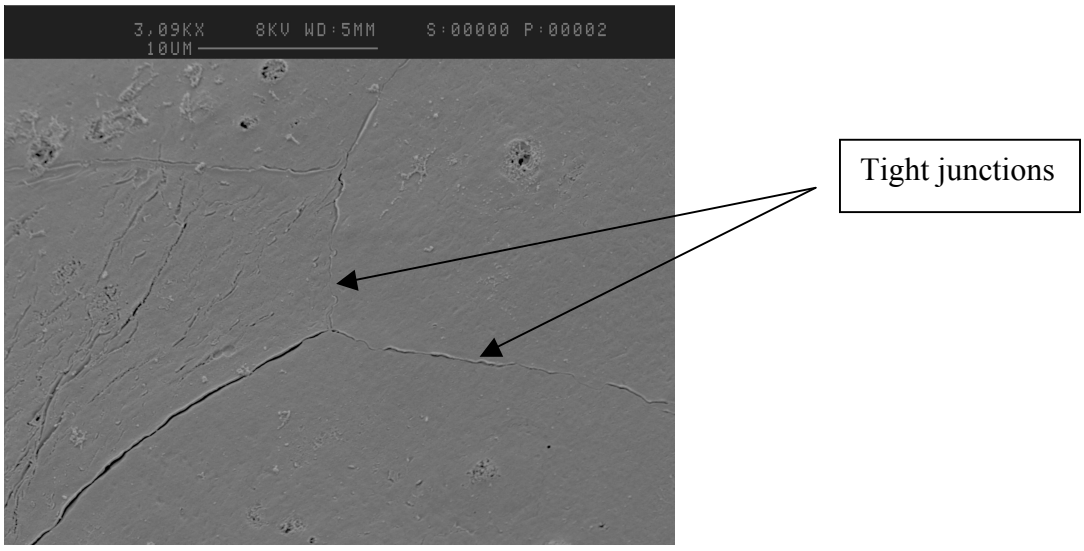


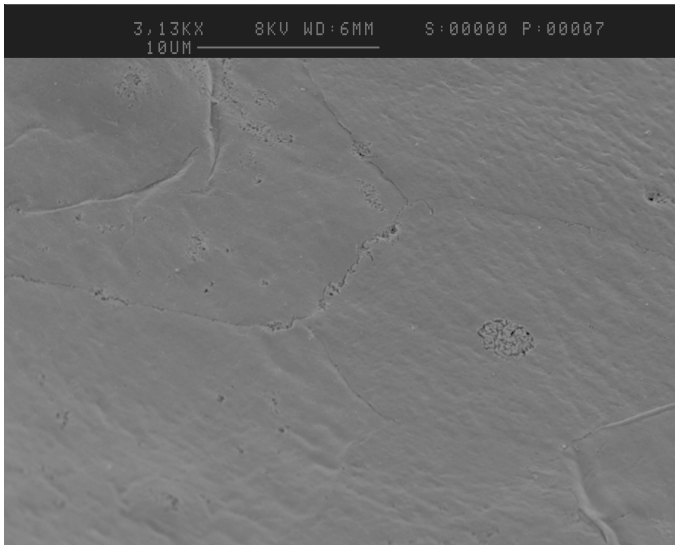
Figure 31. Periodic acid-Schiff staining of primary bronchial epithelial cells cultured at an air-liquid interface from an explanted cystic fibrosis lung

Figure 32. Scanning electron micrographs of primary bronchial epithelial cells from a person with cystic fibrosis cultured at an air-liquid interface, illustrating morphology at different time points and tight junction formation (a), (b) and (c), and microvilli formation (d)

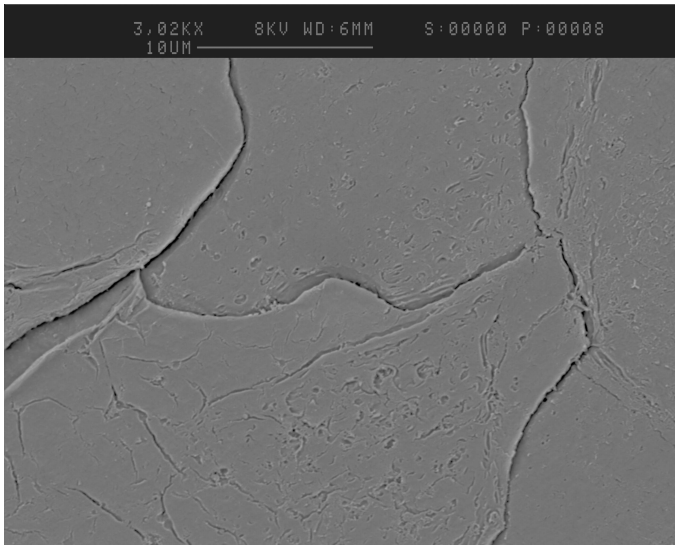
(a) Day 14



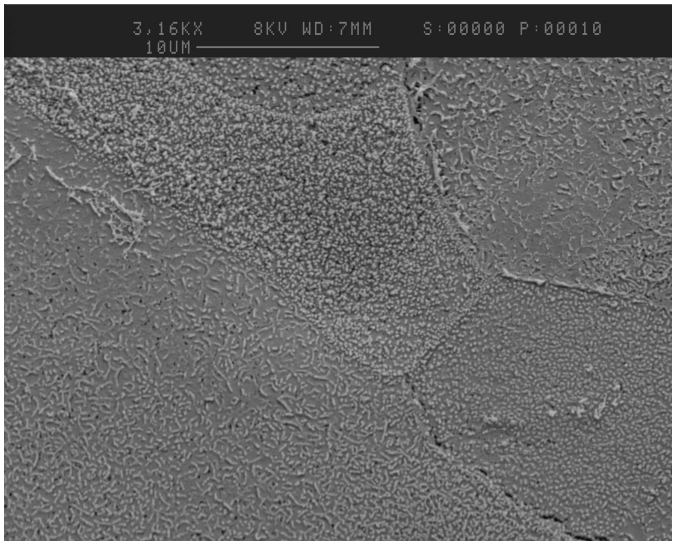
b) Day 21



c) Day 28



d) Microvilli formation



5.6. Discussion

Despite the presence of multidrug resistant microorganisms and copious muco-purulent secretions I have been successful in establishing a programme to culture PBECs from explanted CF lungs in Newcastle. The PBECs were characterised in terms of their morphology (light microscopy and haematoxylin and eosin staining) and cytokeratin staining (immunohistochemistry). In addition, it was possible to perform whole-cell patch clamp experiments to demonstrate the absence of a forskolin-induced CFTR current and the presence of an ionomycin-induced CaCC. Under submerged unstimulated conditions the PBECs produced IL-8, IL-6 and GMCSF.

Experience of culturing PBECs from end-stage CF lung tissue has been previously described in North America. (Karp et al., 2002, Randell et al., 2001, Yamaya et al., 1992) The present study has established an optimised culture method with the novel aspect of using patient-specific combinations of antimicrobials. These were based on up to date knowledge of the organisms cultured from the sputum of patients, in their clinical management pre-transplant. I feel that this has been a significant factor in achieving a favourable success rate (67% overall, 50% using a fixed 'best guess' antimicrobial cocktail and 75% with a patient-specific approach).

In a European setting there are also particular logistical, infrastructure and resource considerations. I have shown that it is possible to utilise tissue from a supra-regional National Health Service clinical transplant programme to set up CF primary culture research. Though mostly generated 'out of hours', tissue was transferred successfully to associated university research facilities, with successful cultures the most common outcome.

It is clearly essential that microorganisms are eliminated from cell cultures and that this is balanced against the potential cytotoxic effects of antimicrobials on epithelial cells. The concentration of each antimicrobial used was guided where available by cytotoxicity data published by Randell *et al.* (2001). Anecdotally, I concur with the finding that the deleterious effects of antimicrobials are manifested by a reduction in growth rate rather than any noticeable effect on cell attachment. (Randell *et al.*, 2001) In

my experience antimicrobials should be removed from the culture medium as early as is practicable to ensure optimal growth rates.

One advantage of an *ex vivo* culture system is that significantly higher and sustained concentrations of antimicrobials can be used compared to those possible in the lungs of patients *in vivo*. Clearly the choice of agents is also not hampered by the frequent allergies encountered clinically in people with end-stage CF lung disease. (Parmar and Nasser, 2005) In patients where cell culture was unsuccessful this was universally due to early overgrowth of cultures with organisms known to colonise the lungs pre-transplant once antimicrobials had been withdrawn from the medium. It has previously been demonstrated that undifferentiated airway epithelial cells, cultured under submerged conditions, are much less resistant to the internalisation of *P. aeruginosa* than differentiated cells that form a tight epithelium. (Plotkowski et al., 1999, Plotkowski et al., 2001) One potential explanation for the recrudescence of infection is the persistence of bacteria in epithelial cells. (Garcia-Medina *et al.*, 2005) In the context of bronchial brushings from clinically stable patients post-lung transplantation it has been noted that respiratory pathogens are sometimes cultured from epithelial cell cultures when bronchoalveolar lavage fluid bacterial culture is negative. (Forrest *et al.*, 2005)

The culture of PBECs from explanted CF lungs is demanding, both in terms of effort and expense, and is limited by their finite lifespan, however, ultimately it yields a model system that is likely to more accurately recapitulate the situation *in vivo* than immortalised cell lines. (Karp *et al.*, 2002) In people with CF there is a diverse spectrum of disease and often a weak correlation between CFTR genotype and clinical lung disease phenotype. (Rowntree and Harris, 2003) It is important to remember that PBECs are likely to reflect this inherent biological heterogeneity in terms of their function. This is illustrated by the variable levels of the cytokines IL-8, IL-6 and GMCSF produced by cells from different donors that I found in the work described in this chapter. Indeed, for some experiments the homogeneity of an immortalised cell line along with isogenic controls may be preferable. Clearly, cultured PBECs from people with CF may be used to establish valuable novel immortalised cell lines. (Fulcher *et al.*, 2009)

Flagellin is the principal component of the flagellum of bacteria and is an agonist of TLR-5 along with a number of other receptors. (Vijay-Kumar and Gewirtz, 2009) In proof-of-principle experiments stimulation of the cultured PBECs with flagellin resulted in a significant increase in IL-8 production and also in cells from some donors IL-6 and GMCSF although this was not a consistent phenomenon. The increase in IL-8 production is in keeping with the findings of others in the published literature and has been demonstrated to involve the NFκB pathway. (Pena *et al.*, 2009) It is biologically plausible that the stimuli that I used of 1-100ng/mL of flagellin are supraphysiological compared to the situation in the airway *in vivo*, however such concentrations are orders of magnitude lower than those employed by other researchers in *ex vivo* experiments. (Kunzelmann *et al.*, 2006) When considering Figure 25 it would seem likely that further experiments, for example using 0.1 and 0.01ng/mL flagellin, would be necessary to establish the threshold concentration of flagellin required to elicit an IL-8 response. In work performed by Schmeck *et al.* (2007) investigating the inflammatory response of immortalised A549 alveolar epithelial cells to infection by the flagellated bacterium *Legionella pneumophila* it was found that infection resulted in production of several pro-inflammatory mediators, including IL-8 and IL-6, but not GMCSF. (Schmeck *et al.*, 2007)

Further experiments involving an increased number of donors would be necessary to robustly conclude about the effects of flagellin stimulation on IL-6 and GMCSF responses on PBECs from people with advanced CF lung disease. There are numerous other pathogen-associated molecular patterns that could be studied using this cellular model. (Govan and Deretic, 1996) This includes lipopolysaccharides produced by *P. aeruginosa* and Burkholderia cepacia complex that are highly relevant to the pathophysiology of advanced CF lung disease. (De Soyza et al., 2008, Pier, 2007) Another important area for potential future experiments is investigation of the signalling pathways involved in the transduction of pro-inflammatory responses by PBECs. The careful use of tools such as receptor-blocking antibodies or antagonism of specific steps by small molecules or small interfering RNAs would allow further dissection of the pathways involved. (Chakraborty and Mann, 2010, Murphy, 2008)

PBECs represent an important resource, particularly so in the new emerging era of targeted therapies for the molecular defect associated with specific CFTR mutations. (O'Sullivan and Freedman, 2009) The problems outlined earlier in this thesis with the

murine models of CF lung disease clearly limit their utility in drug discovery. (Scholte *et al.*, 2004) Although immortalised cell lines are extremely valuable in the early stages of high-throughput screening, as evidenced by the recent identification of novel compounds, primary cells are vital to confirm and validate any initial findings in a cell line prior to more advanced stages of analysis (Figure 10). (Pedemonte et al., 2005, Van Goor et al., 2006, Ma et al., 2002, O'Sullivan and Freedman, 2009)

An alternative approach to performing electrophysiology experiments is to use cells grown to confluence and forming a tight epithelium on Transwell inserts in an Ussing chamber system. (Illek et al., 2010) This would allow more complex and prolonged experimentation and is certainly an area worthy of future exploration using the CF PBECs described in this chapter.

In addition to the scientific challenges of culturing PBECs from end-stage CF lungs, the establishment of a programme to utilise explanted lungs has relied on excellent working relationships with the entire multidisciplinary transplant team. This includes obtaining the appropriate ethical approval to perform such work and clearly the informed consent of the transplant recipients themselves. In my experience it is ideal if individuals are approached for consent at the time of visiting the transplant centre for assessment and not during the emergency admission prior to a potential transplant. Furthermore, the helpful cooperation of cardiothoracic surgeons, transplant coordinators and theatre staff is essential in order to ensure that the research team is informed. Due to the unpredictable nature of transplantation in my experience an ‘on-call’ commitment is essential to facilitate and ensure the appropriate timely procurement of tissue in the event of a transplant occurring.

A key consideration when using primary tissue in research is that the clinical care of patients is not compromised. The method described in this chapter allows clinical specimens to be collected from explanted lungs for microbiology and involves only modest dissection thereby enabling a full examination of the lungs to be performed by a pathologist. This is important because rarely clinically significant findings may be identified solely by pathological examination of explanted lungs, such as a previously undetected neoplasm. (Abrahams *et al.*, 2004) Theoretically the risk is lower in CF than some other disease groups that are transplanted however. Conversely, a more radical dissection technique would undoubtedly yield a greater number of cells from each lung.

In this chapter I have described the initial results and experiences in establishing a programme to culture PBECs from CF lungs removed at the time of transplantation. The culture of PBECs from chronically infected lungs poses technical and logistical challenges but ultimately yields a valuable cellular model to study CF lung disease.

6. Chapter 6. Interleukin-17 and advanced cystic fibrosis lung disease

Elements of the work described in this chapter have been previously reported in the form of a presentation at a scientific meeting and a peer-reviewed paper. (Brodlie et al., 2009c, Brodlie et al., 2010c)

6.1. Abstract

Background: Interleukin-17 is pivotal to pulmonary host defence by orchestrating the activity of neutrophils. Neutrophilic inflammation is the dominant pathology in cystic fibrosis lung disease. I investigated interleukin-17 protein expression in the lower airway in advanced cystic fibrosis lung disease, including the cellular immunolocalisation and the effects of interleukin-17 on cystic fibrosis primary bronchial epithelial cells.

Methods: I performed immunohistochemistry for interleukin-17 on explanted cystic fibrosis lungs and compared findings to the non-suppurative condition pulmonary hypertension. Primary bronchial epithelial cell cultures were also generated from some of the explanted cystic fibrosis lungs and treated with interleukin-17.

Results: Immunoreactivity for interleukin-17 was significantly increased in the lower airway epithelium in cystic fibrosis (median 14.1% of surface area) compared to pulmonary hypertension (2.95%, P=0.0001). The number of cells staining positive for interleukin-17 in the lower airway mucosa was also increased (64 compared to 9/mm basement membrane, P=0.0005) and included both neutrophils, which were frequently intra-epithelial, in addition to mononuclear cells. Treatment of primary bronchial epithelial cells with interleukin-17 increased production of interleukin-8, interleukin-6 and granulocyte macrophage colony-stimulating factor.

Conclusion: Interleukin-17 protein expression is raised in the lower airway of people with cystic fibrosis in terms of surface area of the airway epithelium staining postive and number of inflammatory cells staining positive in the mucosa, including both neutrophils and mononuclear cells. Interleukin-17 increases production of pro-neutrophilic mediators by primary bronchial epithelial cells from people with cystic fibrosis. The immunolocalisation of IL-17 to neutrophils suggests a novel potential positive feedback loop of neutrophilic inflammation in the CF airway.

6.2. Introduction

As outlined earlier in this thesis, CF is caused by abnormalities in the CFTR gene and is associated with life-long morbidity and premature mortality, principally from lung disease. (O'Sullivan and Freedman, 2009) The CF airway is characterised by neutrophilic inflammation, lymphocytic infiltration of the mucosa, retention of mucopurulent secretions and chronic endobronchial infection. (O'Sullivan and Freedman, 2009, Hubeau et al., 2001a)

The cytokine IL-17 plays a central role in pulmonary host defence by orchestrating the accumulation and associated activity of neutrophils in the bronchoalveolar space. (Linden et al., 2005, Aujla et al., 2007) A family of IL-17 cytokines has been described, however IL-17A (referred to as IL-17 onwards in this chapter), and to a lesser extent IL-17F, are the best characterised. (Gaffen, 2009) The orchestrating effect of IL-17 on neutrophils is achieved indirectly via the local release of neutrophil-mobilising factors, including CXC chemokines, from cells resident in the lung. (Linden *et al.*, 2005) Treatment with IL-17 has also been shown to increase expression of the mucin genes MUC5AC and MUC5B by bronchial epithelial cells *in vitro*. (Chen *et al.*, 2003)

IL-17 is the signature cytokine produced by T_H-17 lymphocytes and therefore represents a strategic link between acquired and innate immunity via its orchestrating effect on neutrophils. (Glader *et al.*, 2010) T_H-17 cells are not the only source of IL-17 identified however. IL-17 is also known to be produced by γδ T-cells and natural killer T-cells. (Michel et al., 2007, Roark et al., 2008) Apart from lymphocytes it has also recently been shown that mast cells express IL-17 in rheumatoid arthritis synovium. (Hueber *et al.*, 2010) Moreover, it has also been suggested in human alcoholic liver disease and rodent models of lipopolysaccharide-induced airway inflammation, perinuclear anti-cytoplasmic neutrophil antibodies (p-ANCA) positive vasculitis and kidney ischaemia-reperfusion injury that neutrophils themselves are a potential source of IL-17. (Lemmers et al., 2009, Ferretti et al., 2003, Li et al., 2010, Hoshino et al., 2008)

IL-17 is therefore linked to neutrophilic inflammation and mucus excess, two cardinal features of CF lung disease, in addition to dysregulation of acquired immunity. (Chen et al., 2003, Bettelli et al., 2007, Aujla et al., 2007) Published human studies of IL-17 in CF are rare but some support the importance of this axis. Increased levels of IL-17 have

been found in bronchoalveolar lavage from children and adults during infective exacerbations, and in the serum of clinically stable adults. (McAllister et al., 2005, Dubin et al., 2007, Aujla et al., 2008, Dufresne et al., 2009) In addition, people with CF who exhibit robust T-cell responses to *P. aeruginosa* manifest more severe lung disease. (Winnie and Cowan, 1991) Such work emphasises the need for further translational studies.

In the work described in this chapter I have examined the hypothesis that increased IL-17 protein expression occurs in advanced CF lung disease. To investigate this hypothesis I used airway samples that I had collected from CF lungs removed at the time of transplantation to perform immunohistochemistry and compared findings to those in the non-suppurative condition pulmonary hypertension (PH). I also analysed airway lavage samples from explanted CF lungs for IL-17. In view of the recent reports of myeloid cells producing IL-17 I also investigated the cellular localisation of the positive IL-17 staining in explanted CF lungs. (Li et al., 2010, Hueber et al., 2010, Ferretti et al., 2003, Lemmers et al., 2009) In addition I generated PBEC cultures from some of the same CF lungs, as outlined in Chapter 5. These were used in proof of concept experiments, which examined the effects of IL-17 stimulation on the production of inflammatory mediators in *ex vivo* PBEC cultures.

6.3. Aims

The aims of the work described in this chapter were to:

- Investigate the protein expression of interleukin-17 in the lower airway of people with advanced cystic fibrosis lung disease using
    - immunohistochemistry of the airway epithelium in comparison to the non-suppurative condition pulmonary hypertension.
    - airway lavage fluid in comparison to healthy volunteers
- Describe the cellular localisation of interleukin-17 in the lower airway of people with advanced cystic fibrosis lung disease.
- Investigate the effects of stimulation with interleukin-17 on the production of the pro-neutrophilic mediators interleukin-8, interleukin-6 and granulocyte monocyte colony-stimulating factor by *ex vivo* primary bronchial epithelial cell cultures from people with advanced cystic fibrosis lung disease.

6.4. Hypothesis

Interleukin-17 is pivotal in the orchestration and accumulation of neutrophils in pulmonary host defence and neutrophilic inflammation is the dominant pathology in cystic fibrosis lung disease. I therefore hypothesised that expression of interleukin-17 would be raised in the lower airway of people with advanced cystic fibrosis lung disease. Furthermore I also hypothesised that interleukin-17 may be localised to cells other than lymphocytes in the lower airway of people with cystic fibrosis and that stimulation with interleukin-17 would increase the production of pro-neutrophilic mediators by *ex vivo* cultures of primary bronchial epithelial cells from people with advanced cystic fibrosis lung disease.

6.5. Results

6.5.1. *Detection of interleukin-17 in airway lavage fluid from explanted cystic fibrosis lungs*

I performed a standardised airway lavage of 15mL phosphate buffered saline on freshly explanted CF lungs as outlined in section 4.2.2. This involved a total lavage volume of 30mL/patient. I then assayed the lavage fluid supernatant from 28 CF lungs for IL-17 using an ultrasensitive IL-17 ELISA kit with a lower limit of detection of 0.2pg/mL (MesoScale Discovery). Brief clinical details of the people with CF whose explanted lung lavage fluid was assayed for IL-17 are provided in Table 10. The standard curve for the IL-17 ELISA is displayed in Figure 33. Figure 34 displays the concentrations of IL-17 detected in airway lavage fluid from each CF lung (n=28, median 56.9pg/mL, interquartile range 34.9-79.7pg/mL). Comparison is made with data from the previous work of Dr Des Murphy in Newcastle where IL-17 was undetectable in bronchoscopic BAL (3x60mL) fluid from 4 healthy volunteers. (Murphy, 2008) Dr Murphy used the same IL-17 ELISA kit manufactured by MesoScale Discovery along with a kit with a higher limit of detection manufactured by R&D Systems. (Murphy, 2008)

Table 10. Clinical details of the people with cystic fibrosis that bronchoalveolar lavage fluid was assayed for interleukin-17

Age*	CFTR Variant[$]	FEV_1[^]	Sputum microbiology
32	Unknown	25	*Pseudomonas aeruginosa,* *Burkholderia vietnamiensis*
25	p.Phe508del Unknown	22	*P. aeruginosa,* *Staphylococcus aureus*
23	Unknown	21	*Scedosporium* sp., *Alcaligines* sp., *S. Aureus*
39	p.Phe508del p.Phe508del	14	*P. aeruginosa*
27	p.Phe508del p.Phe508del	22	*P. aeruginosa*
43	p.Phe508del p.Arg560Thr	15	*Stenotrophomonas maltophilia,* *Acinetobacter* sp.
28	p.Phe508del p.Asp443fs	17	*P. aeruginosa*
29	p.Phe508del p.Phe508del	20	*P. aeruginosa,* *A. fumigatus*
30	p.Phe508del p.Phe508del	30	*P. aeruginosa*
40	Unknown	24	*P. aeruginosa*
23	p.Phe508del p.Gly551Asp	30	*P. aeruginosa,* *A. fumigatus*
46	Unknown	30	*P. aeruginosa*
29	p.Phe508del p.Gln685fs	17	*B. multivorans,* *A. fumigatus,* *Geosmithia argillacae*
22	p.Phe508del p.Phe508del	23	*P. aeruginosa*
20	p.Phe508del p.Phe508del	21	*P. aeruginosa*
29	p.Phe508del c.317+10kbC>T	29	*P. aeruginosa,* *S. maltophilia*
28	Unknown	20	*P. aeruginosa,* Methicillin-Resistant *S. aureus*
48	p.Phe508del Unknown	29	*P. aeruginosa,* *S. aureus*
33	Unknown	23	*P. aeruginosa*
22	p.Phe508del p.Phe508del	25	*P. aeruginosa,* *A. fumigatus*
24	p.Phe508del p.Phe508del	26	*P. aeruginosa*
21	Unknown	25	*P. aeruginosa*
45	p.Phe508del p.Phe508del	21	*P. aeruginosa,* *A. fumigatus*
33	Unknown	17	*P. aeruginosa,* *Mycobacterium gordonae*

26	Unknown	25	*P. aeruginosa*
22	Unknown	20	*B. multivorans*
29	p.Phe508del p.Phe508del	19	*P. aeruginosa,* *S. maltophilia,* *Achromobacter* sp., *M. avium,* *A. fumigatus,* *G. argillacea*
23	Unknown	27	*P. aeruginosa,* *P. putida,* *A. fumigatus,* *A. xylosoxidans,* *Paecilomyces* sp., *M. avium* complex

Abbreviations: *Age in years at time of transplantation. $Cystic fibrosis transmembrane conductance regulator genotype (where known). ^Percentage predicted forced expiratory volume in 1 second pre-transplant.

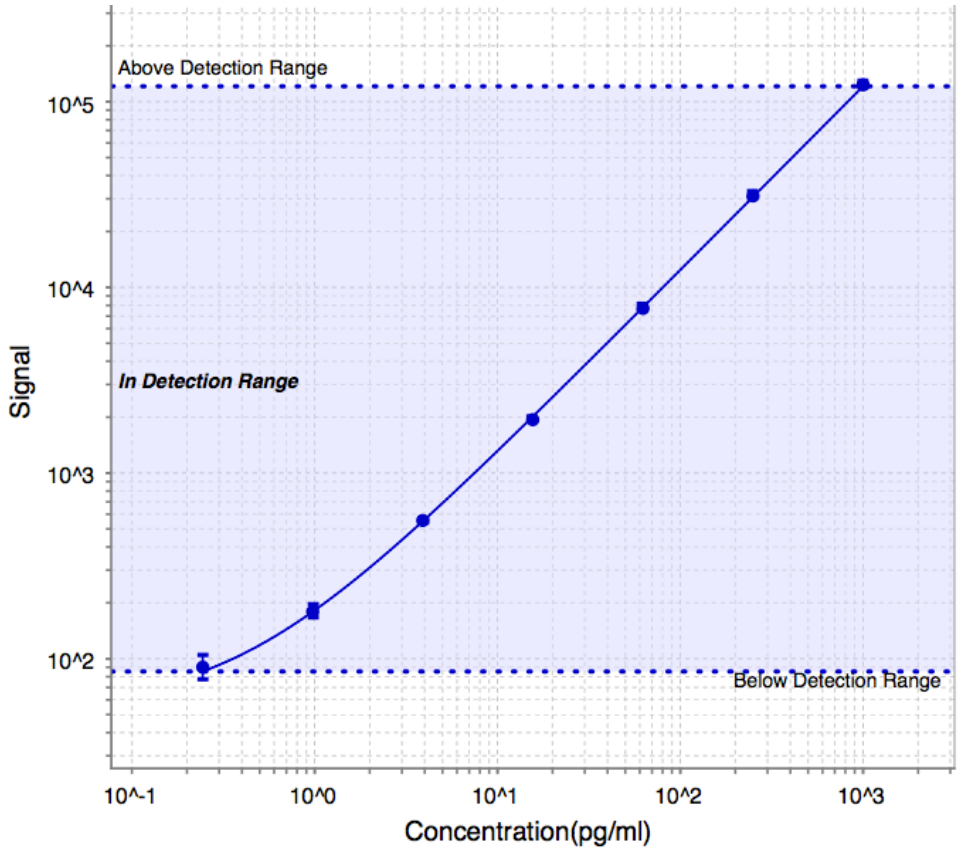


Figure 33. Standard curve for interleukin-17 enzyme-linked immunosorbent assay

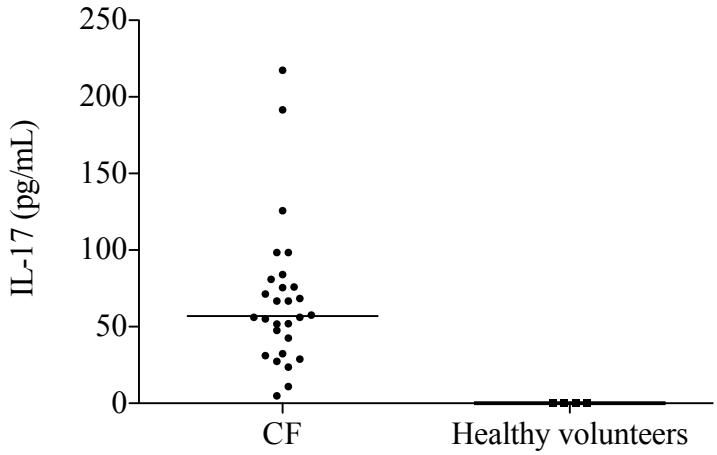


Figure 34. Interleukin-17 in airway lavage fluid from explanted cystic fibrosis lungs

Each symbol represents an individual patient and concentrations account for a dilution factor of 30 in the cystic fibrosis group.
The horizontal line represents the median (56.9pg/mL).
The lower limit of detection was 0.2pg/mL.
The healthy volunteer data relates to 180mL bronchoalveolar lavages performed and assayed by Dr Des Murphy in Newcastle where IL-17 was universally undetectable. (Murphy, 2008)

6.5.2. *Measurement of interleukin-23 in airway lavage fluid from explanted cystic fibrosis lungs*

Following on from the detection of IL-17 in lavage fluid from explanted CF lungs I performed an ELISA for IL-23 on the same samples. The rationale behind this was that IL-23 is known to be essential for the maintenance of T_H-17 cells. (Wilson et al., 2007, Volpe et al., 2008, Cosmi et al., 2008) IL-23 is principally produced by dendritic cells and macrophages in the lung. (Nembrini *et al.*, 2009) The ELISA kit used (Quantikine human IL-23 immunoassay, R&D Systems) had a limit of detection of 6.8pg/mL. The standard curve for the IL-23 ELISA is displayed in Figure 35. Figure 36 shows the BAL fluid results, IL-23 was undetectable in 18 of the 26 patient samples assayed.

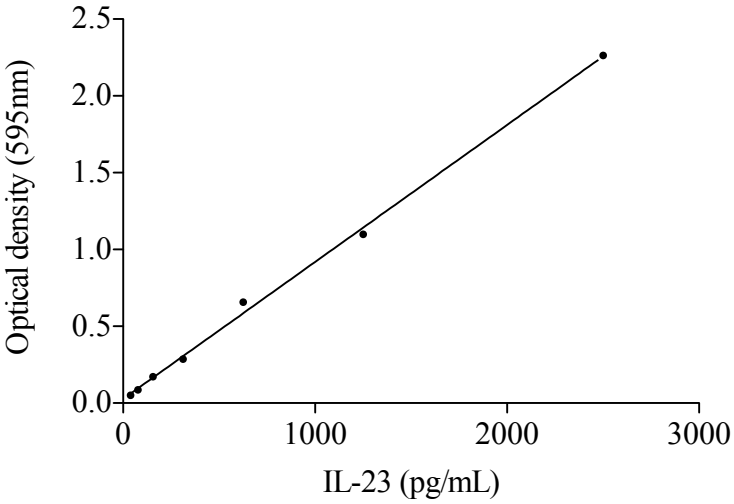


Figure 35. Standard curve for interleukin-23 enzyme-linked immunosorbent assay

y=0.009x+0.027. R square 0.998

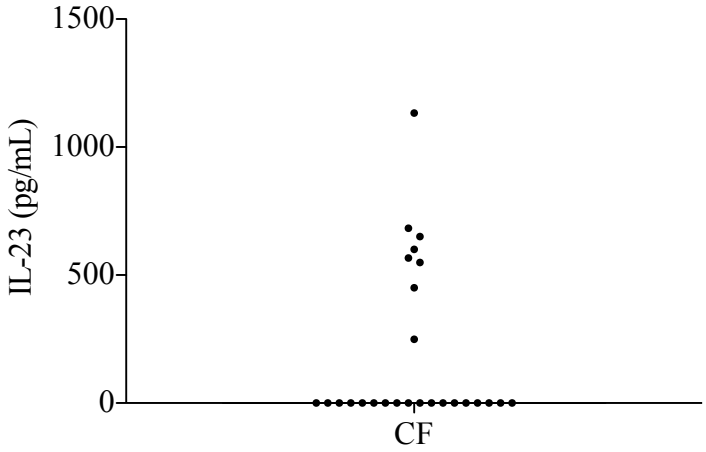


Figure 36. Interleukin-23 in airway lavage fluid from explanted cystic fibrosis lungs

Each symbol represents an individual patient and concentrations account for a dilution factor of 30.
The median concentration of IL-23 detected was 0pg/mL (undectable in 18/26 samples).
The lower limit of detection of the assay was 6.8pg/mL.

6.5.3. *Immunoreactivity for interleukin-17 is increased in the lower airway epithelium of people with advanced cystic fibrosis lung disease*

Immunohistochemistry was performed on sections of medium-large lower airway from explanted lungs removed at the time of transplantation from 19 people with advanced CF lung disease and 8 with PH. Brief details of the 27 patients used for immunohistochemistry are provided in Table 11.

The percentage area of lower airway epithelium positive for IL-17 (mean of 5 randomly selected high power fields) in each individual is displayed in Figure 37**.** Staining for IL-17 was significantly higher in the CF group, median 14.1%, compared to PH, 3.0% (P=0.0001). Figure 38 shows representative IL-17 staining, in an explanted CF (a) and PH (b) lung, and negative control (c).

Table 11. Brief clinical details of patients undergoing lung transplantation used for interleukin-17 immunohistochemistry and stimulation of primary bronchial epithelial cells

Patient number	Diagnosis	Pre-operative sputum microbiology	IHC		PBEC culture
			IL-17	Neu.	
1	CF	*Pseudomonas aeruginosa*	+	-	-
2	CF	*P. aeruginosa*, *Staphylococcus aureus*	+	-	-
3	CF	*P. aeruginosa*, *S. aureus*, *Stenotrophomonas maltophilia*, *Aspergillus fumigatus*	+	+	-
4	CF	*P. aeruginosa*, *S. aureus*	+	+	-
5	CF	*P. aeruginosa*, *Aspergillus fumigatus*	+	+	-
6	CF	*P. aeruginosa*, *Stenotrophomonas maltophilia*	+	-	-
7	CF	*P. aeruginosa*, Methicillin-Resistant *S. aureus*	+	-	-
8	CF	*P. aeruginosa*, *Burkholderia vietnamiensis*	+	+	-
9	CF	*P. aeruginosa*	+	-	+
10	CF	*Scedosporium aspiospermum*, *S. aureus*, *Alcaligines* sp.	+	-	+
11	CF	*P. aeruginosa*	+	+	-
12	CF	*P. aeruginosa*, *S. aspiospermum*	+	-	-
13	CF	*P. aeruginosa*, *A. fumigatus*	+	-	+
14	CF	*P. aeruginosa*	+	+	-
15	CF	*P. aeruginosa*	+	-	-
16	CF	*P. aeruginosa*	+	-	+
17	CF	*P. aeruginosa*, *S. aureus*	+	-	-
18	CF	*P. aeruginosa*, *S. aureus*	+	-	-
19	CF	*P. aeruginosa*	+	+	
20	CF	*Stenotrophomonas maltophilia*, *Acinetobacter* sp.	-	-	+
21	CF	*P. aeruginosa*	-	-	+
22	CF	*P. aeruginosa*	-	-	+
23	CF	*P. aeruginosa*, *A. fumigatus*	-	-	+
24	CF	*P. aeruginosa*, *A. fumigatus*	-	-	+

25	PH	Negative	+	-	-
26	PH	Negative	+	-	-
27	PH	Negative	+	-	-
28	PH	Negative	+	-	-
29	PH	Negative	+	-	-
30	PH	Negative	+	-	-
31	PH	Negative	+	-	-
32	PH	Negative	+	-	-

Abbreviations: IHC patient used for immunohistochemistry, Neu patient used for percentage of neutrophils positive for IL-17, PBEC patient used for primary bronchial epithelial cell culture, CF cystic fibrosis, PH pulmonary hypertension

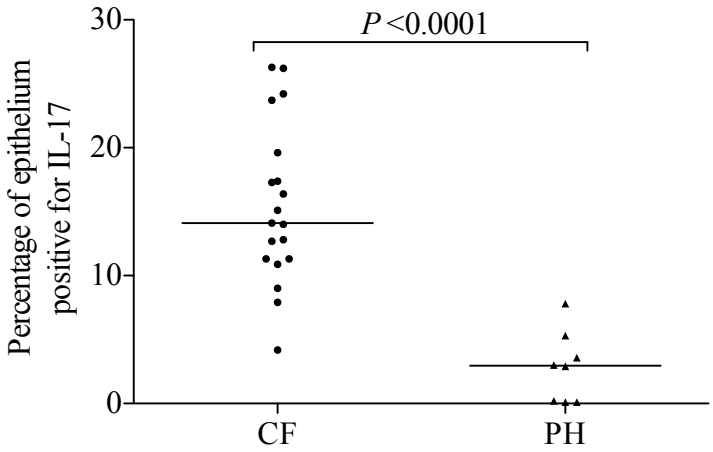


Figure 37. Percentage of epithelium surface area staining positive for interleukin-17 in people with advanced cystic fibrosis lung disease compared to pulmonary hypertension

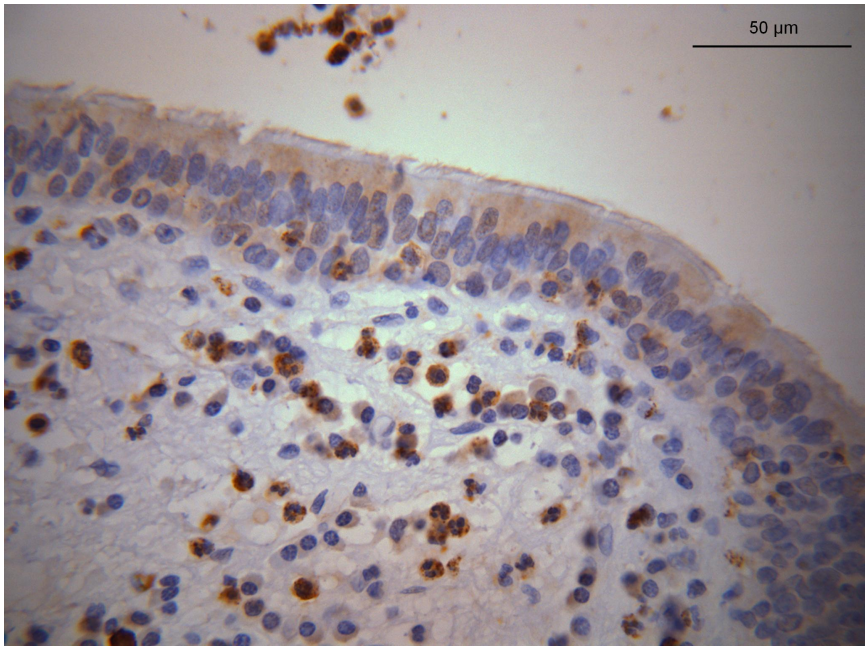
Each symbol represents an individual patient and represents the mean from 5 high-power fields.

Horizontal bar indicates median for each group.

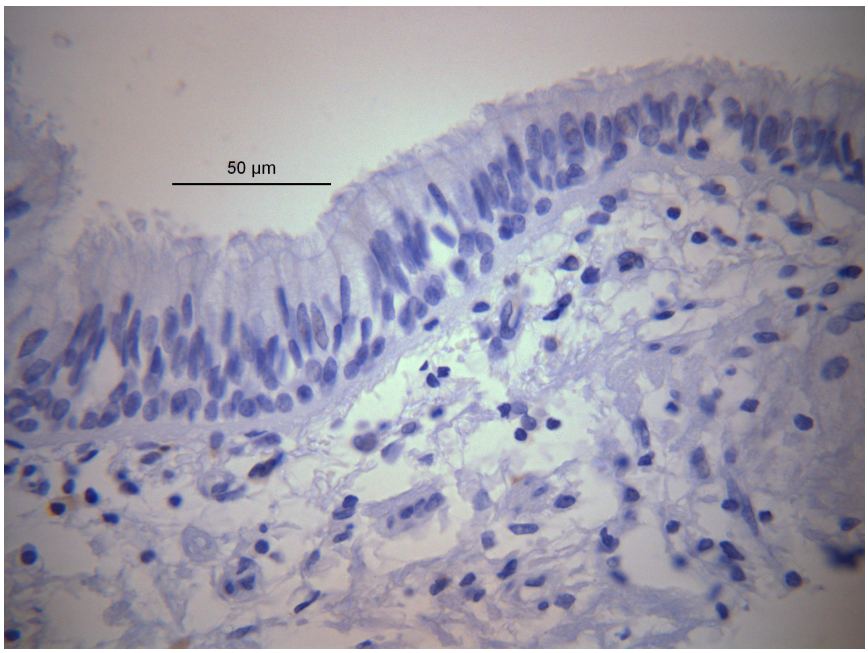
Mann-Whitney test used to compare the two groups.

Figure 38. Representative immunohistochemistry for interelukin-17 in the lower airway mucosa of an explanted lung from a person with a) advanced cystic fibrosis lung disease, b) pulmonary hypertension and c) negative control (normal goat immunoglobulins)

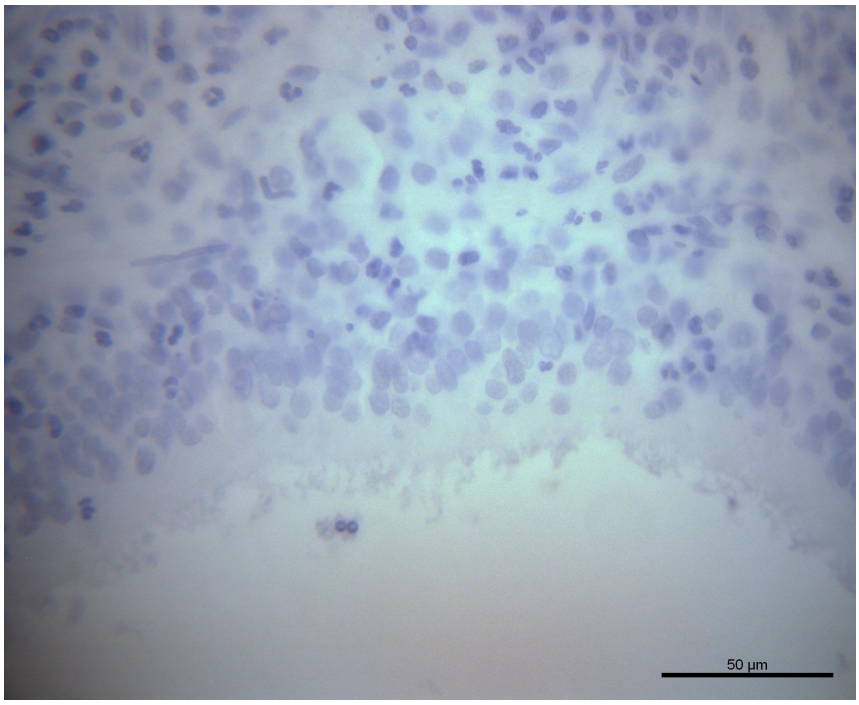
a) Advanced cystic fibrosis lung disease



b) Pulmonary hypertension



c) Negative control (normal goat immunoglobulins)



6.5.4. *Interleukin-17 positive cells are increased in the lower airway mucosa in cystic fibrosis and include neutrophils*

The number of IL-17 positive cells in the mucosa was significantly increased in the CF group, median 64/mm of basement membrane and included both neutrophils and mononuclear cells, compared to PH, 9/mm basement membrane (P=0.0005, Mann-Whitney test). The mean number of positive cells per mm of basement membrane in each individual is displayed inFigure 39. In the CF group substantial numbers of inflammatory cells staining positive for IL-17 were found in the epithelium, of note this frequently included neutrophils (Table 12 and Figure 40) in addition to mononuclear cells (Figure 41). In the PH group IL-17 positive inflammatory cells were absent from the epithelium (Table 12).

The percentage of mucosal neutrophils staining positive for IL-17 was quantified in a subset of 7 people with CF (identified in Table 11). The percentage of neutrophils staining positive for IL-17 in these individuals is displayed in Figure 42, median 38% (interquartile range 34).

I also performed immunohistochemistry for the transcription factor RORγ on sections of lower airway mucosa from people with advanced CF lung disease. RORγ is a transcription factor that is encoded by the retinoic acid receptor-related orphan receptor C (RORC) gene. RORC expression is known to be important in production of IL-17 by TH-17 cells and mast cells. (Bettelli et al., 2007, Hueber et al., 2010) Figure 43 displays a low power view of the lower airway mucosa from an explanted lung from a person with advanced CF lung disease showing several positive cells for RORγ in the mucosa. At higher power, as shown in Figure 44, it is evident that the positive cells are neutrophils. Staining was universally absent in isotype negative controls (normal rabbit immunoglobulins), as exemplified in Figure 45.

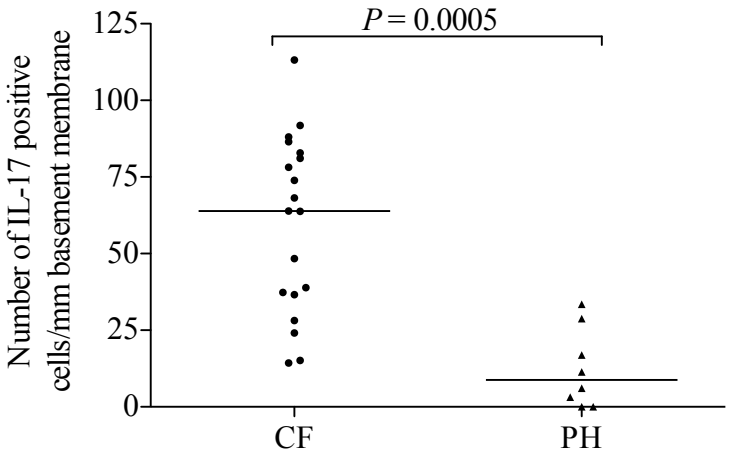


Figure 39. Number of interleukin-17 positive cells/mm basement membrane in the lower airway mucosa in cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and represents the mean from 5 high-power fields.
The horizontal bar indicates the median for each group.
Mann-Whitney test used to compare the two groups.

	Cystic fibrosis	Pulmonary hypertension
Median IL-17 positive cells/mm basement membrane (mucosa) [IQR]	63.9** [37.0-81.9]	8.8 [2.4-19.9]
Median IL-17 positive cells/mm basement membrane (epithelium) [IQR]	17.6 [8.5-23.8]	0
Median IL-17 positive cells/mm basement membrane (lamina propria) [IQR]	41.1* [19.0-59.9]	8.8 [2.4-19.9]

Table 12. Number of interleukin-17 positive cells in the mucosa, epithelium and lamina propria in lungs explanted from people with cystic fibrosis and pulmonary hypertension

Abbreviations: **P*=0.032, ***P*=0.0005, IQR Inter-quartile range.
Mann-Whitney test used to compare the two groups.

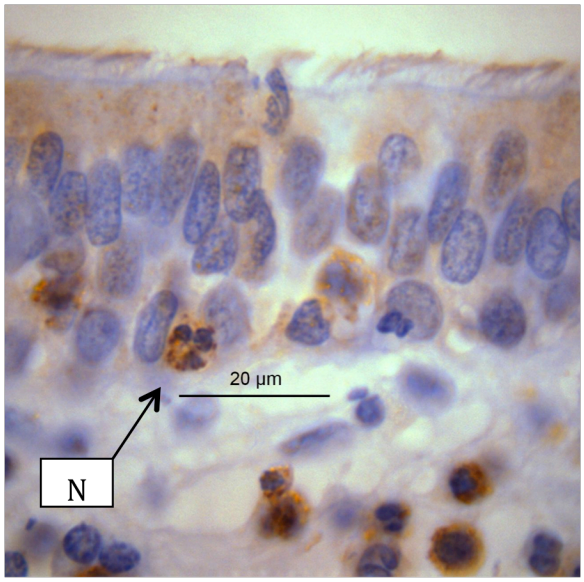


Figure 40. Example of neutrophil (N) staining positive for interleukin-17 in the epithelium of an explanted lung from a person with advanced cystic fibrosis lung disease

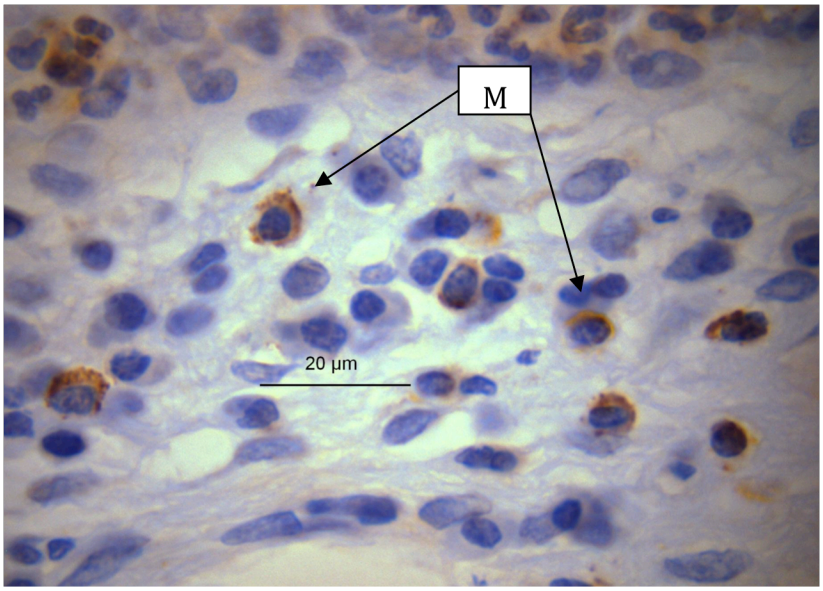


Figure 41. Example of mononuclear cells (M) staining positive for interleukin-17 in the explanted lung of a person with advanced cystic fibrosis lung disease

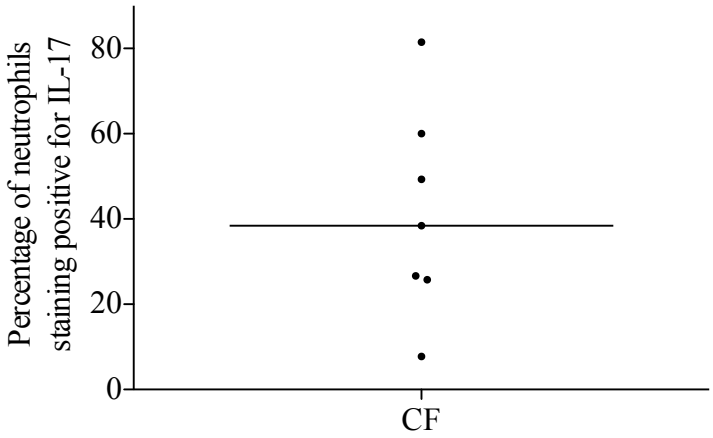


Figure 42. Percentage of neutrophils staining positive for interleukin-17 in the lower airway mucosa of explanted cystic fibrosis lungs (n=7)

Mean of 5-high power fields.

Horizontal line indicates the median.

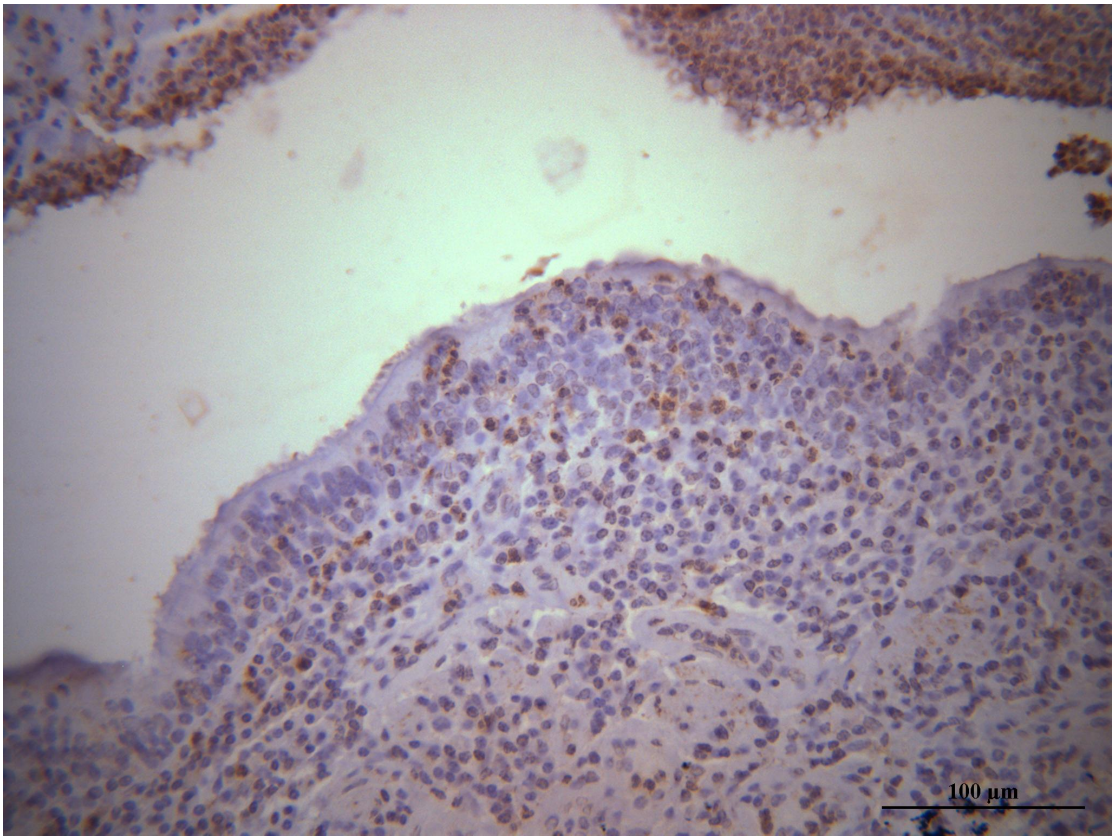


Figure 43. Immunohistochemistry for retinoic acid receptor-related orphan receptor gamma in advanced cystic fibrosis lung disease lower airway (x20 objective)

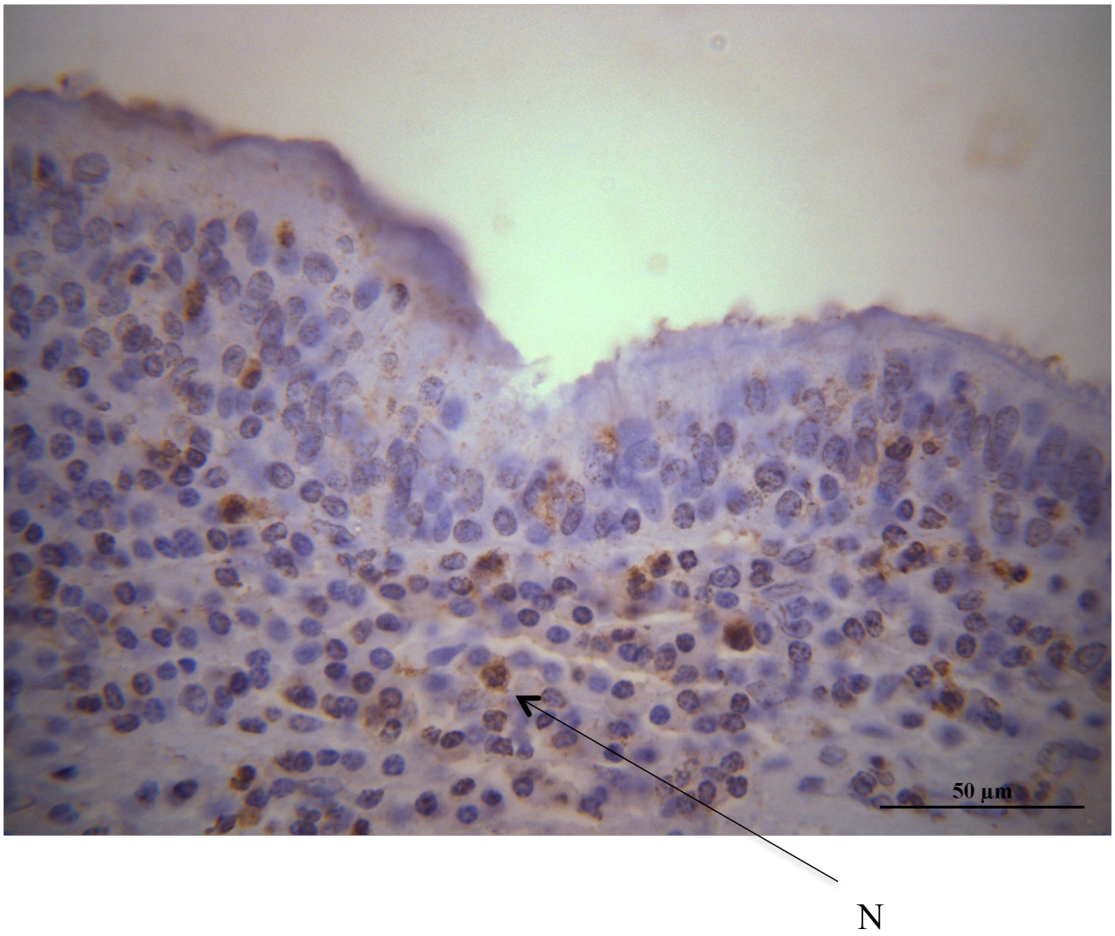


Figure 44. Immunohistochemistry for retinoic acid receptor-related orphan receptor gamma in advanced cystic fibrosis lung disease lower airway (higher power, x40 objective), including evidence of positive staining in neutrophils (N)

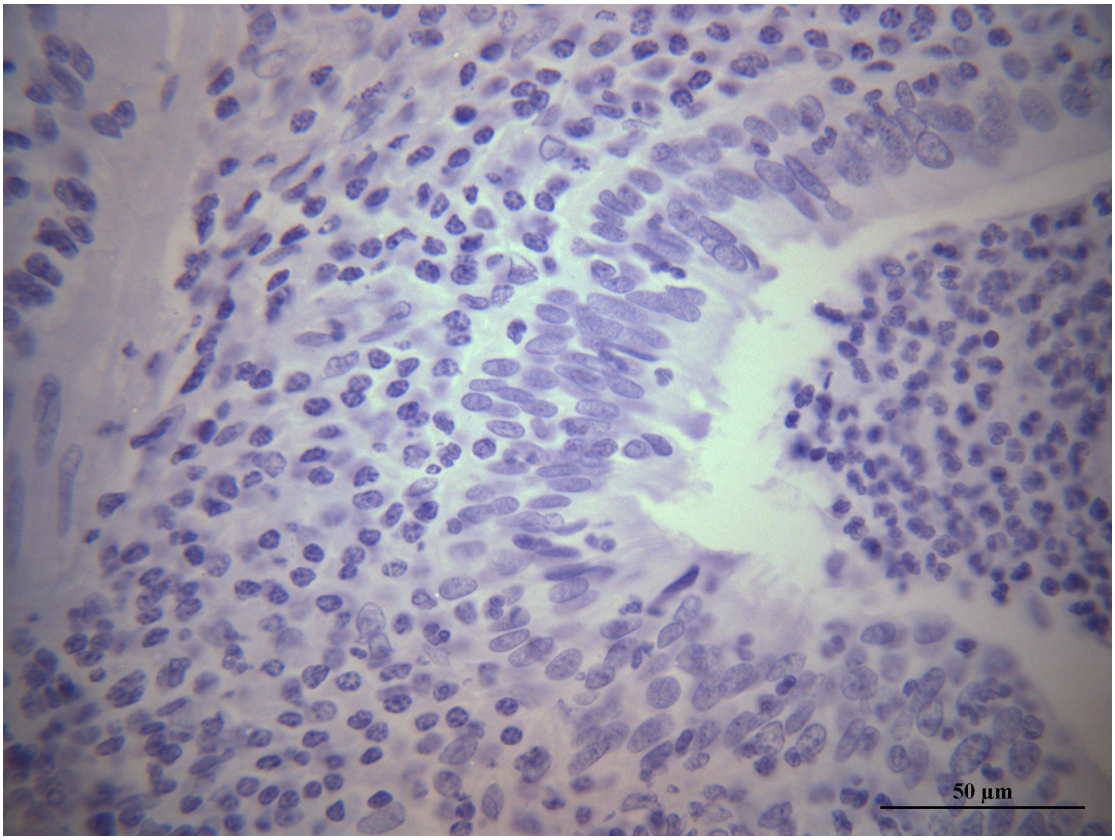


Figure 45. Example of isotype negative control (normal rabbit immunoglobulins) for retinoic acid receptor-related orphan receptor gamma immunohistochemistry cystic fibrosis airway

6.5.5. *Stimulation of primary bronchial epithelial cells isolated from people with advanced cystic fibrosis lung disease with interleukin-17 increases production of the pro-neutrophilic mediators interleukin-8, interleukin-6 and granulocyte macrophage colony-stimulating factor*

The lower airway epithelium is the predominant site of neutrophilic inflammation in CF lung disease. (Hubeau *et al.*, 2001a) Airway epithelial cells are increasingly recognised to operate as 'effector' cells that produce a wide range of inflammatory cytokines and growth factors. (Ward et al., 2009, Brodlie et al., 2009a) The cytokines IL-8, IL-6 and GMCSF are important in neutrophil activation, recruitment and longevity. (Gabay, 2006, Standiford et al., 1993, Barreda et al., 2004) I therefore investigated the effects of IL-17 on the production of these mediators by *ex vivo* cultures of PBECs from people with CF.

PBEC cultures were established from people with CF as outlined in Chapter 3. Table 11 provides brief clinical details of the 9 individual donors, some of whom were also used for immunohistochemistry. There was a significant increase in IL-8 production from control conditions following stimulation with 1 (P=0.033), 10 (P=0.009) and 100ng/mL (P=0.009) of IL-17 (Figure 49, n=9 individual patient donors). There was also a statistically significant increase in IL-6 production (Figure 50) from control following stimulation with 10 and 100ng/mL of IL-17 (P=0.009). There was a clear trend towards increased production of IL-6 following stimulation with 1ng/mL of IL-17 although this was not statistically significant (P=0.076). Increase in production of GMCSF (Figure 51) was statistically significant following stimulation with IL-17 100 ng/mL only (P=0.013). There was a non-significant trend towards increased levels following stimulation with IL-17 at 1 and 10ng/mL (P=0.407 and 0.193).

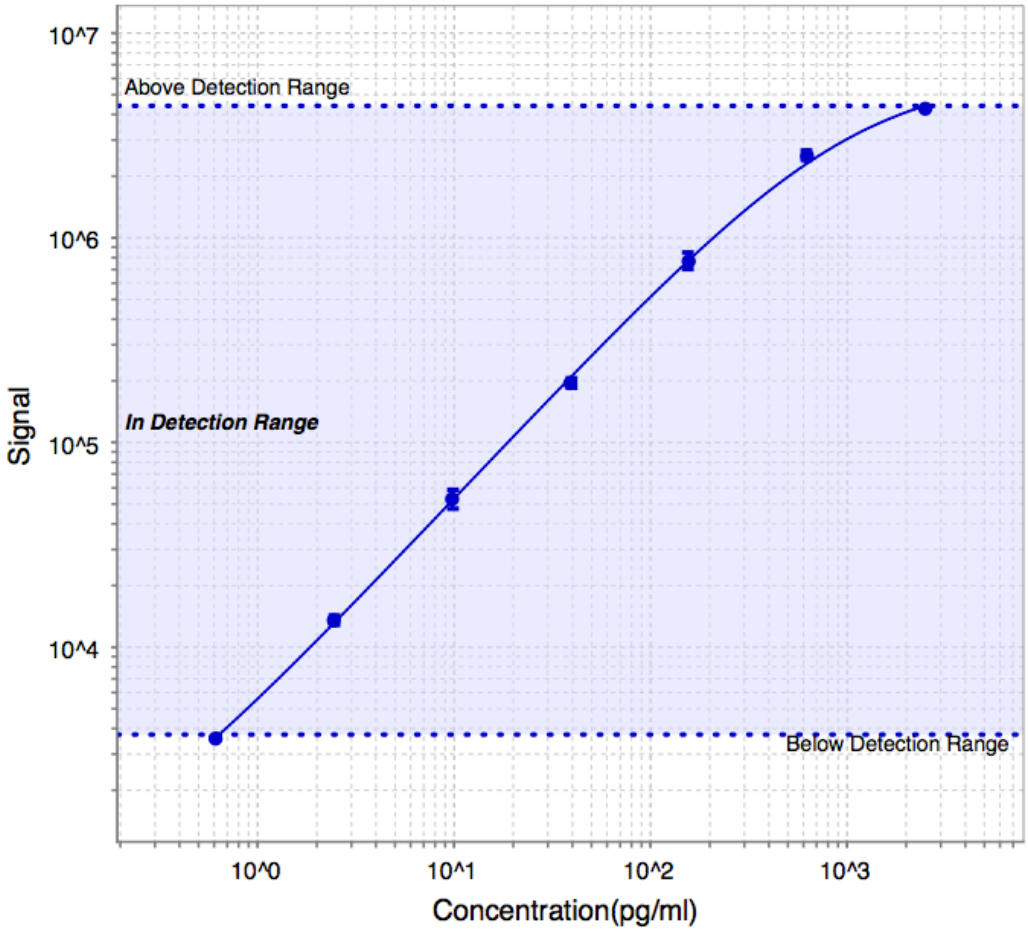


Figure 46. Standard curve for interleukin-8 enzyme-linked immunosorbent assay

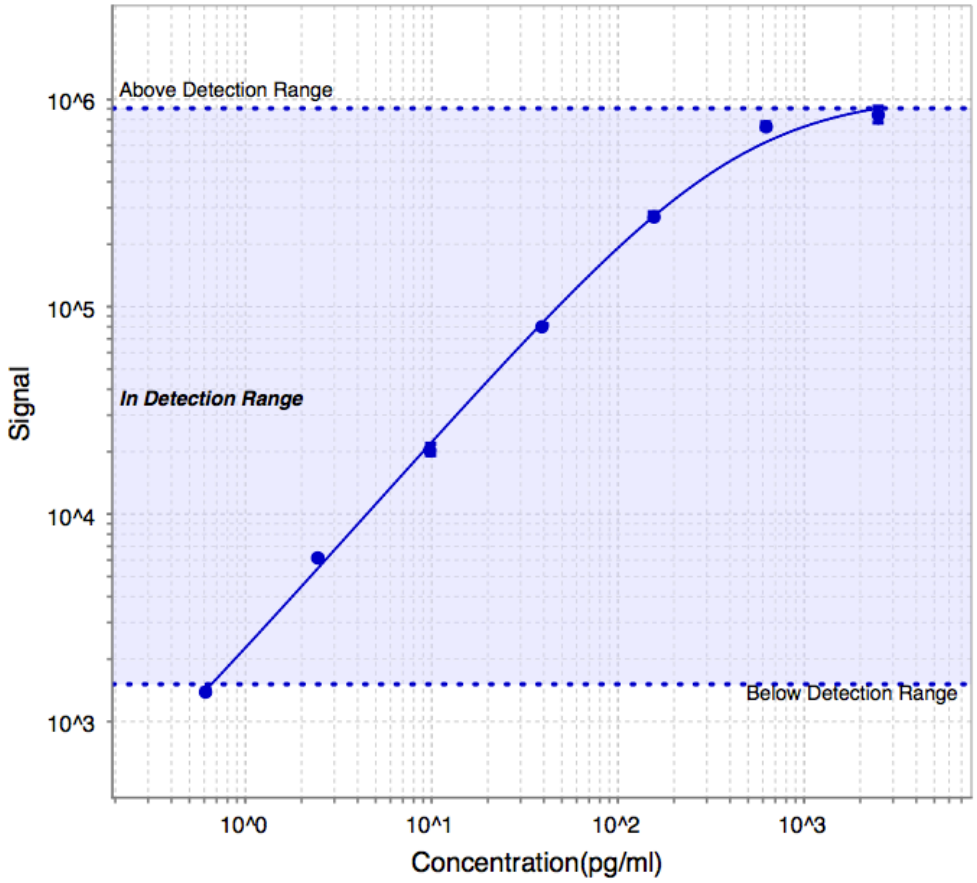


Figure 47. Standard curve for interleukin-6 enzyme-linked immunosorbent assay

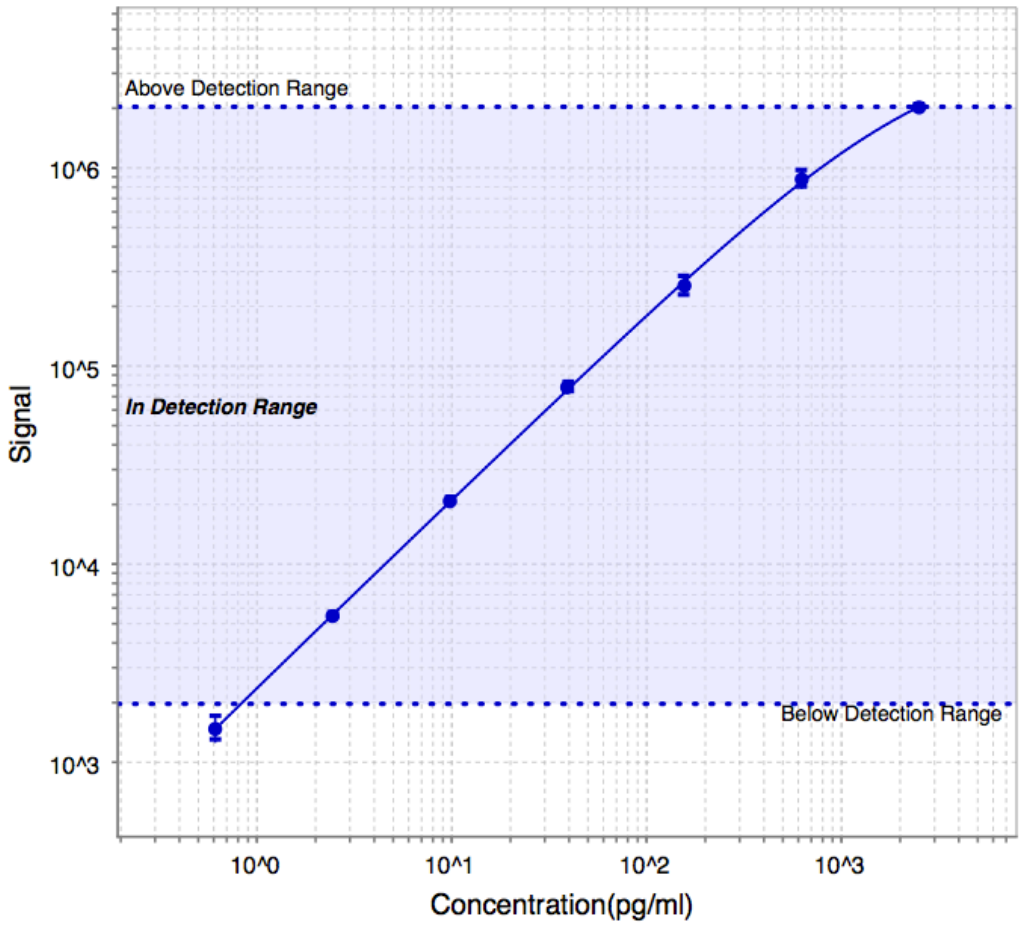


Figure 48. Standard curve for granulocyte macrophage colony-stimulating factor enzyme-linked immunosorbent assay

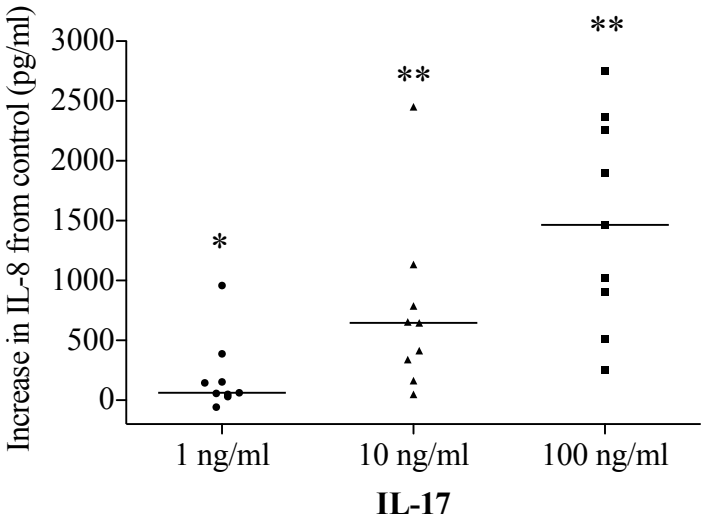


Figure 49. Increase in interleukin-8 production by primary bronchial epithelial cells from people with advanced cystic fibrosis lung disease from control following stimulation with 1, 10 and 100ng/mL interleukin-17.

Each symbol represents an individual patient and is the mean of 3 replicate experiments (n=9 individual patient donors).
Median basal IL-8 production 242pg/mL.
**P*=0.033, ***P*=0.009.
Wilcoxon signed-rank test comparing each concentration of IL-17 to basal production. (Wilcoxon, 1945) Holm-Bonferonni correction applied. (Holm, 1979)

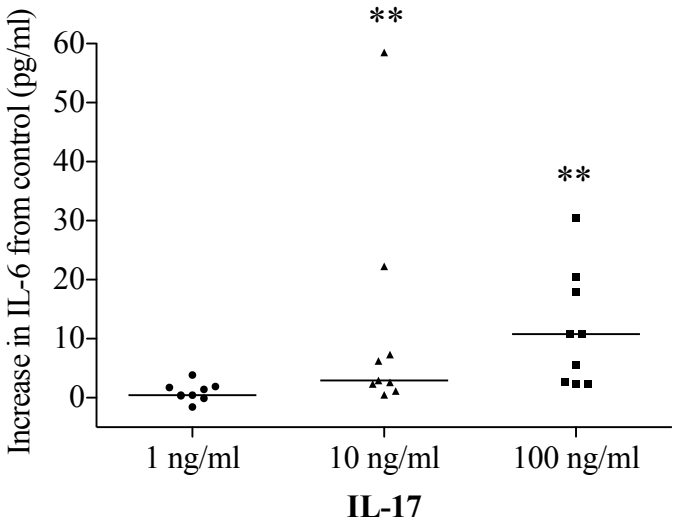


Figure 50. Increase in interleukin-6 production by primary bronchial epithelial cells from people with advanced cystic fibrosis lung disease from control following stimulation with 1, 10 and 100ng/mL interleukin-17.

Each symbol represents an individual patient and is the mean of 3 replicate experiments (n=9 individual patient donors).
Median basal IL-6 production 2.04pg/mL.
***P*=0.009.
Wilcoxon signed-rank test comparing each concentration of IL-17 to basal production (1ng/mL not significant). (Wilcoxon, 1945) Holm-Bonferonni correction applied. (Holm, 1979)

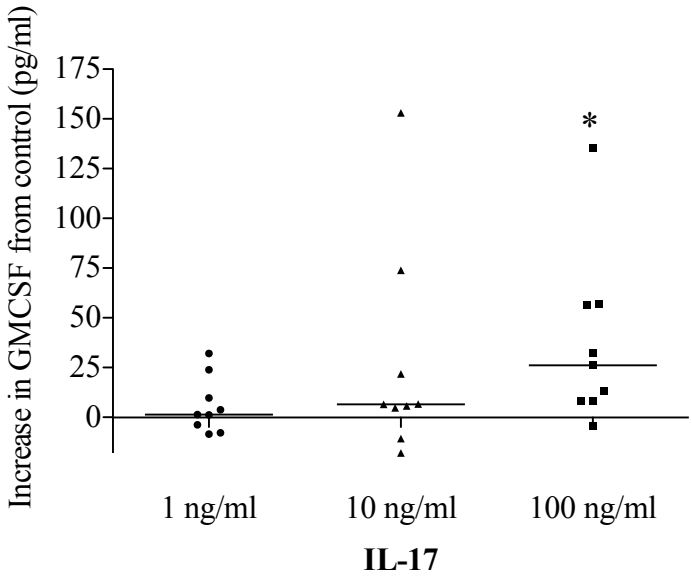


Figure 51. Increase in granulocyte macrophage colony-stimulating factor production by primary bronchial epithelial cells from people with advanced cystic fibrosis lung disease from control following stimulation with 1, 10 and 100ng/mL interleukin-17.

Each symbol represents an individual patient and is the mean of 3 replicate experiments (n=9 individual patient donors).
Median basal GMCSF production 8.08pg/mL.
*P=0.013.
Wilcoxon signed-rank test comparing each concentration of IL-17 to basal production (only 100ng/mL significant). (Wilcoxon, 1945) Holm-Bonferonni correction applied. (Holm, 1979)

6.6. Discussion

In this chapter I have demonstrated that IL-17 is expressed and raised in the lower airway of people with advanced CF lung disease, using immunohistochemistry compared to the non-suppurative condition PH and measured in airway lavage fluid compared to healthy volunteers. I also identified substantially increased numbers of inflammatory cells staining positive for IL-17 present in the lower airway mucosa of people with CF. Some of these were mononuclear cells, in keeping with the literature indicating that T_H-17 cells are a source of IL-17. (Glader *et al.*, 2010) However, I also consistently identified abundant IL-17 positive neutrophils - a new finding, which may be significant in CF lung disease. In addition I found neutrophils in the lower airway mucosa to stain positively for the transcription factor RORγ.

There are no previous descriptions of which I am aware showing that IL-17 protein is localised to neutrophils in human lung, with a strong emphasis on T_H-17 biology in the current literature. IL-17 staining has been described in neutrophils previously in the context of human liver disease and complicated atherosclerotic plaques, and in the lungs of mice, however. (Lemmers et al., 2009, Ferretti et al., 2003, de Boer et al., 2010) IL-17 has also been co-localised to mast cells in rheumatoid arthritis synovium. (Hueber *et al.*, 2010)

Elegant work, only achievable in a mouse setting, recently showed that renal ischaemia-reperfusion injury is IL-17 dependent, and that IL-17 is produced by neutrophils. (Li *et al.*, 2010) This work used IL-17 knockouts, IL-17 production assays, as well as bone marrow transplantation to produce chimeric mice. A series of experiments showed that kidney damage was IL-17 mediated and that this was produced by bone marrow derived neutrophils. Hence, protection from kidney injury was shown in IL-17–/– mice, and this was reversed following transfer of wild type neutrophils, but not IL-17–/– neutrophils. Where reconstituted the injury was in turn attenuated by antibody neutralization of IL-17.

If neutrophils are a source of IL-17, as evidenced by my current human lung work, the aforementioned human liver study and in more detailed animal model work, this suggests that IL-17 mediated injury may have an amplifying positive autocrine feedback element. (Li et al., 2010, Lemmers et al., 2009, Ferretti et al., 2003) IL-17 causes

neutrophil recruitment, which themselves may be potential sources of this key cytokine. (Ferretti *et al.*, 2003) Figure 52 illustrates this potential positive feedback loop of neutrophilic inflammation involving IL-17. IL-17 also has a modulatory post-transcriptional effect on IL-8 and IL-6 responses by epithelial cells in addition to stimulatory effects. (van den Berg *et al.*, 2005)

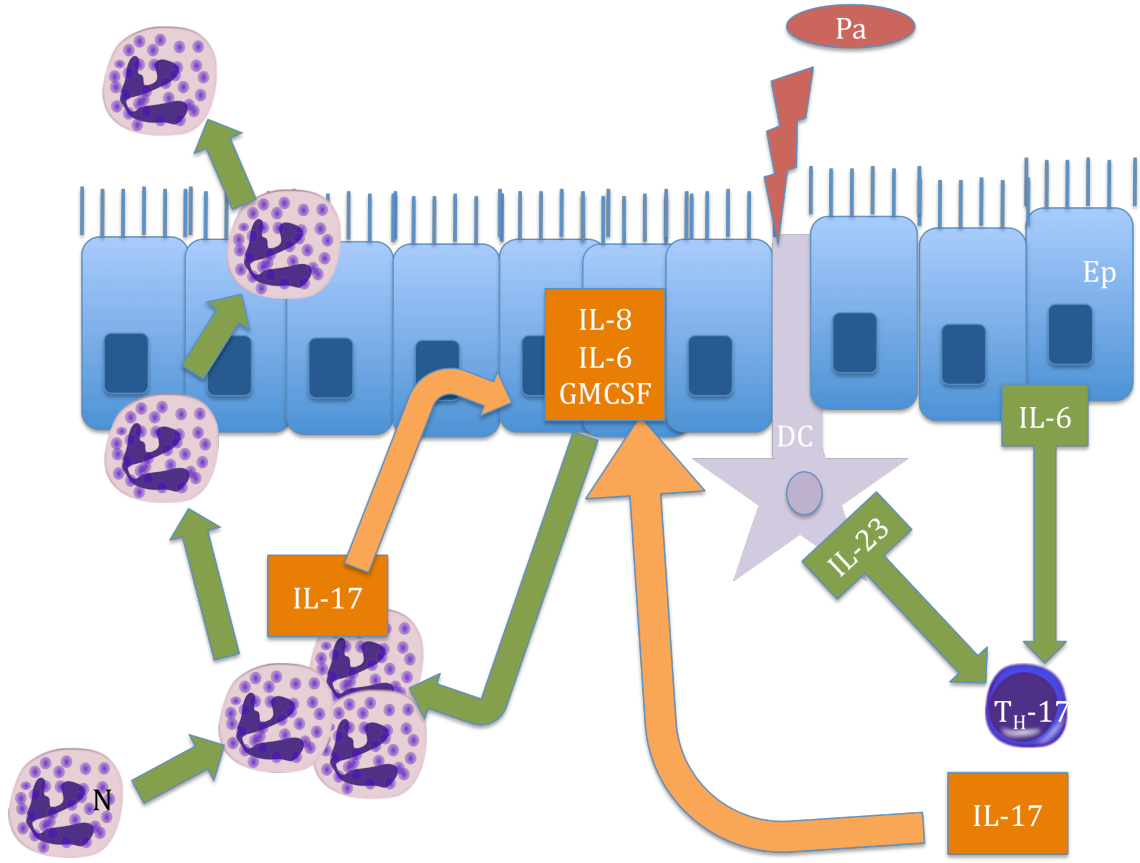


Figure 52. Illustrative diagram of potential positive feedback loop of neutrophilic inflammation involving interleukin-17 in cystic fibrosis airway

Abrreviations: Ep. Epithelium, IL. Interleukin, DC dendritic cell, N neutrophil, Pa *Pseudomonas aeruginosa.*

A potential for positive feedback was further suggested by my proof of concept experiments. In these I assessed the effect of IL-17 stimulation on CF airway epithelial cells. IL-17 is known to exert effects on neutrophil accumulation and activation in the bronchoalveolar space indirectly via the local release of neutrophil-mobilising factors. (Aujla et al., 2007, Linden et al., 2005) I therefore investigated the effects of treatment with IL-17 on the production of the key pro-neutrophilic cytokines and chemokines IL-8, IL-6 and GMCSF by PBEC cultures from people with CF. This showed that stimulation of PBECs with IL-17 resulted in significant increases in the release of these key mediators.

IL-8 is a potent chemoattractant and activator of neutrophils and is the dominant neutrophil chemokine in the sputum of people with CF during an infective exacerbation. (Standiford et al., 1993, McAllister et al., 2005, Downey et al., 2009) A substantial literature also shows that IL-8 is involved in airway angiogenic structural remodelling. (Walters *et al.*, 2008) Stimulation with IL-17 resulted in a significant increase in IL-8 production in all of my CF PBEC experiments. This data is therefore consistent with a potential contributory role in neutrophilic airway inflammation and remodelling in CF. The growth factor GMCSF displays pleiotropic effects on neutrophil proliferation, maturation, activation and inhibition of apoptosis. (Barreda *et al.*, 2004) My finding that treatment of PBECs with IL-17 caused increased GMCSF production, is also therefore consistent with a broad association between IL-17, epithelial cells and neutrophilic inflammation in CF. Furthermore, in the context of myocarditis, there is evidence to suggest that GMCSF plays a key role in the generation and maintenance of T_H-17 cells *via* regulation of IL-23 and IL-6 *in vivo*. (Sonderegger *et al.*, 2008)

Production of IL-6 was also increased significantly following treatment with 10 and 100 ng/mL IL-17 with a clear but non-significant trend towards increased levels at 1 ng/mL. IL-6 plays a key role in the acute phase inflammatory response. (Gabay, 2006) Furthermore, IL-6 induces IL-17 production from human central memory CD4+ T-cells. (Yang *et al.*, 2008) Together with my finding that neutrophils themselves are a source of IL-17, the increase in IL-6 production by PBECs following IL-17 treatment represents a further potential aspect of a positive feedback loop, and augmentation of IL-17 responses. Furthermore, it has recently been shown that following stimulation with IL-17, IL-6 production by bronchial epithelial cells with intact CFTR function is

enhanced when co-cultured with basophils compared to when cultured alone. (Wong *et al.*, 2010)

It was apparent that there was a high degree of variability in the production of IL-8, GMCSF and IL-6 in PBECs isolated from different individuals. This reflects previously described inherent biological heterogeneity in chemokine and cytokine production, and is in keeping with the experiences of other researchers with PBECs. (Becker *et al.*, 2004) Interestingly the findings in my *ex vivo* system resonate with the biological variability seen in the human CF lung disease phenotype, although all of these patients ultimately developed advanced lung disease. (O'Sullivan and Freedman, 2009, Rowntree and Harris, 2003)

The finding that airway wall neutrophils stain positively for IL-17 does not necessarily mean that they are the source of the ligand. Neutrophils express the IL-17 receptor and can bind IL-17. (Ye *et al.*, 2001b) However, this would appear to be an unlikely sole explanation due to the diffuse intra-cellular staining that we identified. If IL-17 is adherent to receptors on neutrophils this may also represent a biologically significant reservoir that is potentially released in to the microenvironment following neutrophil necrosis. (Watt et al., 2004, Haslett, 1999) My finding of positive staining for RORγ in airway mucosa neutrophils provides further evidence to support the hypothesis that neutrophils themselves may be a source of IL-17 in the airway.

Other findings in the published literature pertinent to a role for IL-17 in CF lung disease include the report that immortalised airway epithelial cells that do not express functional CFTR upregulate their innate immune responses following stimulation with IL-17. (Roussel and Rousseau, 2009) In particular, nucleotide-binding oligomerisation domain 1 is increased. (Roussel and Rousseau, 2009) IL-17 has also been demonstrated to modulate bicarbonate secretion in normal airway epithelial cells. (Kreindler *et al.*, 2009)

Although my study includes potentially significant observations, it has limitations. This work was clearly limited to a study of advanced CF lung disease, utilising a well-characterised and substantive tissue archive that I helped establish. However, this precluded any meaningful exploration of clinical associations and did not include any individuals with less severe lung disease. Further work, in earlier stages of CF lung

disease, in less severe phenotypes and in patients with differing microbiology are therefore warranted. Lung tissue from people with non-CF bronchiectasis would also be an important future comparative group.

The suggestion that IL-17 is produced by airway neutrophils was achieved using unambiguous, immunolocalisation using an affinity-purified, specific antibody. (Le Gouvello *et al.*, 2008) I clearly showed IL-17 positive, intra-epithelial neutrophils. My results do not however indicate what the levels of local IL-17 release are at this precise intra-epithelial site. In particular, concentrations at the immunological interface between neutrophils, lymphocytes and epithelial cells are not known.

The detection of IL-17 in airway lavage fluid from explanted CF lungs is an important finding, albeit at relatively low levels. It is particularly significant when considered in the context of the previous work performed by Dr Des Murphy in Newcastle where IL-17 was universally undetectable using the same ultrasensitive ELISA, in addition to a number of other commercially available ELISA kits, in 180ml BALs from healthy volunteers and lung allograft recipients at the time of clinical stability, acute rejection or with broncholitis obliterans syndrome. (Murphy, 2008) (DM Murphy, personal communication) The inevitable difference in lavage technique between the BAL in healthy volunteers performed by Dr Murphy and the smaller volume airway lavages of explanted CF lungs should be noted however.

In agreement with my findings in CF, researchers in Pittsburgh, USA, have found similar levels of IL-17 (in the region of 40-200pg/mL) to my results in BAL from adults and children with CF during an infective exacerbation. (McAllister et al., 2005, Dubin et al., 2007) Lower levels of IL-17 (0.5-1.5pg/mL) have recently been reported by Glader *et al.* in BAL fluid from healthy human volunteers following experimental airway exposure to endotoxin. (Glader *et al.*, 2010)

Interestingly in the same study IL-23 was undetectable in BAL following challenge with vehicle alone but was detectable at low levels (2-20pg/mL) in some samples post-endotoxin challenge. (Glader *et al.*, 2010) I measured detectable levels of IL-23 in BAL from 8 of 26 explanted CF lungs.

The work in this chapter describes elevated IL-17 in the airways of patients with advanced CF lung disease and the first description that neutrophils are a potential source of this key cytokine in human airway. This should stimulate further translational work as well as other approaches including appropriate animal models. Such work may be particularly important in CF, but may also be relevant in other lung pathophysiologies involving neutrophil biology such as chronic obstructive pulmonary disease, asthma, non-CF bronchiectasis, adult respiratory distress syndrome, chronic allograft rejection and viral bronchiolitis. (Alcorn et al., 2010, Tsushima et al., 2009, Robertson et al., 2009, McNamara et al., 2003, Eastham et al., 2004)

7. Chapter 7. Ceramide and cystic fibrosis lung disease

Elements of the work described in this chapter have been previously reported in the form of presentations at medical and scientific meetings and in a peer-reviewed paper. (Brodlie et al., 2009b, Brodlie et al., 2009d, Brodlie et al., 2010b, Brodlie et al., 2010a)

7.1. Abstract

Background: Ceramide accumulates in the airway epithelium of cystic fibrosis transmembrane conductance regulator-deficient mice resulting in susceptibility to *Pseudomonas aeruginosa* infection and inflammation. The primary aim of the work described in this chapter was to quantitatively investigate ceramide levels in the lower airway of people with cystic fibrosis compared to pulmonary hypertension, emphysema and lung donors. In addition I examined relationships between levels of immunoreactive ceramide in the lower airway epithelium and markers of neutrophils and colonisation with *Pseudomonas aeruginosa*.

Methods: Immunohistochemistry was performed on the lower airway epithelium of explanted lungs (8 cystic fibrosis, emphysema and pulmonary hypertension respectively) and 8 donor lungs using ceramide, neutrophil elastase and myeloperoxidase antibodies. High performance liquid chromatography-mass spectrometry was performed on tissue from 5 cystic fibrosis and 5 pulmonary hypertension lungs.

Results: Staining for ceramide was significantly increased in the lower airway epithelium of people with cystic fibrosis (median 14.11% surface area) compared to pulmonary hypertension (3.03%, P=0.0009), unused lung donors (3.44%, P=0.0009) and emphysema (5.06%, P=0.01). Ceramide staining was increased in emphysematous lungs compared to pulmonary hypertension (P=0.0135) and unused donors (P=0.0009). The number of neutrophil elastase and myeloperoxidase positive cells in the airway was positively correlated with the percentage of epithelium staining for ceramide (P=0.001). Ceramide staining was significantly increased in lungs colonised with *Pseudomonas aeruginosa* (10.1%) compared to those not colonised (3.14%, P=0.0106). Significantly raised levels of ceramides C16:0, C18:0 and C20:0 were detected by mass spectrometry in cystic fibrosis lungs compared to pulmonary hypertension. Differences in C22:0 were not significant.

Conclusion: Immunoreactive ceramide is increased in the lower airway epithelium of people with advanced cystic fibrosis. Detected by mass-spectrometry ceramide species C16:0, C18:0 and C20:0 but not C22:0 are increased.

7.2. Introduction

As outlined in earlier chapters of this thesis, CF is a chronic condition caused by abnormalities in the CFTR gene that is associated with life-long morbidity and mortality. (Ratjen, 2009, Davies et al., 2007) Over 95% of morbidity and mortality in CF is associated with lung disease. (Doring *et al.*, 2007) Promising developments in clinical care have yielded an increase in survival for people with CF over recent decades, however, the exact pathogenesis of CF lung disease remains poorly understood. (Dodge et al., 2007, O'Sullivan and Freedman, 2009)

The sphingolipid ceramide is an essential component of plasma membranes and regulates many physiological cellular functions including apoptosis and responses to stress and cytokines. (Uhlig and Gulbins, 2008) There is also growing evidence that sphingolipids play a central role in the pathogenesis of several lung diseases, including acute lung injury, viral infection, asthma, emphysema and CF. (Niessen et al., 2008, Oskeritzian et al., 2007, Teichgraber et al., 2008, Petrache et al., 2005, Grassme et al., 2005, Noe et al., 2009)

Chronic infection with *P. aeruginosa* and intense neutrophilic inflammation are two hallmarks of CF lung disease. (Davidson et al., 1995, Downey et al., 2009) Teichgraber *et al.* (2008) recently demonstrated that CFTR-deficient mice accumulate ceramide in airway epithelial cells. Furthermore, ceramide accumulation triggered chronic pulmonary inflammation, death of airway epithelial cells and extracellular deposition of DNA in the airways, thereby increasing susceptibility to infection with *P. aeruginosa*. (Teichgraber *et al.*, 2008) Treatment with the tricyclic antidepressant amitriptyline normalised pulmonary ceramide and susceptibility to *P. aeruginosa* infection in CFTR-deficient mice. (Becker et al., 2010b, Teichgraber et al., 2008) Ceramide accumulation was also shown in nasal epithelial cells and demonstrated at a qualitative level in three sections of airway from people with CF. (Teichgraber *et al.*, 2008)

In contrast, low plasma levels of ceramide have been reported by Guilbault *et al.* (2008a and 2008b) in people with CF compared to healthy volunteers. The same researchers also found reduced levels of ceramide in the plasma, lungs, pancreas and ileum of CFTR-deficient mice that could be corrected by treatment with fenretinide. (Guilbault et al., 2008b, Guilbault et al., 2008a) The potential broad importance of ceramide biology

to lung homeostasis is indicated by the recent finding that in lung endothelial cells CFTR function is required for stress-induced apoptosis by maintaining ceramide activation. (Noe *et al.*, 2009)

The findings of current research into ceramide and CF pathophysiology are divergent however, and possibly specific to the model systems studied. (Guilbault et al., 2008b, Guilbault et al., 2008a, Teichgraber et al., 2008) Hence, Yu *et al.* found no significant difference in basal ceramide levels in IB3-1 immortalised CF bronchial epithelial cells and lung homogenate from CFTR knock out mice compared to wild type cells and mice. (Yu *et al.*, 2009)

The lower airways are the site of major pathology in human CF lung disease. (Hamutcu *et al.*, 2002) The aim of the work described in this chapter was therefore to investigate the hypothesis that ceramide accumulates in the lower airway of people with advanced CF lung disease. In light of the findings of Teichgraber *et al.* (2008) outlined above, relationships between levels of epithelial ceramide and markers of neutrophilic inflammation and colonisation with *P. aeruginosa* were also investigated.

7.3. Aims

The aims of the work described in this chapter were to:

- Investigate levels of ceramide in the lower airway of people with advanced cystic fibrosis lung disease. More specifically to:
    - Quantify immunoreactive ceramide localised to the lower airway epithelium in advanced CF lung disease compared to pulmonary hypertension, emphysema and, previously healthy, unused lung donors.
    - Quantify the specific ceramide species C16:0, C18:0, C20:0 and C22:0 in whole lung tissue from people with advanced CF lung disease compared to pulmonary hypertension by the independent technique of high performance liquid chromatography-mass spectrometry.
- Correlate the amount of immunoreactive ceramide in the lower airway epithelium in advanced CF lung disease, pulmonary hypertension, emphysema and unused lung donors with levels of neutrophilic inflammaion in the lower airway mucosa, as measured by the number of cells staining positive for neutrophil elastase and myeloperoxidase per mm of basement membrane.
- Investigate the relationship between the amount of immunoreactive ceramide in the lower airway epithelium in advanced cystic fibrosis lung disease, pulmonary hypertension and emphysema, and colonisation with *Pseudomonas aeruginosa*.

7.4. Hypothesis

The work in this chapter examines the hypothesis that ceramide is raised in the lower airway epithelium of people with advanced CF lung disease.

7.5. Results

7.5.1. *Immunohistochemistry for ceramide in the lower airway epithelium in advanced cystic fibrosis lung disease, pulmonary hypertension, emphysema and unused lung donors*

Immunohistochemistry was performed on sections of medium-large lower airway from explanted lungs removed at the time of transplantation from 8 people with advanced CF lung disease, PH and emphysema respectively. The clinical details of these 24 patients are provided in Table 13. Airway sections were also obtained from 8 donor lungs that were assessed but not used for lung transplantation. Haematoxylin and eosin stained sections of airway from these blocks were independently evaluated to be free from fibrosis and significant inflammation as previously described. (Ward *et al.*, 2005)

Historically a lack of appropriate antibodies has limited the study of ceramide and other sphingolipids in biological systems. However, two antibodies have become commercially available in recent years, namely a mouse IgM anti-ceramide monoclonal antibody (Clone MID 15B4, Sigma-Aldrich) and a polyclonal mouse, antiserum enriched for IgM, anti-ceramide antibody (Clone S58-9, Glycobiotech). In lipid overlay assays the polyclonal antibody has been demonstrated to have favourable specificity for ceramide and dihydroceramide compared to the monoclonal antibody. (Cowart *et al.*, 2002) In order to adopt the most thorough approach possible I performed immunohistochemistry using each of the antibodies.

Age*	Transplant†	Diagnosis (CFTR variant)	Sputum microbiology
23§	SSL	Cystic fibrosis (p.Phe508del / p.Phe508del)	*P. aeruginosa, S. maltophilia*
26	SSL	Cystic fibrosis (p.Phe508del / p.Phe508del)	*P. aeruginosa, S. apiospermum*
25§	SSL	Cystic fibrosis (Unknown)	*P. aeruginosa*
57	SSL	Cystic fibrosis (p.Arg117His / p.Arg553X)	*P. aeruginosa*
26§	SSL	Cystic fibrosis (Unknown)	*P. aeruginosa, A. fumigatus*
28§	SSL	Cystic fibrosis (F508del/F508del)	*P. aeruginosa, A. fumigatus*
14	SSL	Cystic fibrosis (Unknown)	*P. aeruginosa, C. parapsilosis*
32	SSL	Cystic fibrosis (Unknown)	*P. aeruginosa, B. vietnamiensis*
53	HL	Pulmonary hypertension	Nil
60	HL	Pulmonary hypertension	Nil
52	HL	Pulmonary hypertension	Nil
25	HL	Pulmonary hypertension	Nil
36	HL	Pulmonary hypertension	Nil
51	HL	Pulmonary hypertension	Nil
37§	HL	Pulmonary hypertension	Nil
52	HL	Pulmonary hypertension	Nil
46	SL	Emphysema	Nil
59	SSL	Emphysema	*P. aeruginosa*
37	SSL	Emphysema	*P. aeruginosa*
51	SSL	Emphysema	*M. catarrhalis, Serratia* sp.
47	SL	Emphysema	Nil
33	SSL	Emphysema	*P. aeruginosa*
54	SSL	Emphysema	*M. catarrhalis*
58	SSL	Emphysema	*P. aeruginosa*

Table 13. Clinical details of the patients undergoing lung transplantation used for ceramide immunohistochemistry

Abbreviations: *Age in years at time of transplant (complete years only). †SSL bilateral single sequential lung transplant, SL single lung transplant, HL heart-lung transplant. §Patient also used for high performance liquid chromatography-mass spectrometry (Table 14).

7.5.2. *Glycobiotech polyclonal mouse, antiserum enriched for IgM, anti-ceramide antibody*

Staining for ceramide using the Glycobiotech antibody was significantly increased in the lower airway epithelium of people with CF (median 14.11%) compared to PH (3.03%, P=0.0009), unused lung donors (3.44%, P=0.0009) and emphysema (5.06%, P=0.01) (Figure 53). Ceramide staining was significantly increased in emphysematous lungs compared to PH (P=0.0135) and unused donors (P=0.0009) (Figure 53). Representative staining using the Glycobiotech antibody is illustrated from people with CF, emphysema, PH and unused lung donors in Figure 54. Appropriate negative controls; no primary antibody added and istotype control are also shown in Figure 54.

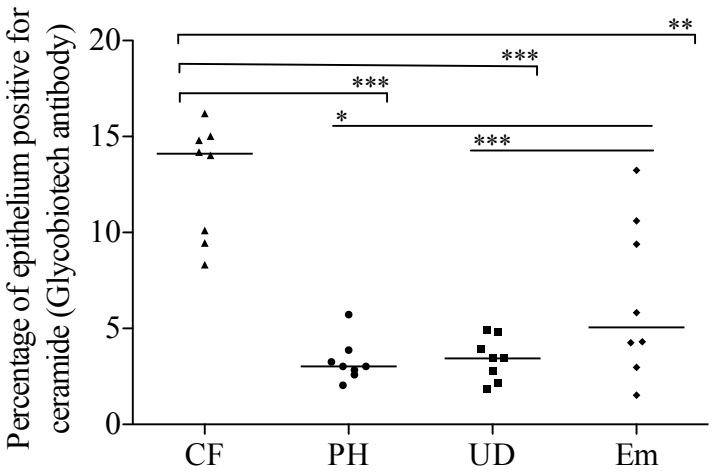
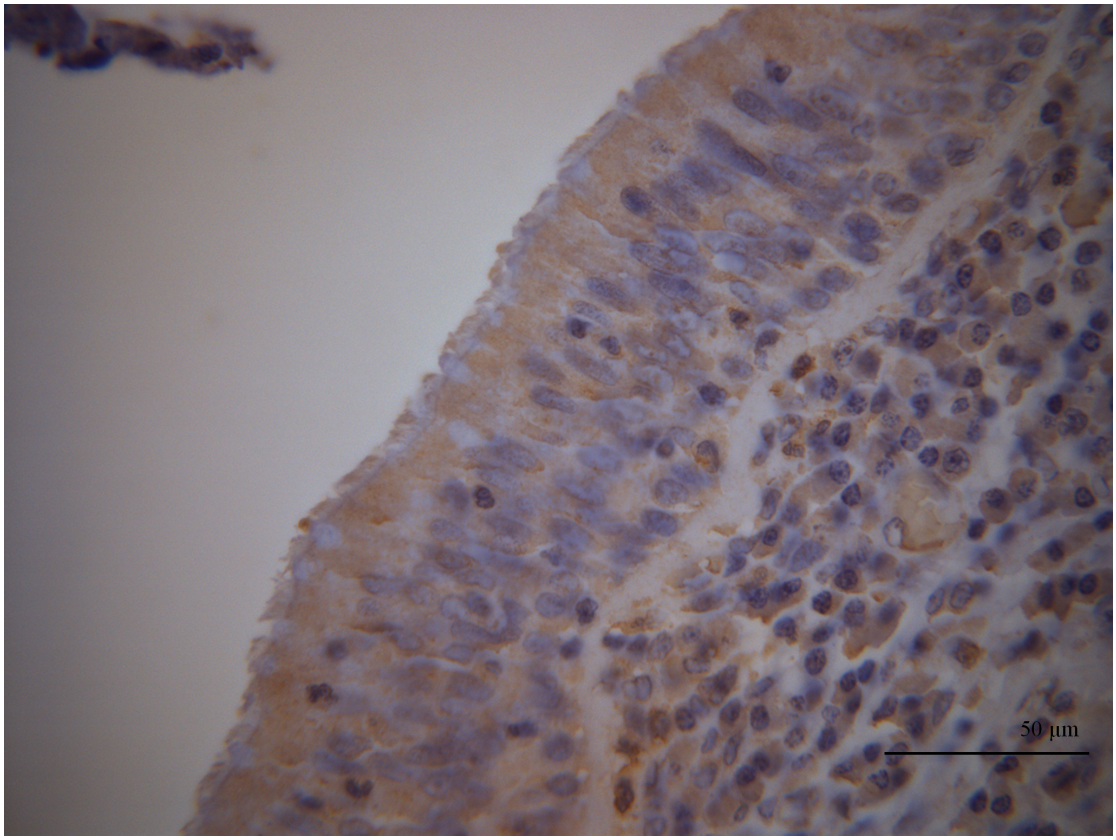


Figure 53. Percentage of epithelium staining positive for ceramide with Glycobiotech antibody.

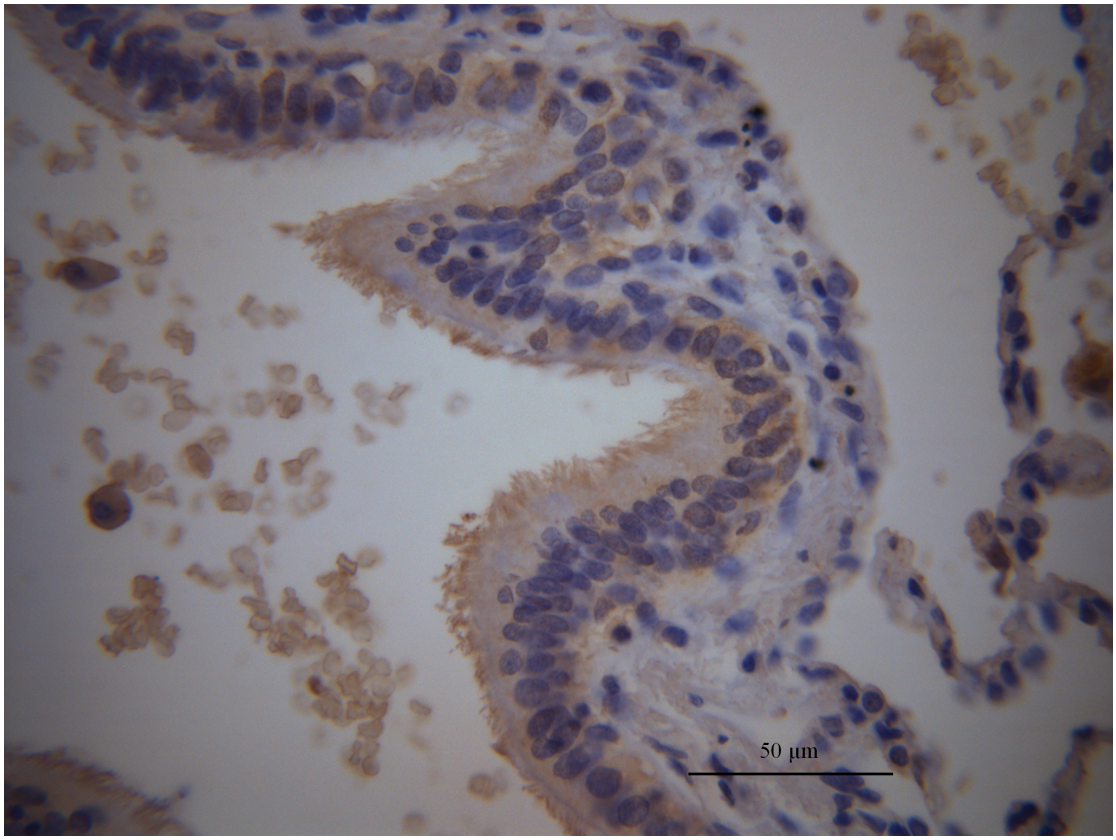
Each symbol represents an individual patient and is the mean of the percentage epithelium staining positive in five randomly selected high power fields.
The horizontal bar represents the median for each group.
Abbreviations: CF cystic fibrosis, PH pulmonary hypertension, UD unused lung donors, Em emphysema.
**P*=0.0135, ***P*=0.01, ****P*=0.0009. Mann-Whitney tests, Holm-Bonferonni correction applied. (Mann and Whitney, 1947, Holm, 1979)

Figure 54. Representative staining for ceramide with Glycobiotech antibody, (a) in a person with advanced cystic fibrosis lung disease, (b) emphysema, (c) pulmonary hypertension, (d) emphysema and (e) negative control (isotype IgM cystic fibrosis airway).

(a)



(b)



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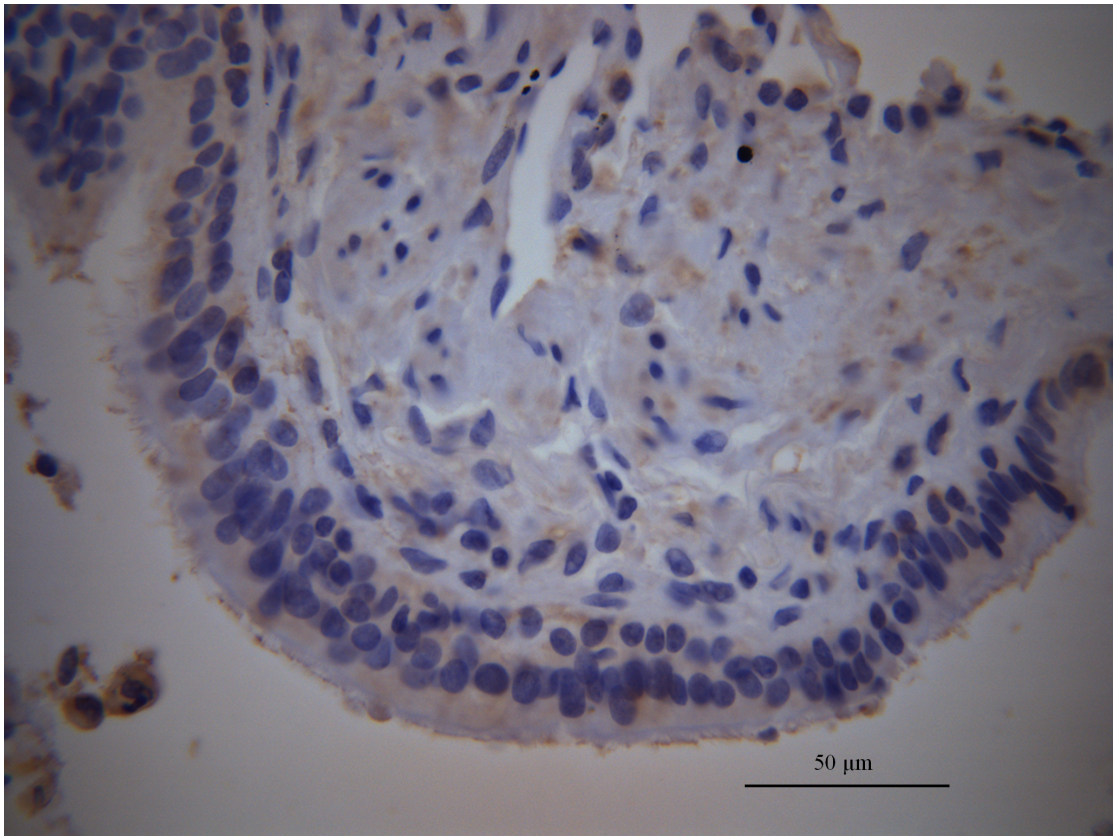
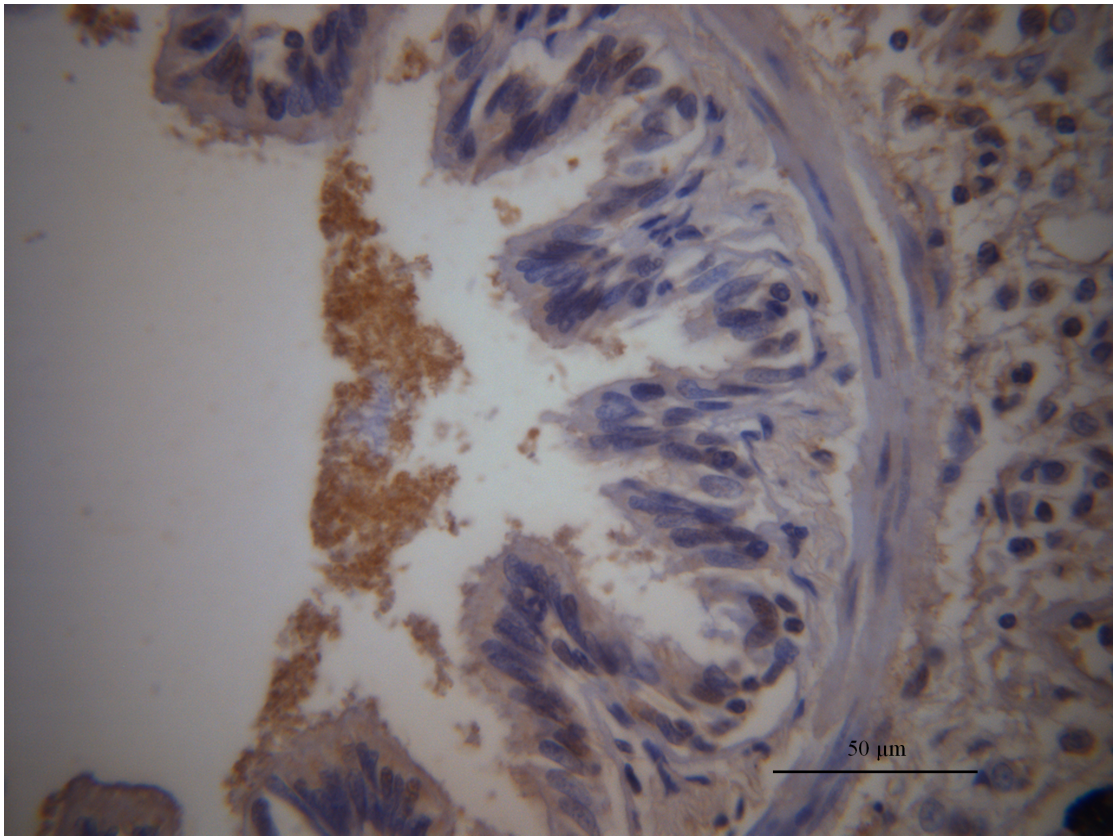
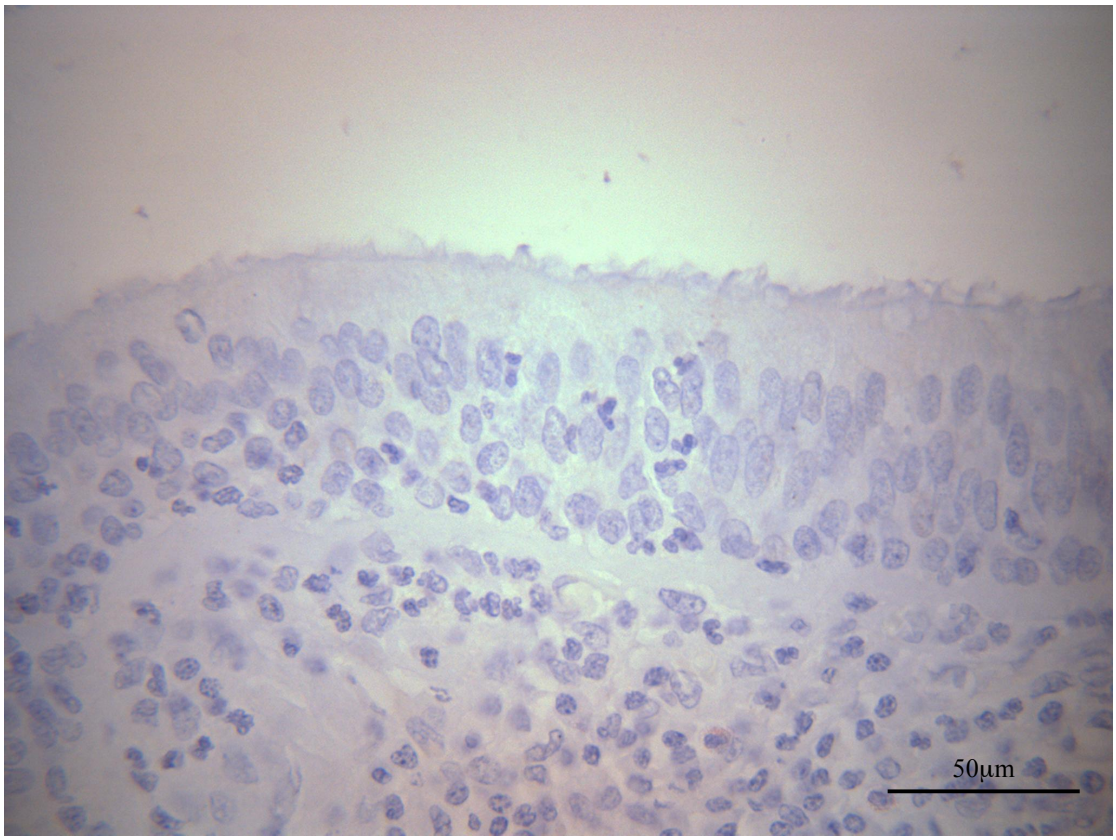


(d)



(e)



7.5.3. *Sigma monoclonal IgM anti-ceramide antibody*

Staining for ceramide using the Sigma antibody was significantly increased in the lower airway epithelium of people with CF (median 13.45%) compared to PH (4.62%, *P*=0.01) and unused lung donors (3.22%, *P*=0.005) (Figure 55). There was no statistically significant difference between people with CF (13.45%) and emphysema (6.35%, *P*=0.1278) using the monoclonal antibody (Figure 55). Staining for ceramide was significantly greater in emphysematous lungs (6.35%) compared to PH (4.62%, *P*=0.0136) and unused donors (3.22%, *P*=0.0009) (Figure 55). Representative staining using the Sigma antibody is illustrated from people with CF, emphysema, PH and unused lung donors in Figure 56. Appropriate negative controls; no primary antibody added and istotype control are also shown in Figure 56.

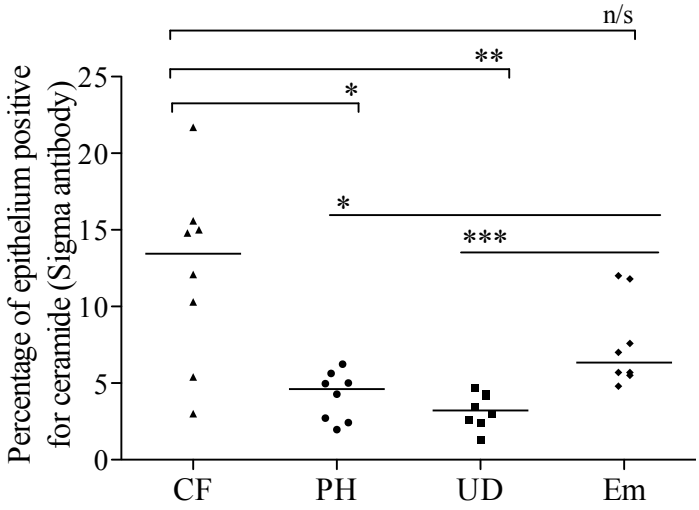
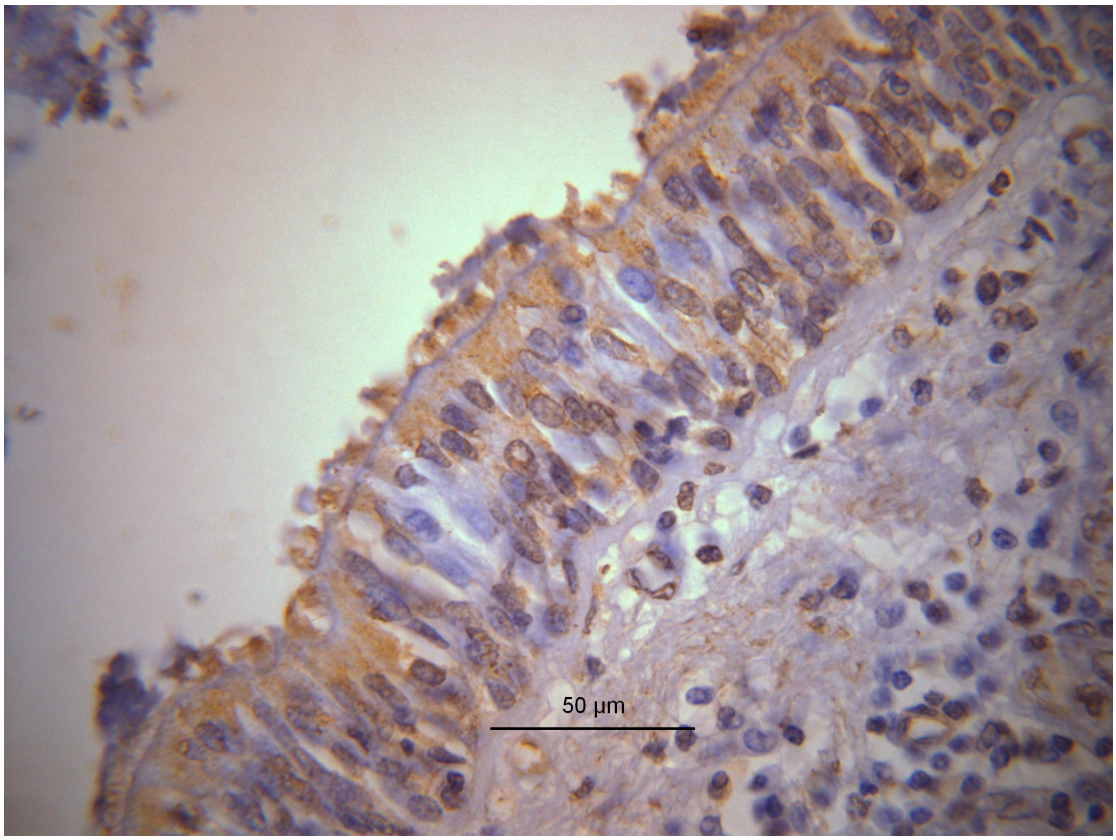


Figure 55. Percentage of epithelium staining positive for ceramide with Sigma antibody.

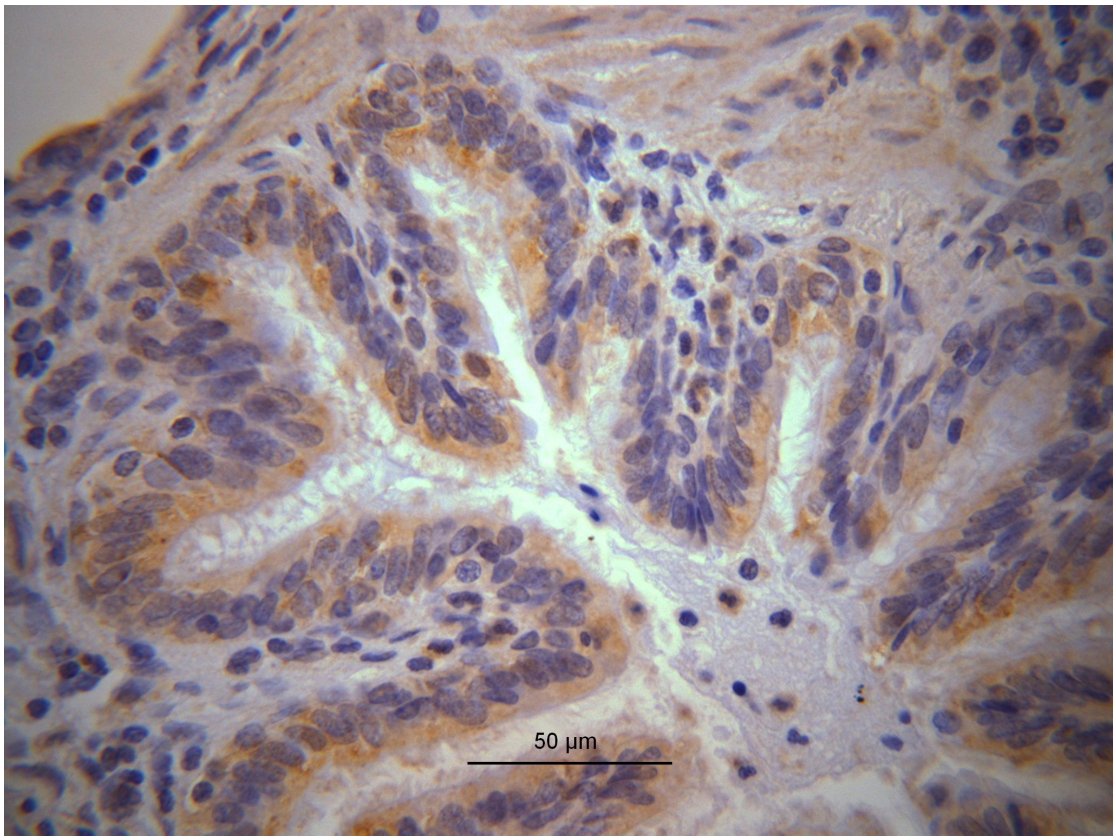
Each symbol represents an individual patient and is the mean of the percentage epithelium staining positive in five randomly selected high power fields.
The horizontal bar represents the median for each group.
Abbreviations: CF cystic fibrosis, PH pulmonary hypertension, UD unused lung donors, Em emphysema.
**P*=0.01, ***P*=0.005, ****P*=0.0009, n/s non-significant. Mann-Whitney tests, Holm-Bonferonni correction applied. (Mann and Whitney, 1947, Holm, 1979)

Figure 56. Representative staining for ceramide with Sigma antibody, (a) in a person with advanced cystic fibrosis lung disease, (b) emphysema, (c) pulmonary hypertension, (d) emphysema and (e) negative control (isotype IgM cystic fibrosis airway).

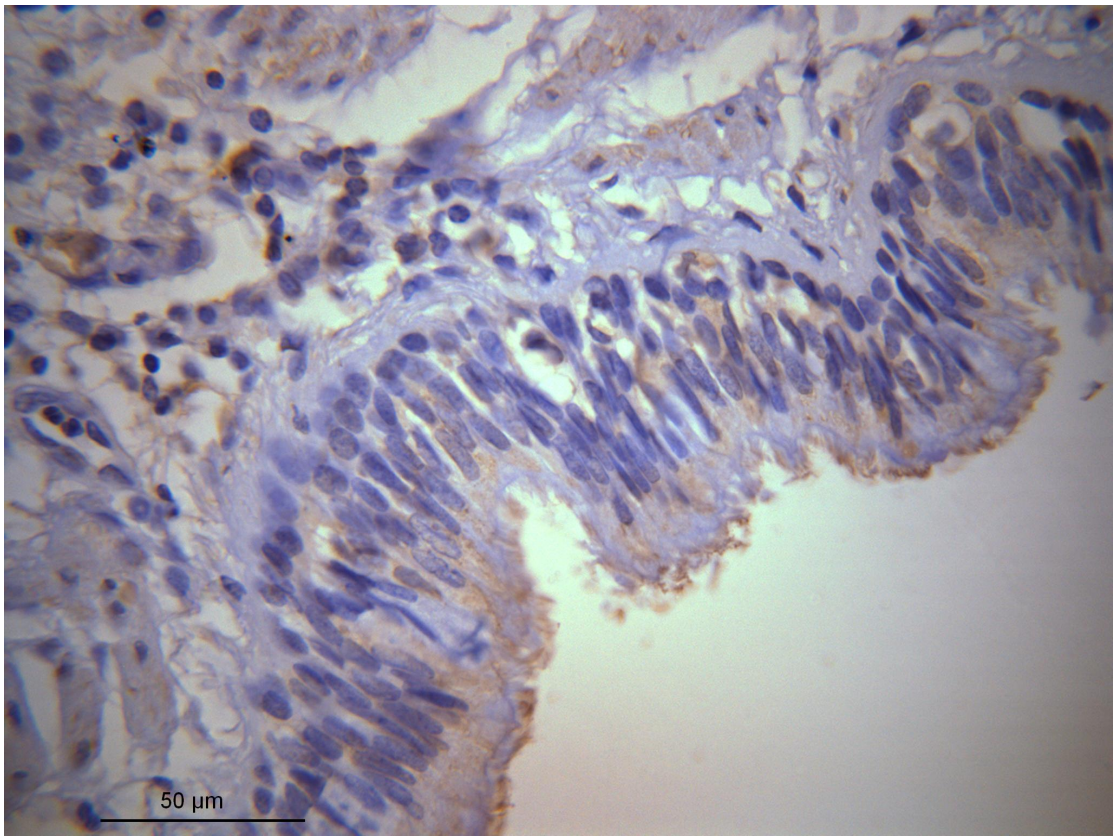
(a)



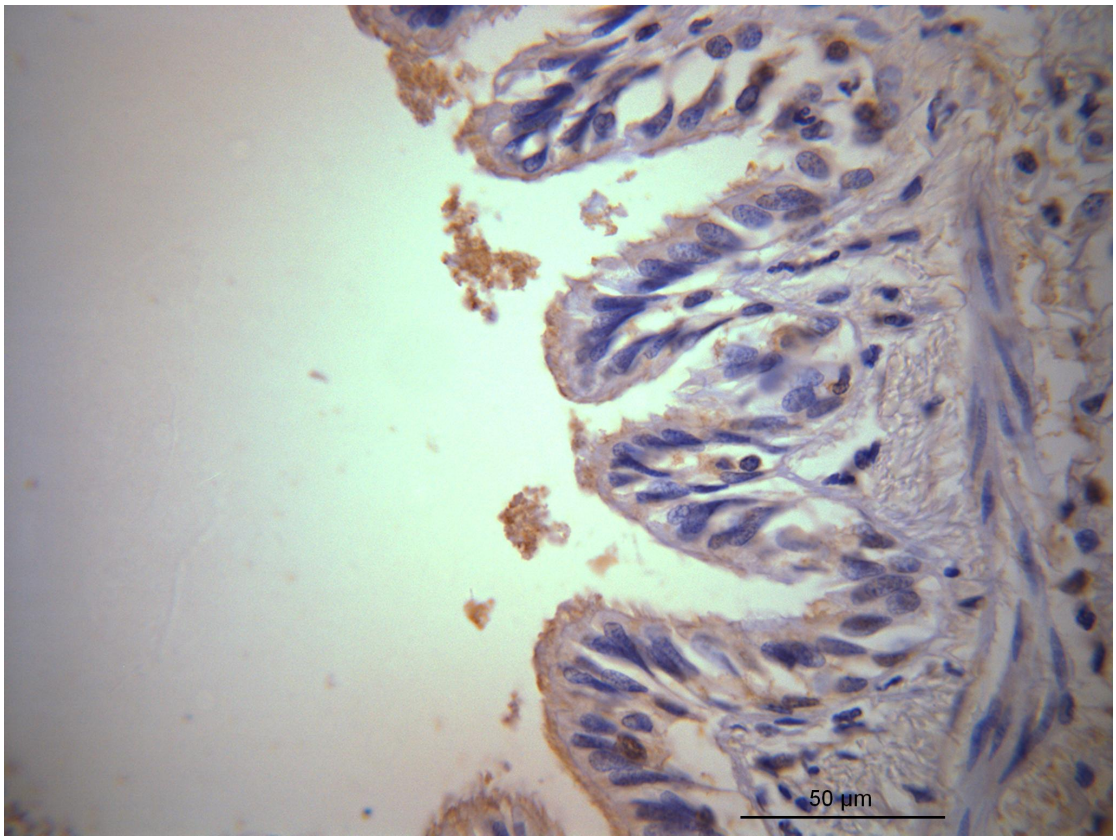
(b)



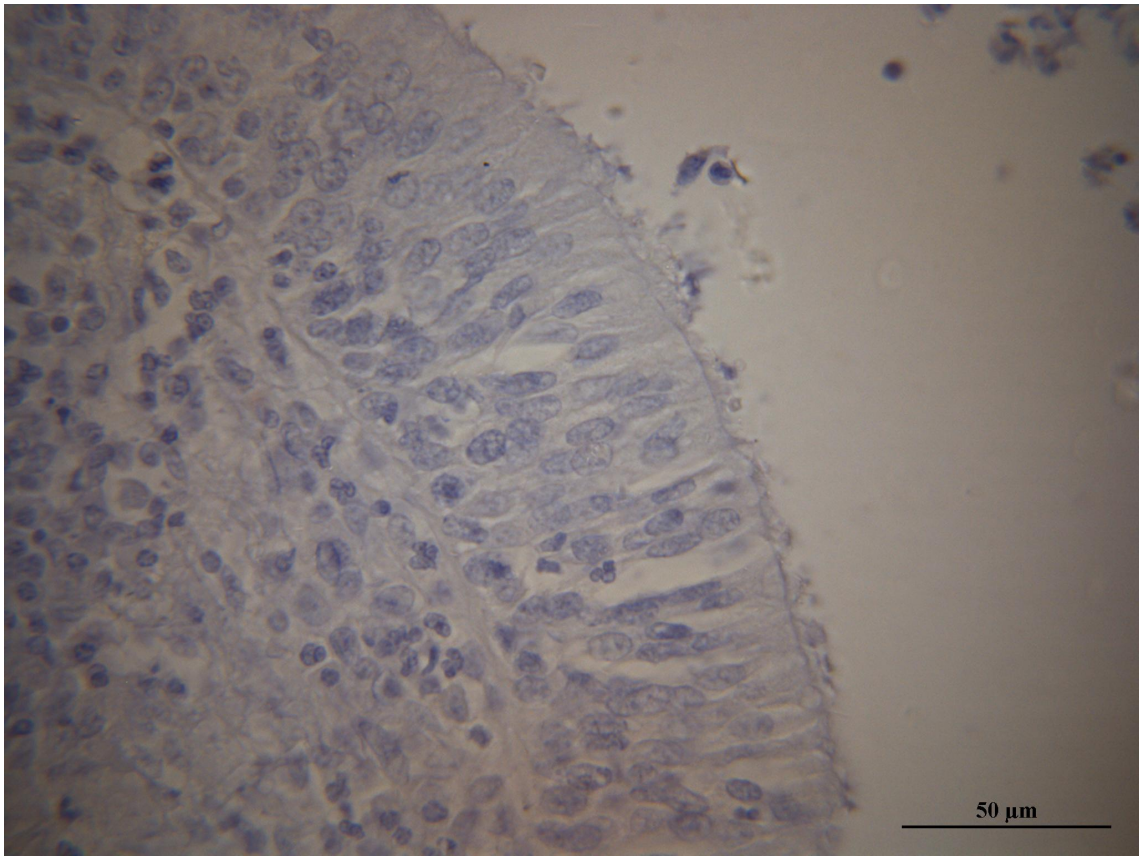
(c)



(d)



(e)



7.5.4. *Comparison of Glycobiotech polyclonal mouse, antiserum enriched for IgM, anti-ceramide antibody and Sigma monoclonal IgM anti-ceramide antibody*

As mentioned earlier in this chapter, in lipid overlay assays the Glycobiotech polyclonal antibody has been demonstrated to have favourable specificity for ceramide and dihydroceramide compared to the Sigma monoclonal antibody. (Cowart *et al.*, 2002) Examination of Figure 54 and Figure 56 allows comparison of the typical staining appearances with each antibody. A Bland-Altman plot of average against difference is shown in Figure 57. (Bland and Altman, 1986) As one would expect there was a degree of variability in staining between the two antibodies. However, there was no systematic bias in the measurements, i.e. one antibody was not systematically providing higher percentage staining compared to the other (bias -0.07742%, standard deviation of bias ±3.164, 95% limits of agreement -6.280% and +6.125%) (Figure 57).

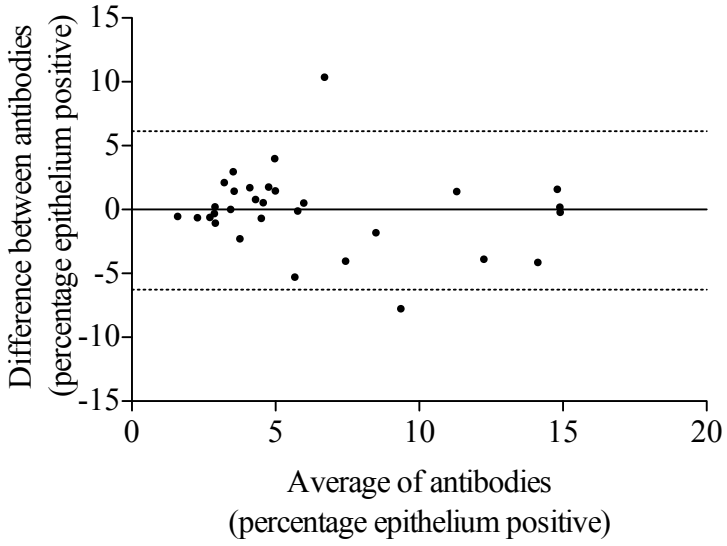


Figure 57. Bland-Altman plot comparing lower airway epithelial staining with the Glycobiotech polyclonal mouse, antiserum enriched for IgM, anti-ceramide antibody and Sigma monoclonal IgM anti-ceramide antibody

Bias -0.07742% (standard deviation of bias ±3.164)
95% limits of agreement (dashed lines) -6.280% and +6.125%

7.5.5. *Investigation of the reproducibility of percentage epithelium staining positive measurements*

In order to investigate the reproducibility of my measurements of the percentage epithelium staining positive I performed a Bland-Altman analysis. I blindly repeated the measurements on a random selection of three patients from each group stained with the Glycobiotech ceramide antibody. Figure 58 displays the Bland-Altman plot. A degree of variability is evident but no systematic bias between each occasion.

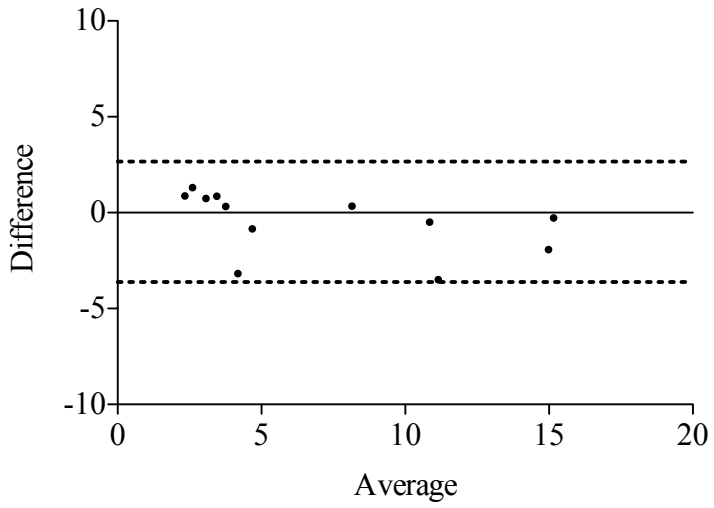


Figure 58. Bland-Altman plot of difference against average for repeat measurements of percentage epithelium positive for ceramide using Glycobiotech ceramide antibody

Bias -0.4813, 95% limits of agreement -3.622 to 2.659 (dotted lines)

7.5.6. *Immunohistochemistry for neutrophil elastase in the lower airway mucosa in advanced cystic fibrosis lung disease, pulmonary hypertension, emphysema and unused lung donors*

The CF airway is characterised by neutrophilic inflammation and as one would expect I found significantly more neutrophil elastase positive cells/millimetre basement membrane in people with CF (median 203.5) compared to PH (2.8, P=0.0002), emphysema (14.9, P=0.0003) and unused donors (9.2, P=0.0002) (Figure 59). (Hays and Fahy, 2006, Downey et al., 2009) Representative staining for neutrophil elastase in each of the respective groups is shown in Figure 60. A negative isotype control is also shown in Figure 60.

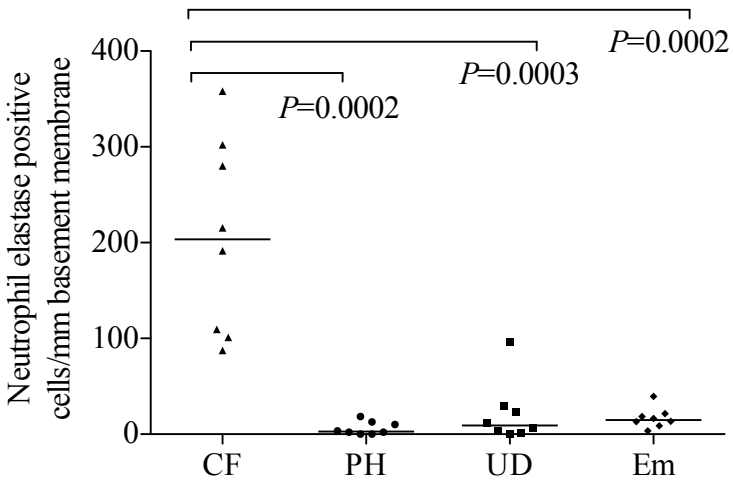
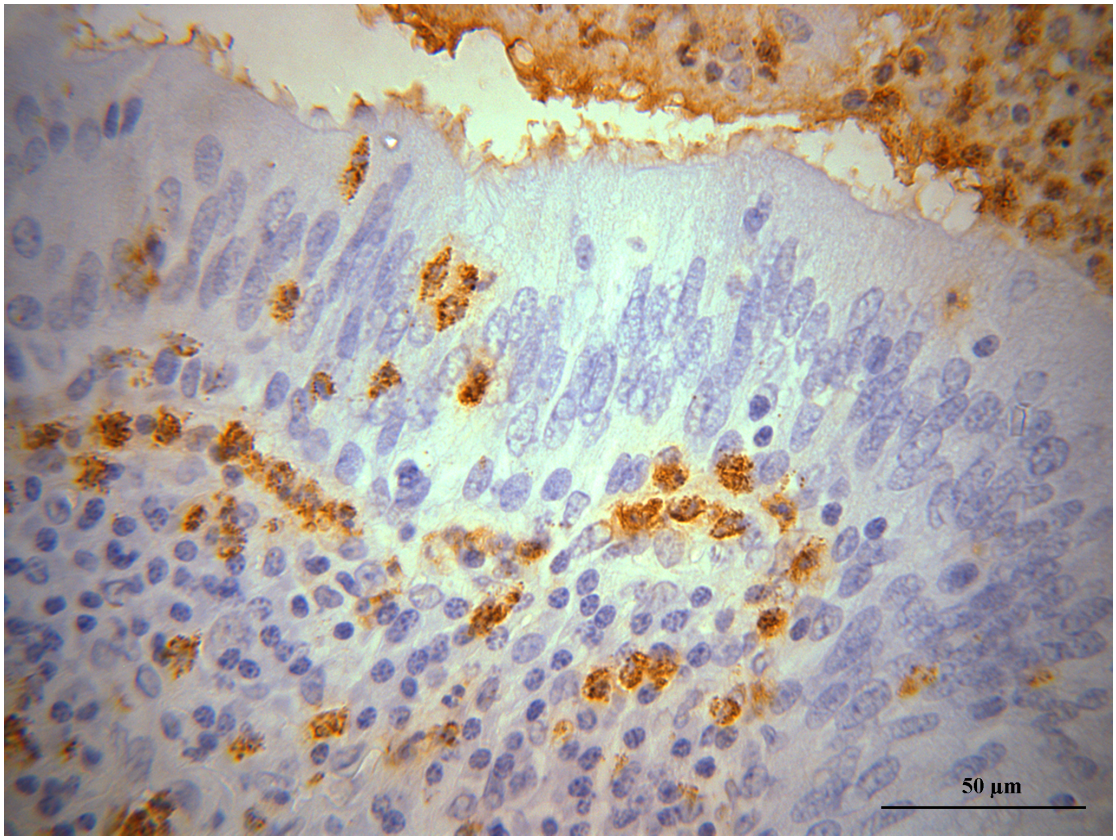


Figure 59. Number of neutrophil elastase positive cells in the lower airway mucosa in advanced cystic fibrosis lung disease, pulmonary hypertension, emphysema and unused lung donors

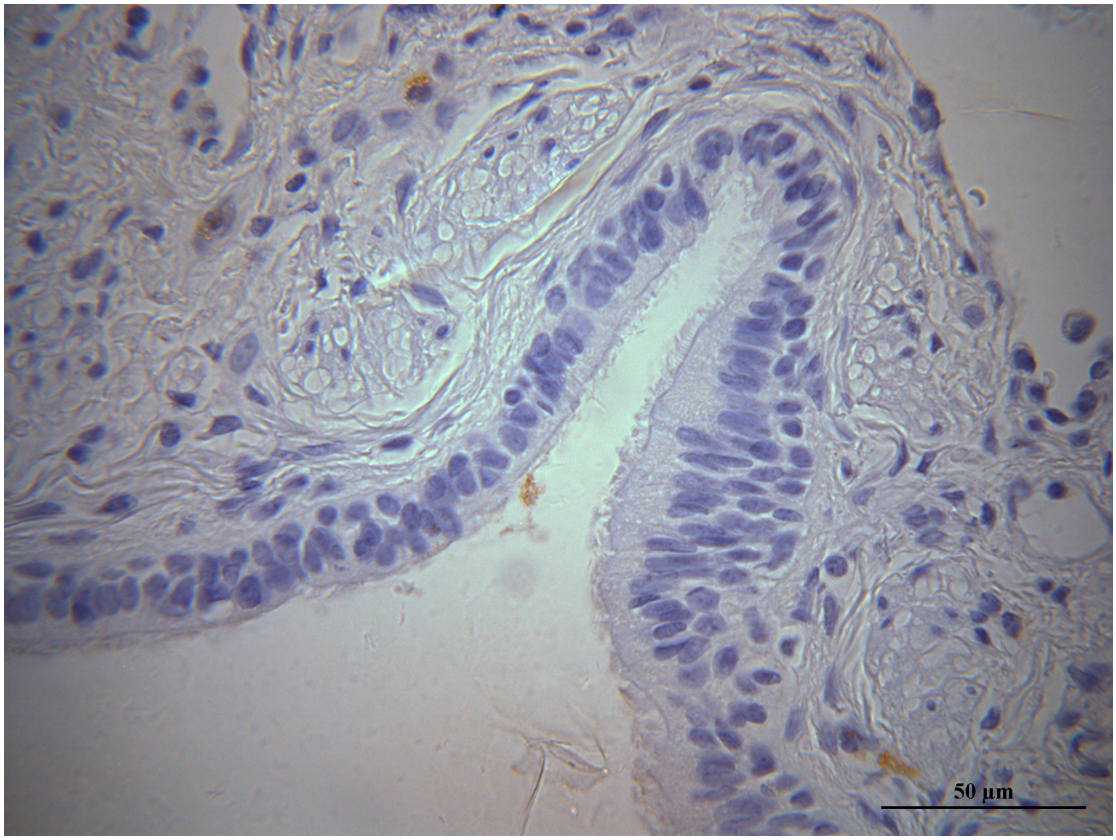
Abbreviations: CF cystic fibrosis, PH pulmonary hypertension, UD unused lung donors, Em emphysema.
Horizontal bars indicate the median for each group.
Mann-Whitney tests, Holm-Bonferonni correction applied. (Mann and Whitney, 1947, Holm, 1979)

Figure 60. Representative staining for neutrophil elastase in the lower airway mucosa in advanced cystic fibrosis lung disease (a), pulmonary hypertension (b), emphysema (c), unused lung donor (d) and immunoglobulin G_1 isotype negative control (e).

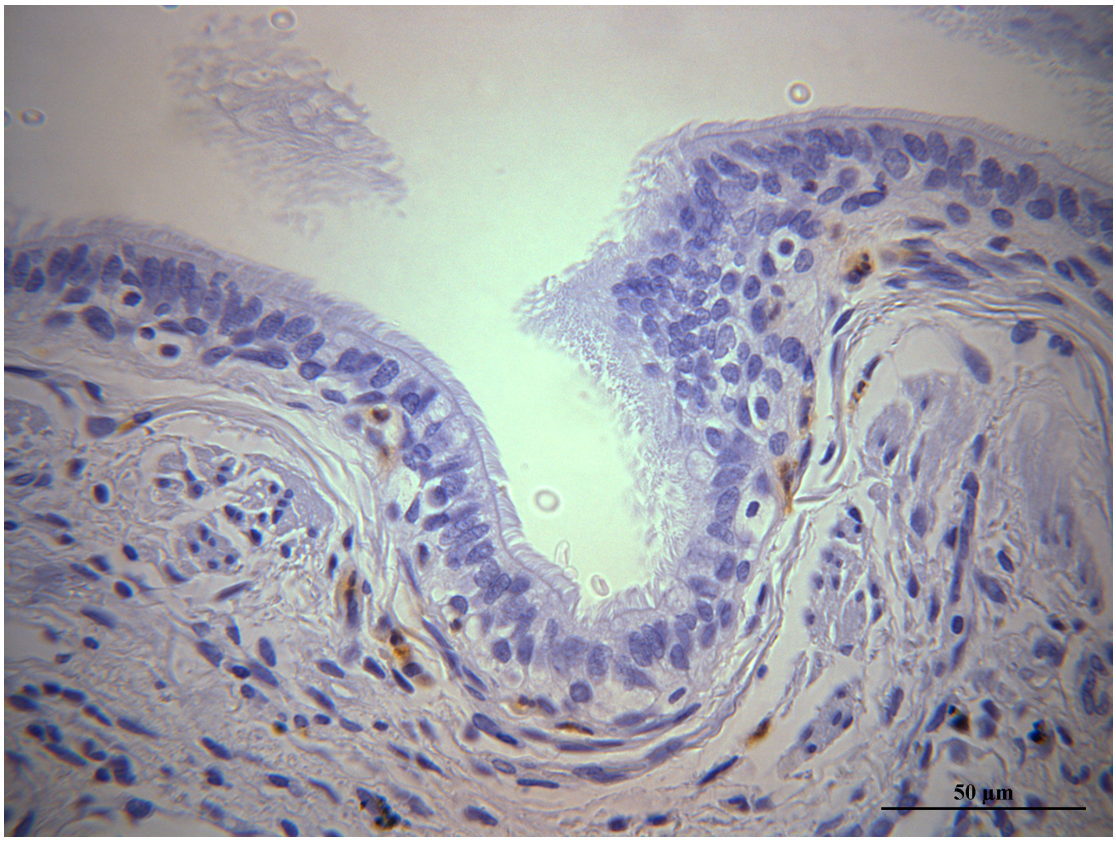
(a)



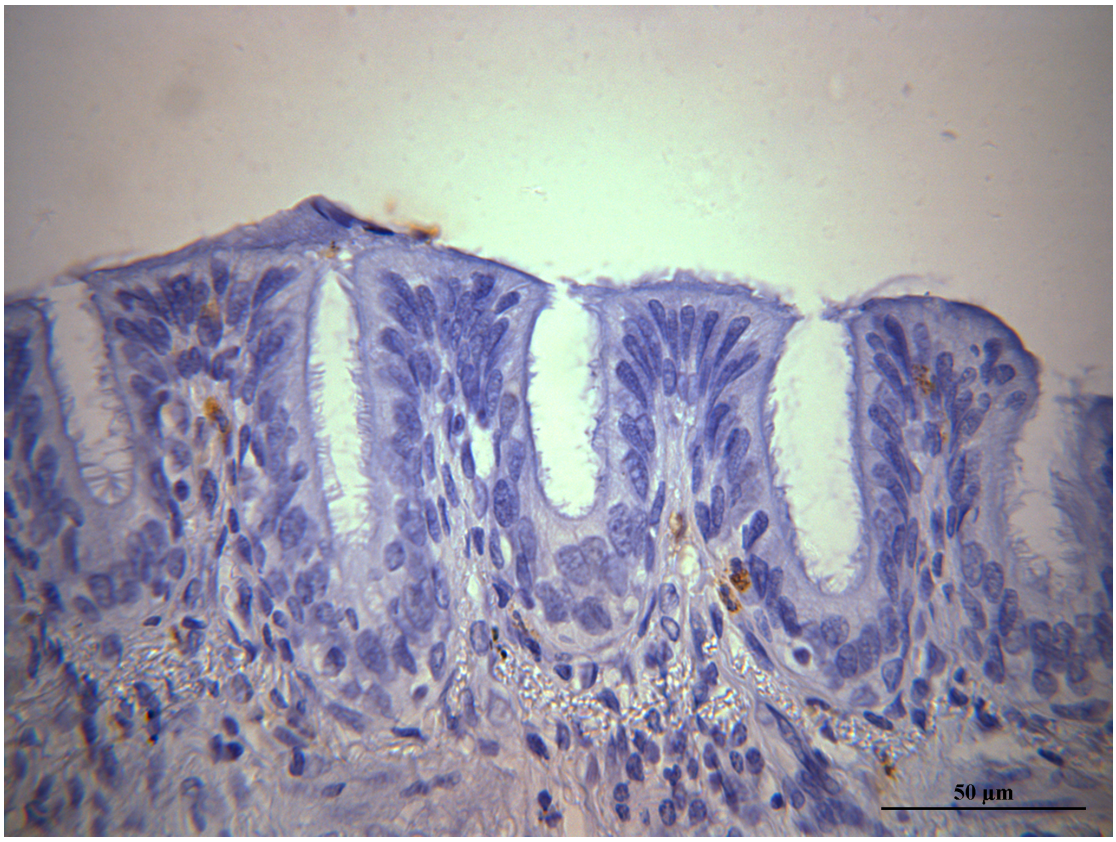
(b)



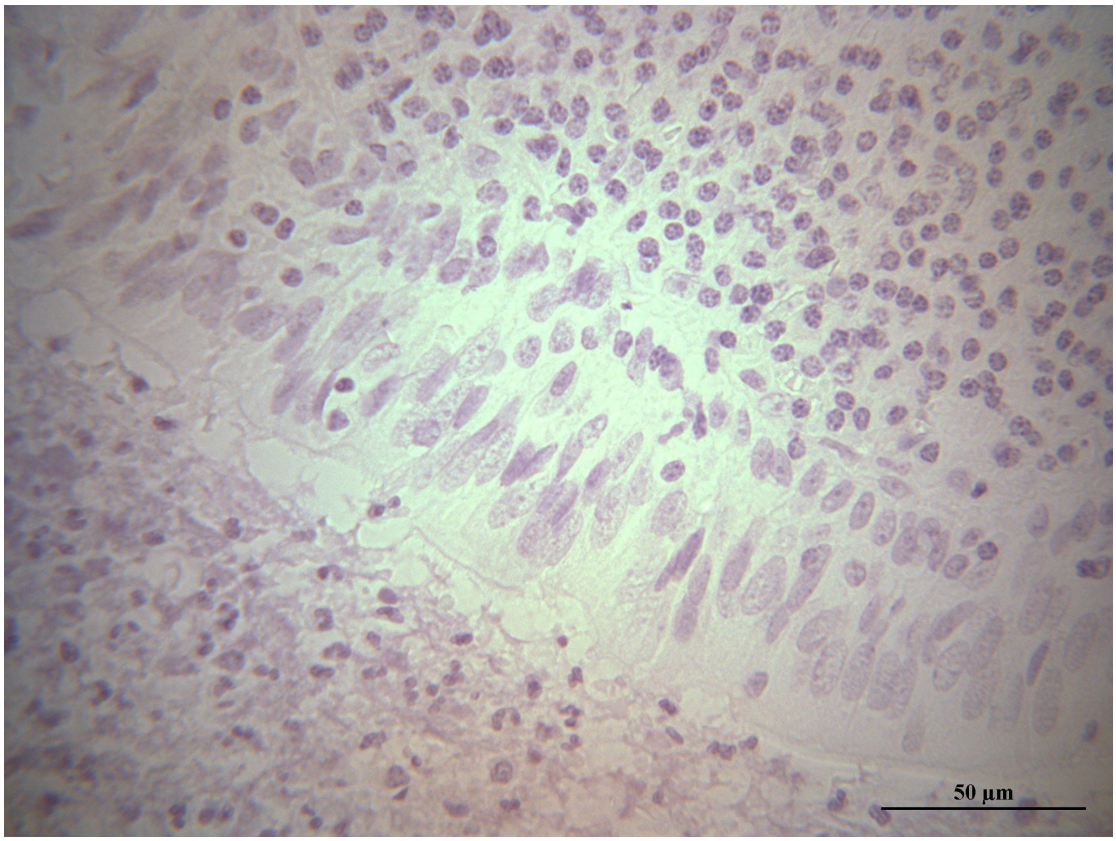
(c)



(d)



(e)



7.5.7. *Immunohistochemistry for myeloperoxidase in the lower airway mucosa in advanced cystic fibrosis lung disease, pulmonary hypertension, emphysema and unused lung donors*

Similarly to neutrophil elastase, I found significantly increased myeloperoxidase positive cells/millimetre basement membrane in people with CF (median 299.3) compared to PH (14.8, *P*=0.0002), emphysema (20.5, *P*=0.0002) and unused lung donors (6.8, *P*=0.0002) (Figure 61). Representative staining for myeloperoxidase in each of the respective groups is shown in Figure 62. A negative isotype control is also shown in Figure 62.

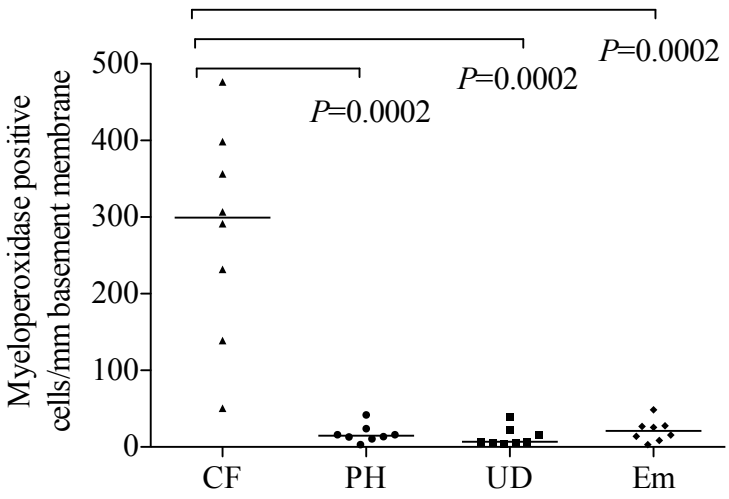
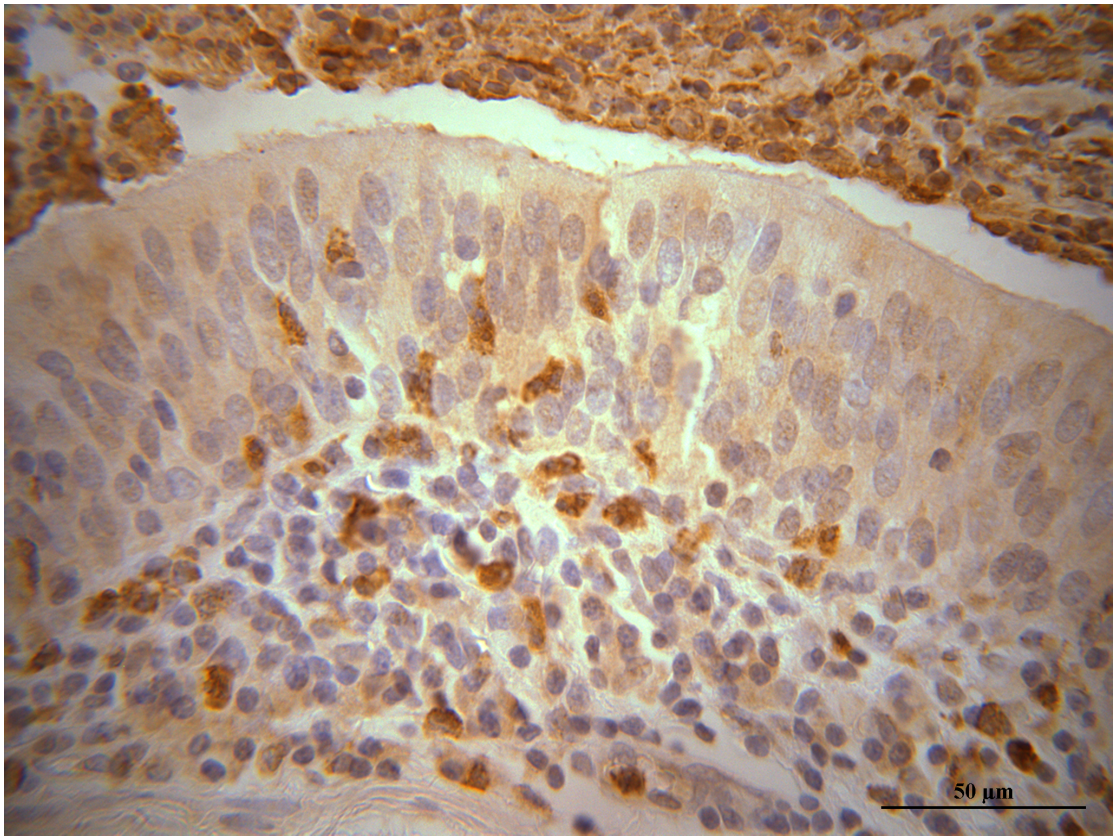


Figure 61. Number of myeloperoxidase positive cells in the lower airway mucosa in advanced cystic fibrosis lung disease, pulmonary hypertension, emphysema and unused lung donors

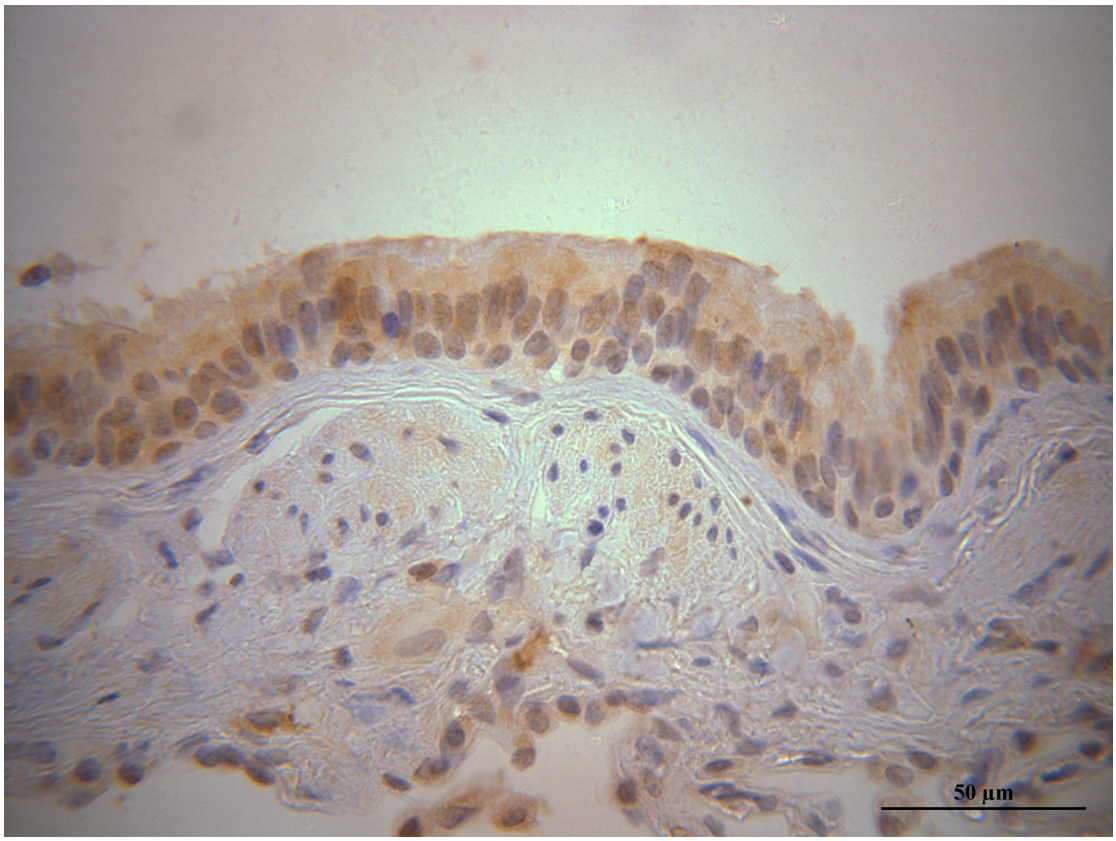
Abbreviations: CF cystic fibrosis, PH pulmonary hypertension, UD unused lung donors and Em emphysema.
Horizontal bars indicate the median for each group.
Mann-Whitney tests, Holm-Bonferonni correction applied. (Mann and Whitney, 1947, Holm, 1979)

Figure 62. Representative staining for myeloperoxidase in the lower airway mucosa in advanced cystic fibrosis lung disease (a), pulmonary hypertension (b), emphysema (c), unused lung donors (d) and rabbit immunoglobulins negative control (e)

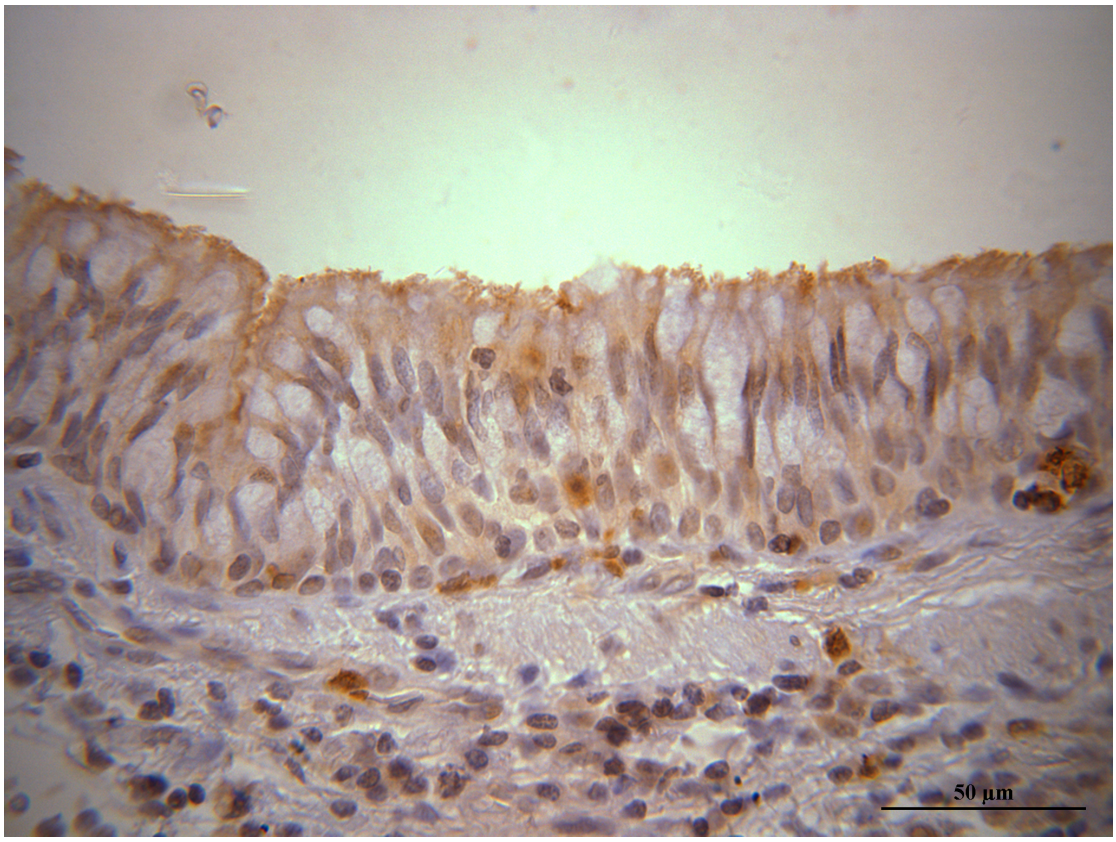
(a)



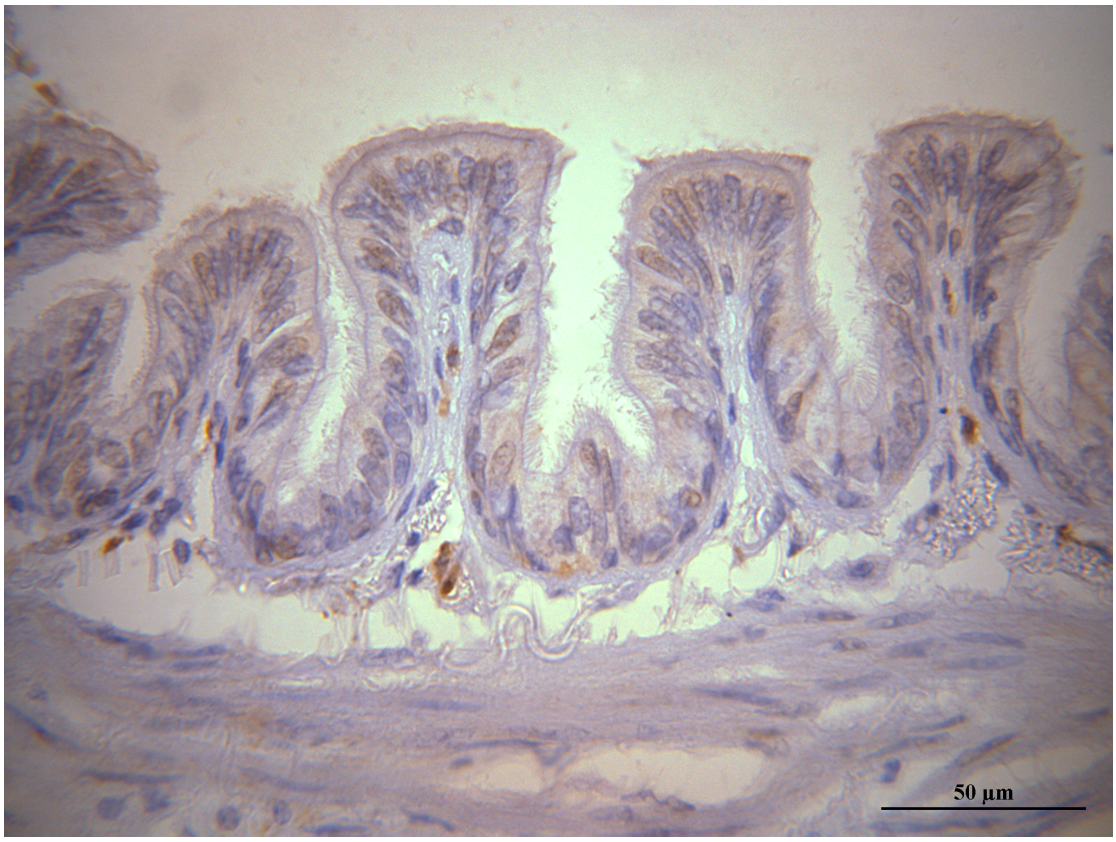
(b)



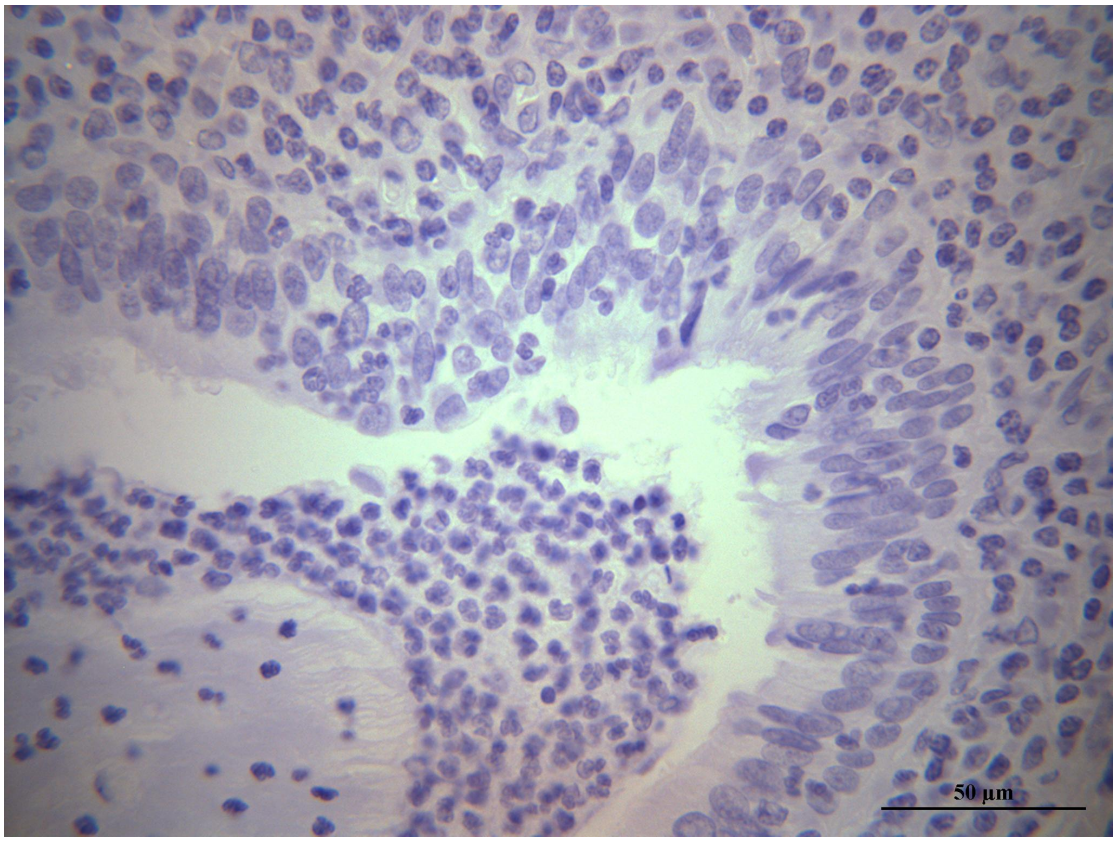
(c)



(d)



(e)



7.5.8. *Correlation between epithelial staining for ceramide and neutrophilic inflammation*

There was a positive correlation between the number of neutrophil elastase positive cells/millimetre basement membrane and percentage of epithelium staining positive for ceramide with the Glycobiotech antibody (Pearson correlation 0.634, $P<0.000$) and the Sigma antibody (Pearson correlation 0.574, $P=0.001$) across all groups (Figure 63 and Figure 64 respectively). Similarly, I found a positive correlation between the number of myeloperoxidase positive cells/millimetre basement membrane and percentage of epithelium staining positive for ceramide with the Glycobiotech antibody (Pearson correlation 0.704, $P<0.000$) and the Sigma antibody (Pearson correlation 0.577 $P=0.001$) across all groups (Figure 65 and Figure 66).

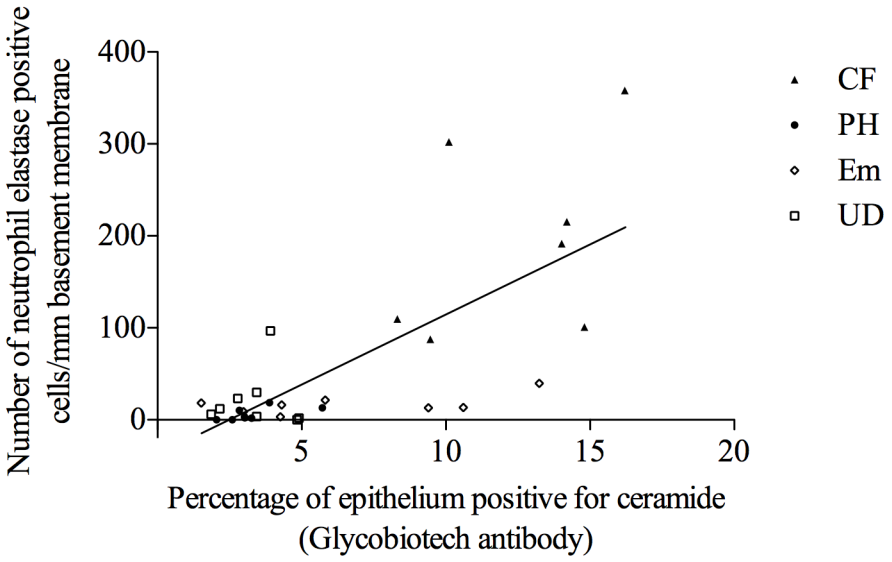


Figure 63. Scatter plot of number of neutrophil elastase positive cells in the lower airway mucosa against percentage of epithelium staining positive for ceramide with Glycobiotech antibody across all groups

Pearson correlation 0.634 (P<0.000)

Abbreviations: CF cystic fibrosis, PH pulmonary hypertension, Em emphysema, UD unused donor

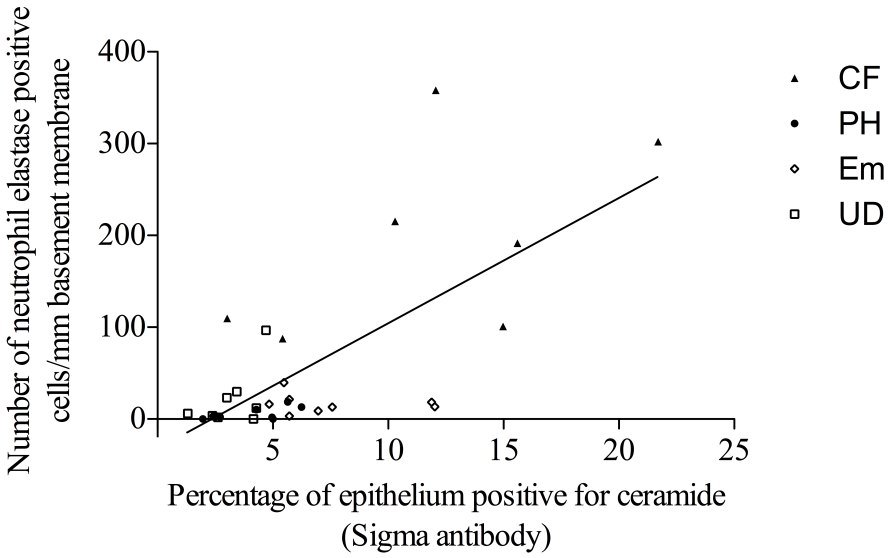


Figure 64. Scatter plot of number of neutrophil elastase positive cells in the lower airway mucosa against percentage of epithelium staining positive for ceramide with Sigma antibody across all groups

Pearson correlation 0.574 (*P*=0.001)

Abbreviations: CF cystic fibrosis, PH pulmonary hypertension, Em emphysema, UD unused donor

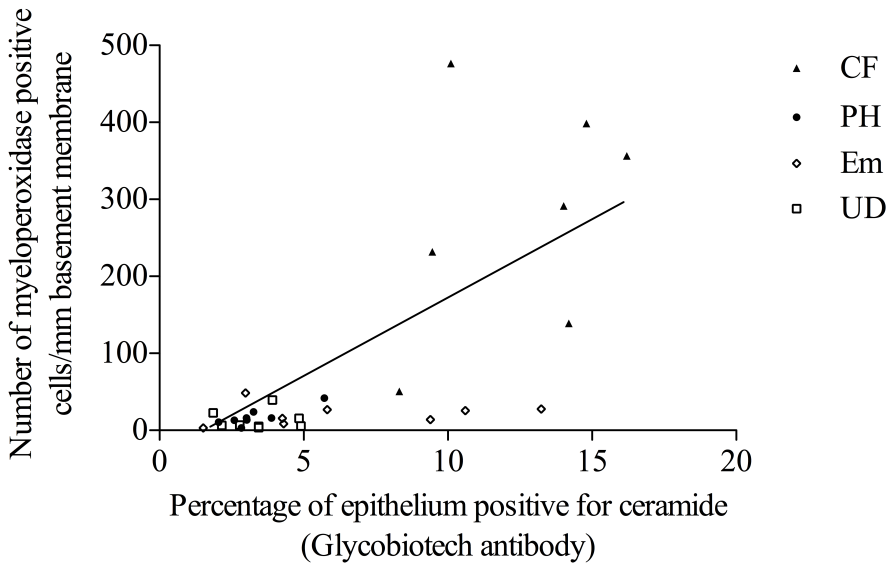


Figure 65. Scatter plot of number of myeloperoxidase positive cells in the lower airway mucosa against percentage of epithelium staining positive for ceramide with Glycobiotech antibody across all groups

Pearson correlation 0.704 (P<0.000)

Abbreviations: CF cystic fibrosis, PH pulmonary hypertension, Em emphysema, UD unused donor

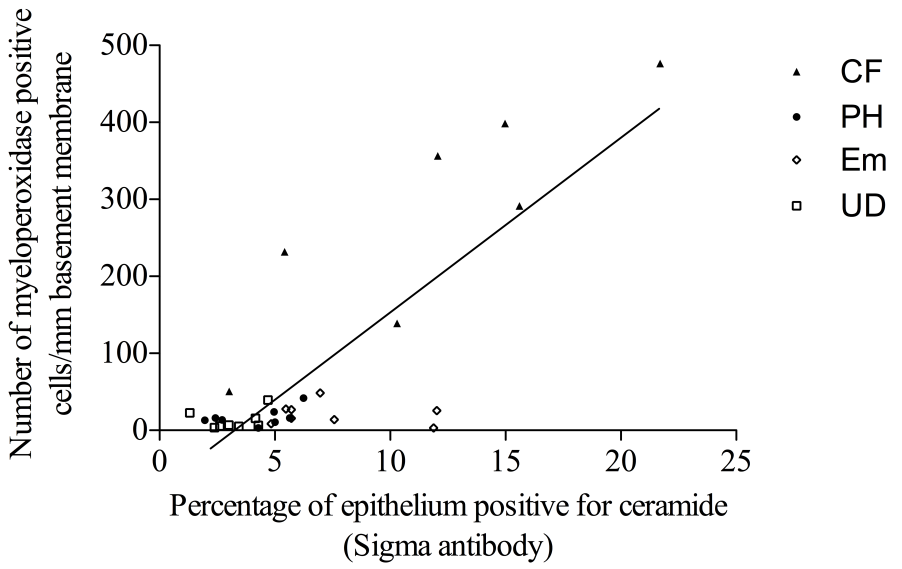


Figure 66. Scatter plot of number of myeloperoxidase positive cells in the lower airway mucosa against percentage of epithelium staining positive for ceramide with Sigma antibody across all groups

Pearson correlation 0.577 (*P*=0.001)

Abbreviations: CF cystic fibrosis, PH pulmonary hypertension, Em emphysema, UD unused donor

7.5.9. *Relationship between epithelial staining for ceramide and colonisation with Pseudomonas aeruginosa*

In view of the finding that ceramide accumulation in CFTR-deficient mice has been shown to increase susceptibility to infection with *P. aeruginosa.* (Teichgraber *et al.*, 2008) I analysed my results for ceramide staining in people with CF, PH and emphysema with regard to the presence or absence of colonisation with *P. aeruginosa*, as judged by standard pre-operative sputum culture (Table 13). (Forrest et al., 2005, Meachery et al., 2008, Corris, 2008) Ceramide staining with the Glycobiotech antibody was significantly increased in lungs colonised with *P. aeruginosa* (median 10.1%) compared to those not colonised across all disease groups (3.1%, P=0.0106) (Figure 67). It was also significantly increased with the monoclonal antibody in lungs colonised with *P. aeruginosa* (median 11.9%) compared to those not colonised (5.3%, P=0.0337) (Figure 68).

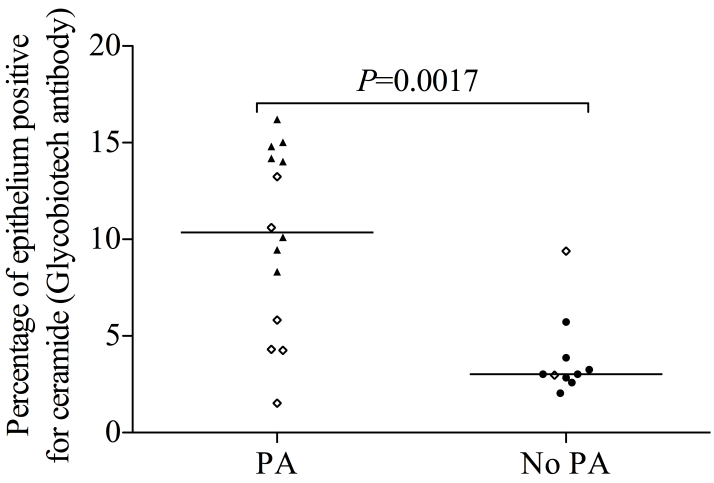


Figure 67. Percentage of epithelium staining positive for ceramide with Glycobiotech antibody in patients colonised with *Pseudomonas aeruginosa* compared to those not colonised across all disease groups

Abbreviations: PA patients colonised with *Pseudomonas aeruginosa*, No PA patients free of *Pseudomonas aeruginosa.*
Legend: ▲ cystic fibrosis, ● pulmonary hypertension, ◇ emphysema
Mann-whitney test comparing each group.
The horizontal lines indicate the median in each group.

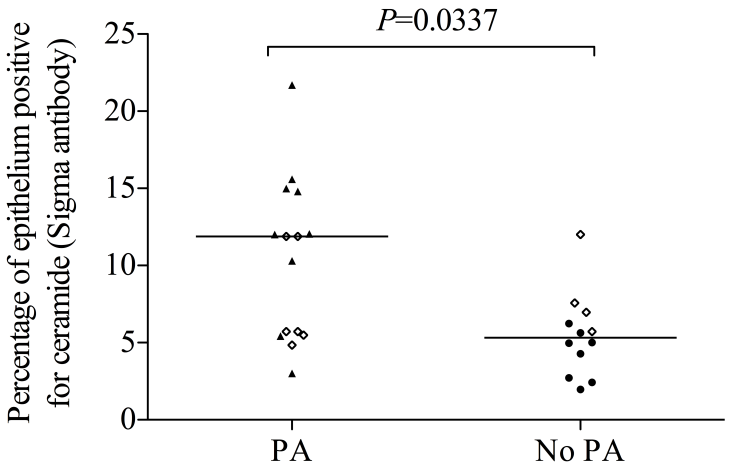


Figure 68. Percentage of epithelium staining positive for ceramide with Sigma antibody in patients colonised with *Pseudomonas aeruginosa* compared to those not colonised across all disease groups

Abbreviations: PA patients colonised with *Pseudomonas aeruginosa*, No PA patients free of *Pseudomonas aeruginosa.*
Legend: ▲ cystic fibrosis, ● pulmonary hypertension, ◇ emphysema
Mann-whitney test comparing each group.
The horizontal lines indicate the median in each group.

7.5.10. *Quantification of specific ceramide species in lung tissue from people with advanced cystic fibrosis lung disease and pulmonary hypertension by high-performance liquid chromatography mass spectrometry*

When considered in more detail ceramides are in reality a family of more than 50 molecularly distinct molecules. (Novgorodov and Gudz, 2009) Each specific ceramide consists of sphingosine and a fatty acid. (Hannun and Obeid, 2008) It is highly probable that specific species of ceramide are of particular importance to disease, as highlighted by the work of Guilbault *et al*. in the plasma of people with CF and by Hamai *et al*. in immortalised epithelial cell lines that express defective CFTR. (Guilbault et al., 2008a, Hamai et al., 2009) I therefore also used the independent technique of HPLC-MS to quantify levels of the specific ceramide species C16:0, C18:0, C20:0 and C22:0 in homogenates of 5 explanted CF lungs and 5 PH lungs. The clinical details of these patients are provided in Table 14.

Age*	Patient number[§]	Transplant†	Diagnosis (CFTR genotype)	Sputum microbiology
15	1	HL	Pulmonary hypertension	Negative
37‡	2	HL	Pulmonary hypertension	Negative
45	3	HL	Pulmonary hypertension	Negative
32	4	HL	Pulmonary hypertension	Negative
32	5	HL	Pulmonary hypertension	Negative
26‡	6	SSL	Cystic fibrosis (Unknown)	*Alcaligines xylosoxidans* *Aspergillus fumigatus*
22	7	SSL	Cystic fibrosis (Unknown)	*Burkholderia multivorans*
25‡	8	SSL	Cystic fibrosis (Unknown)	*Pseudomonas aeruginosa*
28‡	9	SSL	Cystic fibrosis (F508del/F508del)	*Pseudomonas aeruginosa* *Aspergillus fumigatus*
23‡	10	SSL	Cystic fibrosis (F508del/F508del)	*Pseudomonas aeruginosa* *Stenotrophomonas maltophilia*

Table 14. Clinical details of the patients undergoing lung transplantation used for high performance liquid chromatography-mass spectrometry

*Age in years at time of transplant (completed years only). †SSL: bilateral single sequential lung transplant, HL: heart-lung transplant. ‡Patient also used for immunohistochemistry (Table 13). [§]See Table 16 and Table 17.

7.5.11. *Generation of calibration curves for ceramides C16:0, C18:0, C20:0 and C22:0*

Calibration curves were constructed from triplicate readings of a mixture of standards of the 4 ceramides at 5, 0.5, 0.05 and 0.005 pmoles. Table 15 shows the calibration data for each of the specific ceramides. Figures 16 to 19 show the calibration curves for ceramides C16:0, C18:0, C20:0 and C22:0 respectively.

Concentration (pmoles)	Peak area under curves			
	C16:0	**C18:0**	**C20:0**	**C22:0**
5	321454794	338757912	386878421	461716911
5	309895960	394520533	394408476	394636261
5	337470071	391280778	378286076	385015360
0.5	50499758	56725146	80340968	81024680
0.5	53036558	68302685	81008837	77468080
0.5	59397105	76549954	75690065	75153958
0.05	5169044	5194721	8324085	7497218
0.05	5453865	7215912	8551650	8618399
0.05	6695481	8077005	7970736	8654490
0.005	256436	324888	464547	279308
0.005	378237	448890	510001	414620
0.005	463414	516844	535174	440815

Table 15. Calibration data for high performance liquid chromatography-mass spectrometry ceramide standards

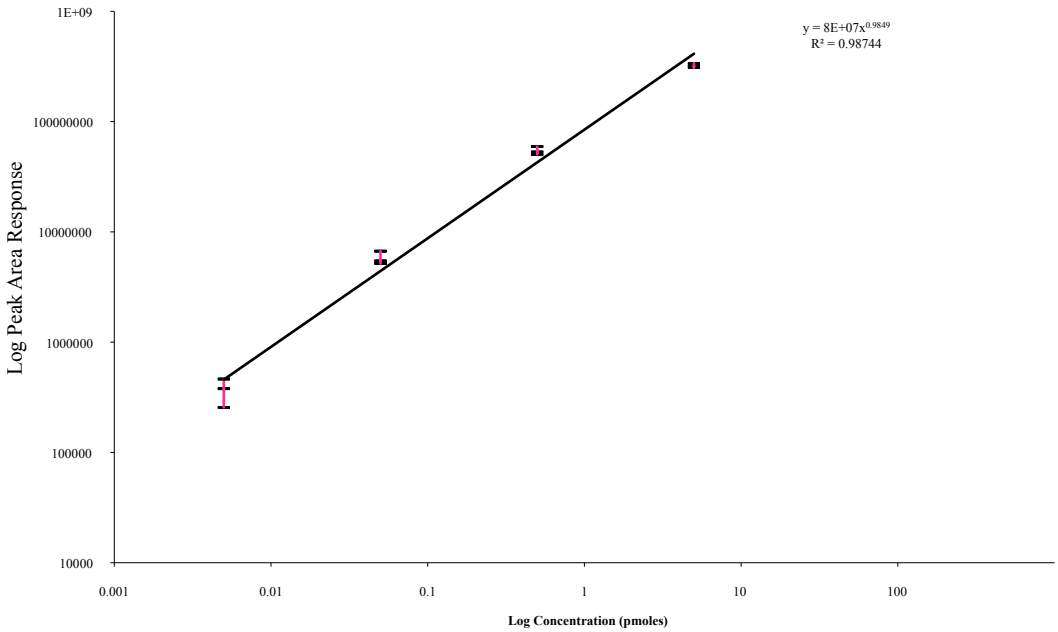


Figure 69. Calibration plot for ceramide C16:0 standard

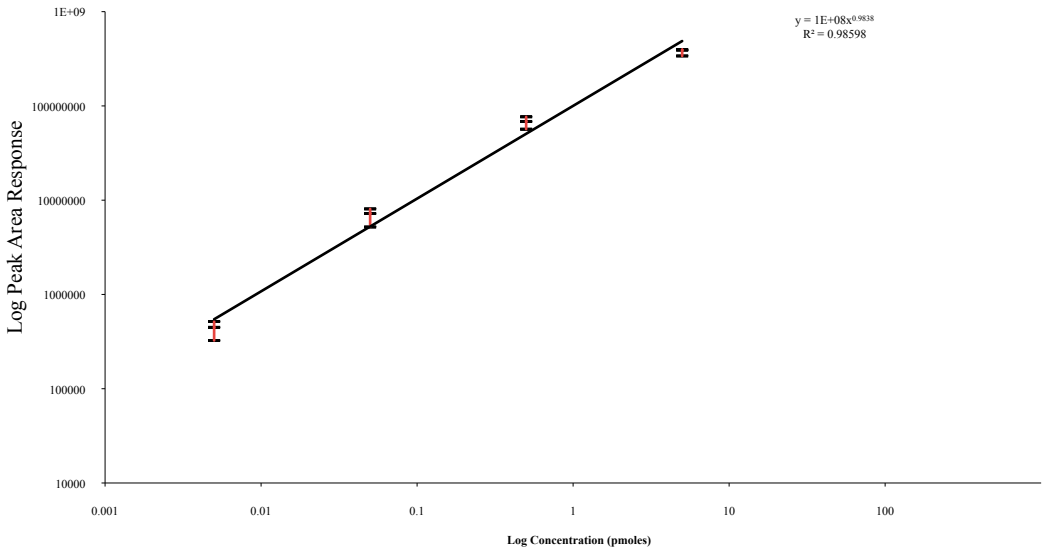


Figure 70. Calibration plot for ceramide C18:0 standard

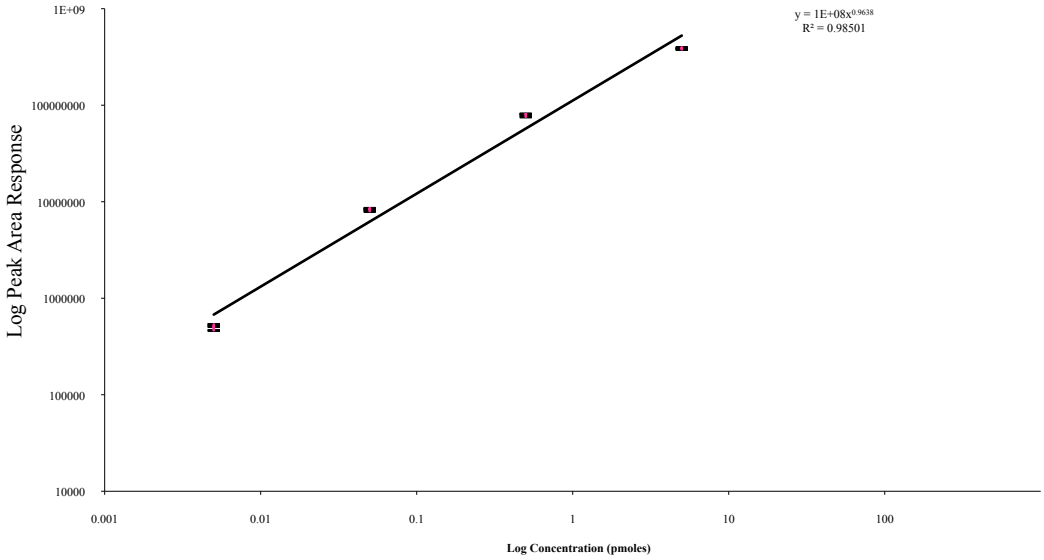


Figure 71. Calibration plot for ceramide C20:0 standard

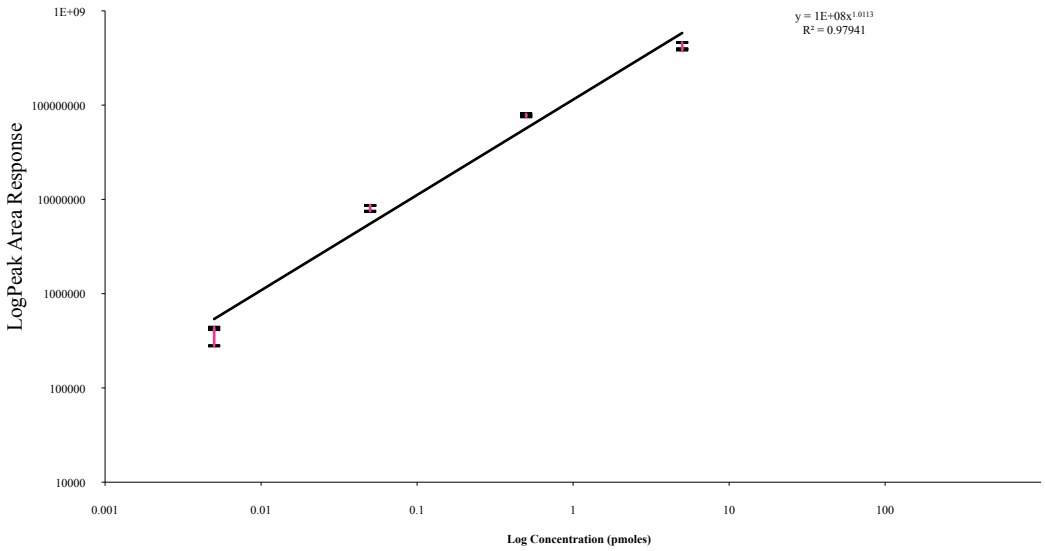


Figure 72. Calibration plot for ceramide C22:0 standard

7.5.12. *Analysis of homogenates of explanted cystic fibrosis and pulmonary hypertension lungs*

7.5.13. *Amount of ceramide per mass of wet tissue*

Figure 73 to Figure 76 show the amounts of C16:0, C18:0, C20:0 and C22:0 respectively measured in each lung per mass of wet tissue. The median levels were significantly increased in the CF lungs compared to PH for C16:0 (10.01 and 2.92 pmoles/mg, P=0.0216), C18:0 (1.09 and 0.31 pmoles/mg, P=0.0367) and C20:0 (0.70 and 0.17 pmoles/mg, P=0.0216) ceramides. The difference was not significant for C22:0 (2.90 and 1.82pmoles/mg, P=0.1437).

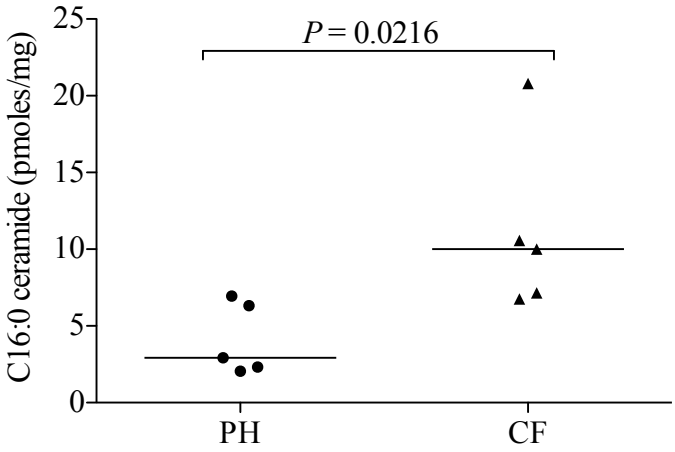


Figure 73. Amount of C16:0 ceramide per mass of wet tissue in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar.
Abbreviations: PH pulmonary hypertension, CF cystic fibrosis.
Mann-whitney test.

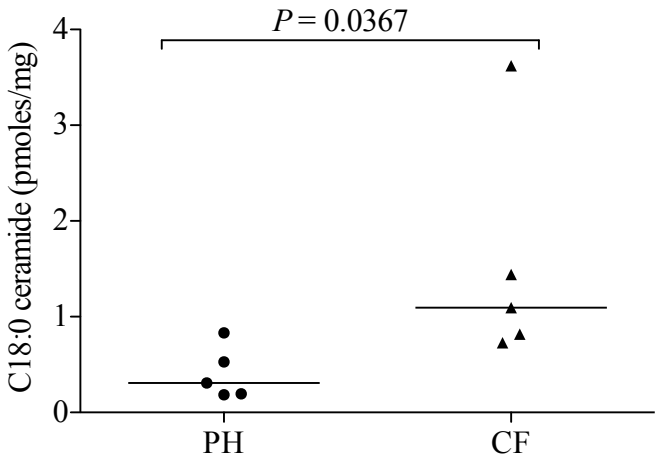


Figure 74. Amount of C18:0 ceramide per mass of wet tissue in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar.
Abbreviations: PH pulmonary hypertension, CF cystic fibrosis
Mann-whitney test.

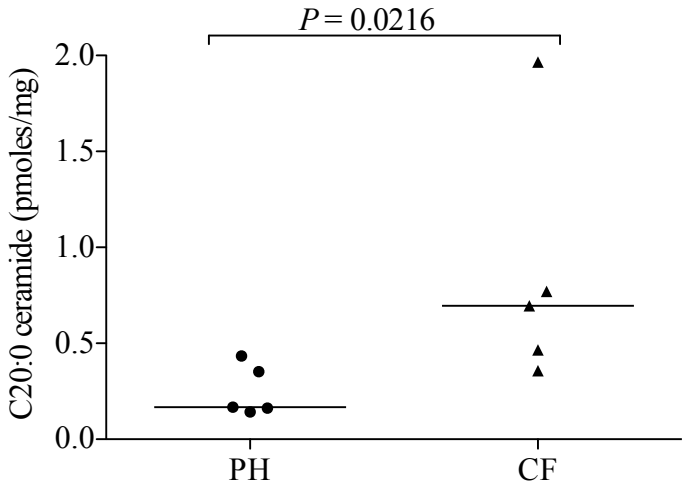


Figure 75. Amount of C20:0 ceramide per mass of wet tissue in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar.
Abbreviations: PH pulmonary hypertension, CF cystic fibrosis
Mann-whitney test.

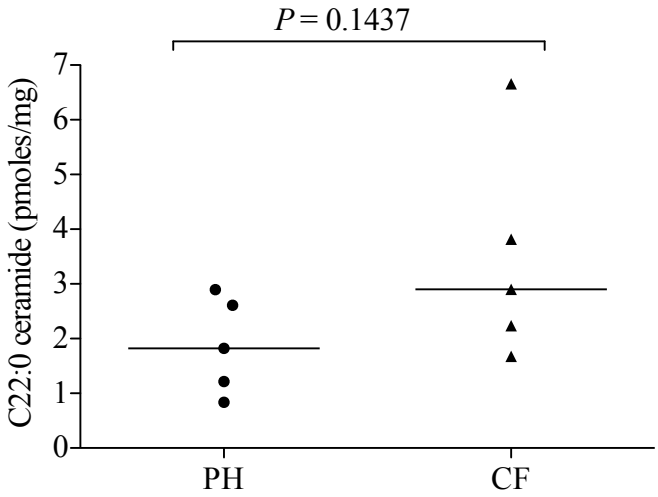


Figure 76. Amount of C22:0 ceramide per mass of wet tissue in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar.
Abbreviations: PH pulmonary hypertension, CF cystic fibrosis.
Mann-whitney test.

7.5.14. *Amount of ceramide per micromole of phosphate*

I performed a further analysis using the concentration of phosphate in the lung homogenate as the denominator. The phosphate levels were quantified using the PiBlue assay described in Chapter 4. The standard curve for the assay is shown in Figure 77. The concentrations of phosphate in each homogenate are presented in Table 16. The data for the individual patients is displayed for ceramides C16:0, C18:0, C20:0 and C22:0 in Figure 78 to Figure 81 respectively. Despite trends towards increased levels in CF compared to PH with each ceramide species none of these differences were statistically significant (Figure 78 to Figure 81).

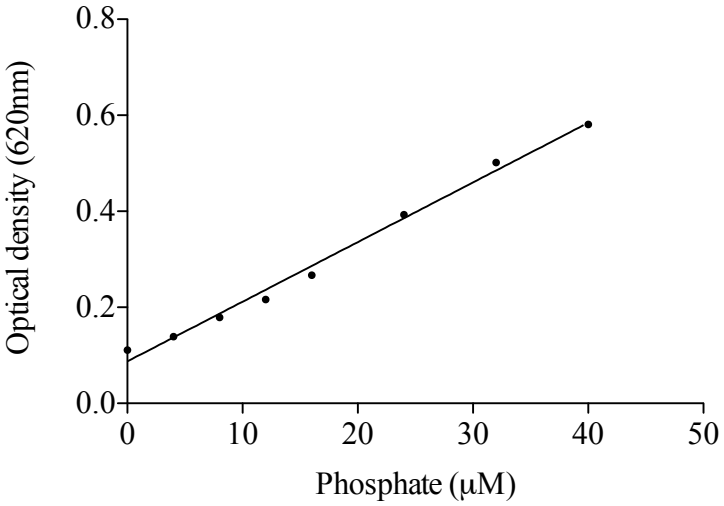


Figure 77. Phosphate assay standard curve

Each symbol represents the mean of triplicate measurements

Patient number	Phosphate* (μM)
1	7.59
2	11.98
3	6.86
4	9.52
5	8.27
6	9.00
7	5.90
8	4.97
9	38.93
10	6.20

Table 16. Phosphate concentration for each lung homogenate

*mean of triplicate measurements

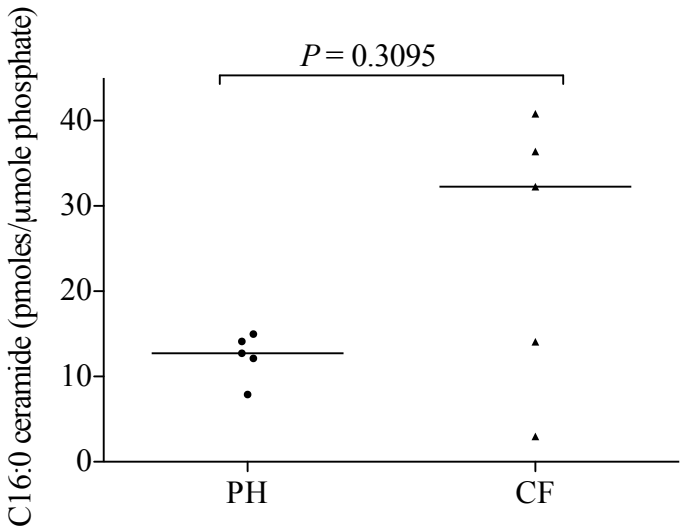


Figure 78. Amount of C16:0 ceramide per micromole of phosphate in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar. Abbreviations: PH pulmonary hypertension, CF cystic fibrosis

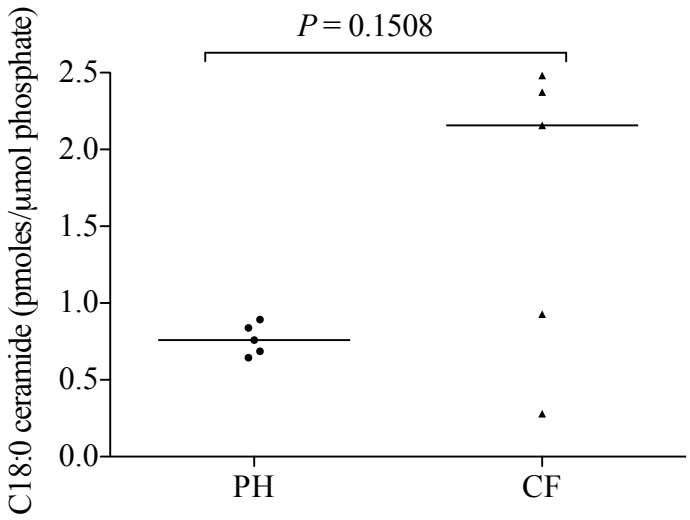


Figure 79. Amount of C18:0 ceramide per micromole of phosphate in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar. Abbreviations: PH pulmonary hypertension, CF cystic fibrosis

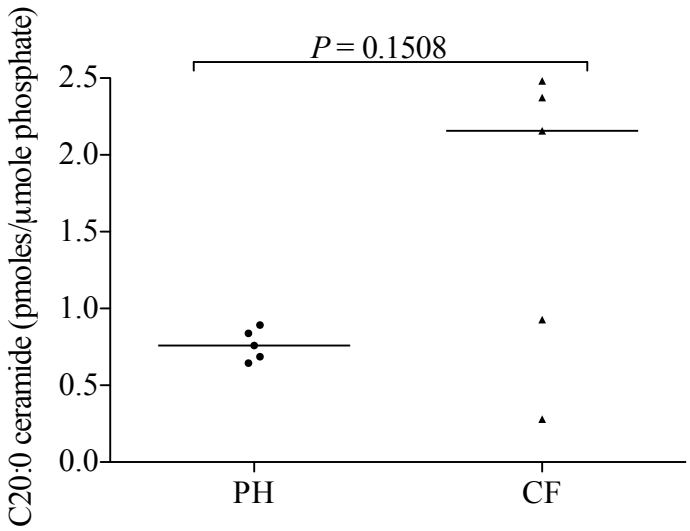


Figure 80. Amount of C20:0 ceramide per micromole of phosphate in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar. Abbreviations: PH pulmonary hypertension, CF cystic fibrosis

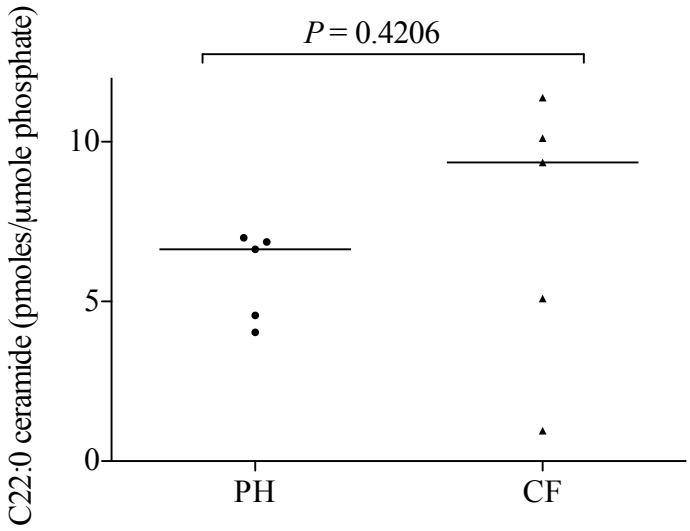


Figure 81. Amount of C22:0 ceramide per micromole of phosphate in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar. Abbreviations: PH pulmonary hypertension, CF cystic fibrosis

7.5.15. *Amount of ceramide per microgram of protein*

I performed a further analysis using the concentration of protein in the lung homogenate as the denominator. The protein levels were quantified spectroscopically using a Bradford protein assay as described in Chapter 4. The standard curve for the assay is shown in Figure 82. The concentrations of protein in each homogenate are presented in Table 17. The data for ceramides C16:0, C18:0, C20:0 and C22:0 per microgram of protein in each patient are displayed in Figure 83 to Figure 86 respectively. Despite trends towards increased levels in CF compared to PH with each ceramide species none this was only statistically significant in the case of C18:0.

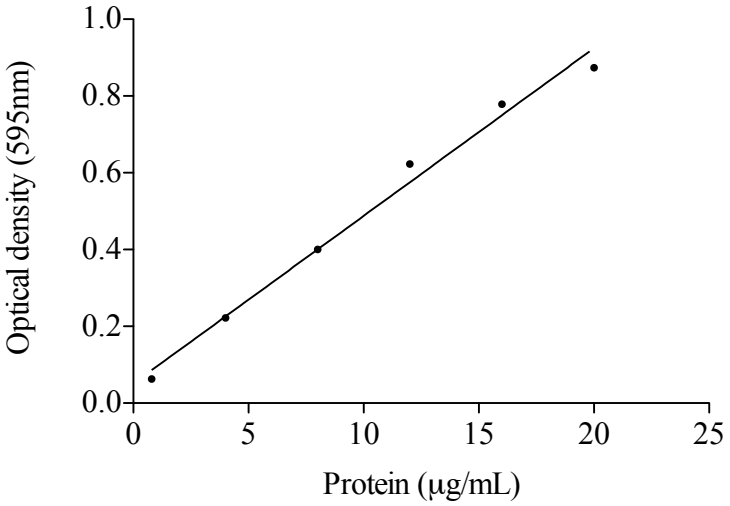


Figure 82. Protein assay standard curve

Each symbol represents the mean of triplicate assays.

Patient number	Protein* (μg/mL)
1	6.46
2	10.49
3	11.45
4	29.16
5	13.87
6	12.86
7	9.48
8	13.28
9	12.86
10	10.21

Table 17. Protein concentration for each lung homogenate

*mean of triplicate measurements

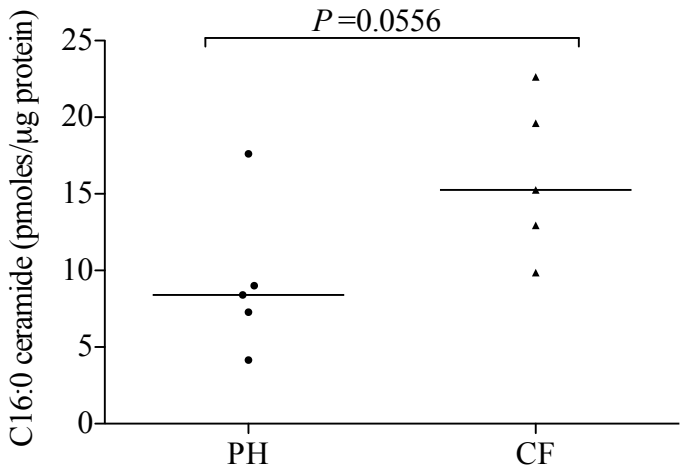


Figure 83. Amount of C16:0 ceramide per microgram of protein in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar. Abbreviations: PH pulmonary hypertension, CF cystic fibrosis

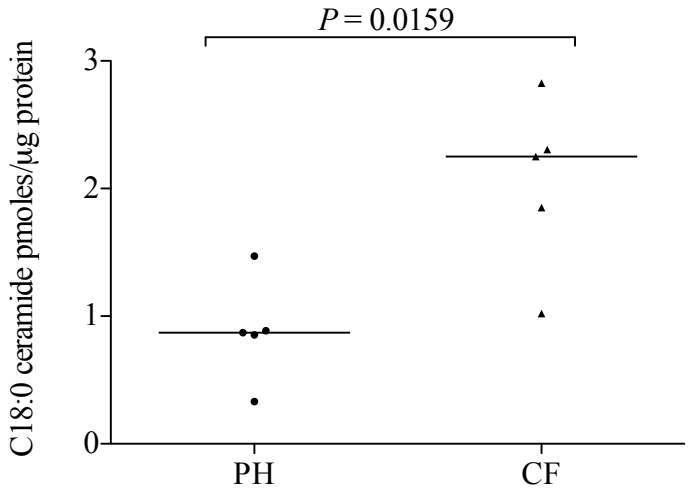


Figure 84. Amount of C18:0 ceramide per microgram of protein in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar. Abbreviations: PH pulmonary hypertension, CF cystic fibrosis

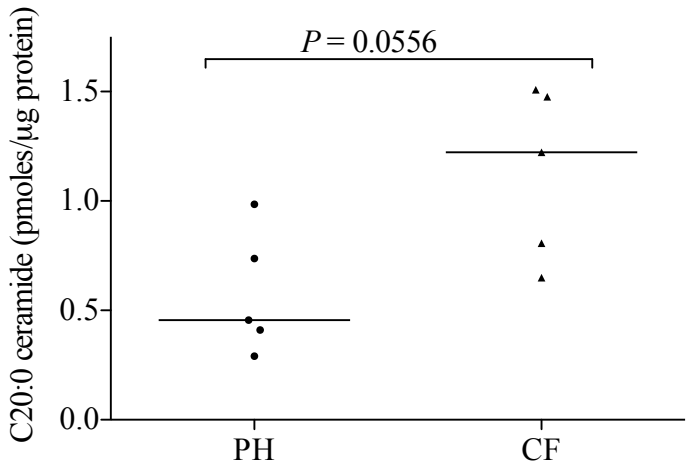


Figure 85. Amount of C20:0 ceramide per microgram of protein in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar. Abbreviations: PH pulmonary hypertension, CF cystic fibrosis

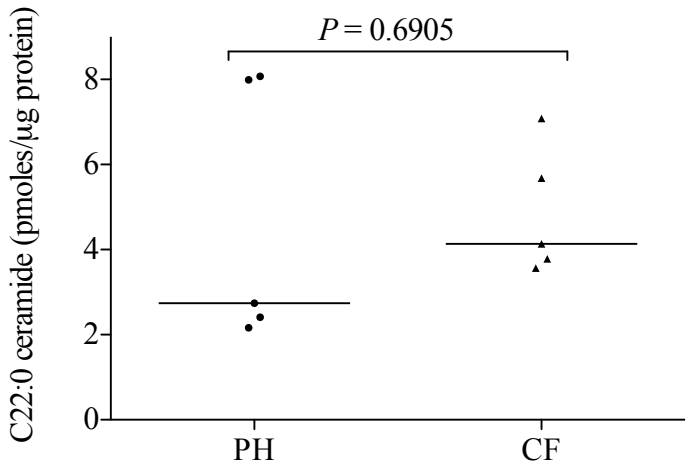


Figure 86. Amount of C22:0 ceramide per microgram of protein in the parenchyma of explanted cystic fibrosis and pulmonary hypertension lungs

Each symbol represents an individual patient and is the mean of three independent measurements. The median of each group is represented by a horizontal bar. Abbreviations: PH pulmonary hypertension, CF cystic fibrosis

7.6. Discussion

The data presented in this chapter provides the first quantitative immunohistochemical evidence to support the hypothesis that ceramide accumulates in the lower airway epithelial cells of people with CF, agreeing with previous descriptive data. (Teichgraber *et al.*, 2008) It was of some interest that I found increased ceramide staining in emphysematous lungs compared to PH and unused lung donors, although this was at lower levels than in the people with CF. This is in keeping with previous work that has identified ceramide as a mediator in the development of emphysema, thought to act by inducing oxidative stress and apoptosis of alveolar endothelial and epithelial cells. (Petrache et al., 2005, Uhlig and Gulbins, 2008, Petrache et al., 2008)

The precise role of epithelial ceramide accumulation in the pathogenesis of CF lung disease is yet to be fully elucidated. The work performed by Teichgräber *et al.* (2008) in mice suggests that ceramide accumulation occurs in a constitutive, age-dependent fashion in the airway epithelial cells of CFTR-deficient animals and promotes pro-inflammatory cytokine release, apoptosis, DNA deposition in the airway and susceptibility to *P. aeruginosa* infection. It has previously been elegantly demonstrated that ceramide-enriched membrane platforms are central to epithelial defence against *P. aeruginosa*. (Grassme et al., 2003, Nieuwenhuis et al., 2002) Physiological levels of ceramide appear to be essential for the homeostasis of cells and inflammatory responses and therefore it is plausible that too little ceramide may be equally as deleterious to epithelial physiology as a situation where ceramide accumulation occurs. (Guilbault *et al.*, 2008a)

A proposed mechanism for ceramide accumulation is that defective CFTR leads to inappropriate alkalinisation of intracellular vesicles, in particular pre-lysosomes and lysosomes, resulting in an increase in acid sphingomyelase and reduction in acid ceramidase activity and subsequent ceramide accumulation. (Teichgraber *et al.*, 2008) However, the role of CFTR in the acidification of lysosomes has been hotly debated recently with apparently contradictory results published by different investigators. (Haggie and Verkman, 2009a, Haggie and Verkman, 2009b, Di et al., 2006, Teichgraber et al., 2008, Poschet et al., 2002, Noe et al., 2009)

I found that staining for ceramide was not significantly increased in the lower airway epithelium in CF compared to end-stage emphysema using the Sigma monoclonal antibody. This may make a defective CFTR-specific mechanism for the accumulation of ceramide as a sole explanation, less likely. I would however emphasise that low numbers were used in this study and that it was not designed to investigate mechanisms of ceramide dysregulation.

I found a positive association between the number of cells staining for the neutrophil products neutrophil elastase and myeloperoxidase in the airway and epithelial ceramide staining across all patients. This suggests a potential link between epithelial ceramide levels and neutrophilic inflammation in humans similar to that proposed in mice. (Teichgraber *et al.*, 2008) In the lungs of CFTR-deficient mice Teichgräber *et al.* found a constitutive increase in the expression of IL-1 and keratinocyte-derived chemokine (KC), the mouse homolog of human IL-8. (Teichgraber *et al.*, 2008) Inhibition of acid sphingomyelinase activity normalised levels of IL-1 and KC suggesting that cytokine upregulation is linked to ceramide accumulation. It is known that children with CF who have *P. aeruginosa* infecting their lower airway have higher levels of IL-8, neutrophils and neutrophil elastase in bronchoalveloar lavage fluid compared to children free of lower respiratory tract *P. aeruginosa* infection. (Sagel *et al.*, 2009)

In contrast to my results and those of Teichgräber *et al.*, Guilbault *et al.* found low plasma levels of ceramide in people with CF compared to healthy volunteers and similarly low levels in the plasma and organs of CFTR-deficient mice that could be corrected by treatment with fenretinide. (Guilbault et al., 2008b, Guilbault et al., 2008a, Teichgraber et al., 2008) Specifically, Guilbault *et al.* found decreased levels of several ceramide sphingolipid species in the plasma of people with CF, namely C14:0, C20:1, C22:0, C22:1, C24:0 and dihydroxy ceramide. (Guilbault *et al.*, 2008a) One explanation proposed by the Gulbins group for the apparently discrepant results in mice is that CFTR-deficient strains that require high fat feeds, such as Peptamen®, develop artificially high cholesterol levels that subsequently reduce acid sphingomyelinase activity. (Becker et al., 2010b, Pier, 2008, Teichgraber et al., 2008) However, Guilbault *et al.* did not find low levels of ceramide in the plasma or lungs of CFTR-deficient mice after prolonged ingestion of Peptamen®. (Guilbault *et al.*, 2008b)

The prospective focus for my study was to evaluate levels of ceramide specifically in the lower airway epithelium of people with CF. As outlined in the introduction of this chapter, I did this because it is the site of the major pathology in CF lung disease. (Hamutcu *et al.*, 2002) By using immunohistochemistry coupled with image analysis, quantification of ceramide was possible along with architectural resolution to the specific tissue compartment of interest, in human patients with CF. Correlations with neutrophil numbers, also determined by immunohistochemistry were also possible, which is potentially important given the salient importance of neutrophilic inflammation to the disease. (Downey *et al.*, 2009) To strengthen my approach, 2 antibodies were used in the study.

To confirm my findings and to allow quantification of specific ceramide species, namely C16:0, C18:0, C20:0 and C22:0, I used the independent technique of HPLC-MS on lung tissue from people with CF. Clearly analysis of whole lung homogenates using HPLC-MS does not allow the localisation to specific tissue compartments of interest in the lung, however. A number of other ceramide species, for example C24:0 and C24:1, are known to be biologically important and abundant. (Haus *et al.*, 2009) It may be that specific species are of particular importance to disease, as highlighted by the work of Guilbault *et al*. in the plasma of people with CF and by Hamai *et al*. in immortalised epithelial cell lines that express defective CFTR. (Guilbault et al., 2008a, Hamai et al., 2009) The HPLC-MS method used in this study was not designed or optimised to characterise all species of ceramide, and I was therefore unable to measure the abundance of all potentially relevant ceramides in the lungs of people with advanced CF lung disease. This underlines a need for further translational studies in this area of research.

A major strength of my work is that it involved human tissue, dissected directly from the complex milieu present in the CF lung. (Brodlie et al., 2010f) The correlation of findings in cellular or animal work to the actual human disease has been stated as vital. (Doring *et al.*, 2007) Given the discrepant results regarding ceramide obtained in different models and compartments of CF disease, I feel that the additional human airway data provided by this study is a useful contribution to the current debate, offering a translational perspective to compliment the available science.

The number of individuals included in this study was modest. This unfortunately precludes analysis relating to CFTR genotype. Lung transplantation is an intervention for end-stage disease and therefore the span of ages was also insufficient to address the important question of whether or not ceramide accumulates in an age-dependent manner in people with CF, as has been described in CFTR-deficient mice. (Teichgraber *et al.*, 2008)

At a basic level I was able to examine the level of ceramide staining in people with CF, PH and emphysema with consideration of the presence or absence of colonisation with *P. aeruginosa*. The finding that ceramide staining was increased in those colonised with *P. aeruginosa* concurs with the work of Teichgräber *et al.* in mice and suggests a putative link between epithelial ceramide accumulation and *P. aeruginosa* infection. (Teichgraber *et al.*, 2008) Importantly however, *P. aeruginosa* colonisation is frequent in people with end-stage CF lung disease and indeed was universal in my study cohort. There was also a high prevalence of colonisation with *P. aeruginosa* in people with emphysema in this study. This is likely to reflect the severity of disease present in those who come forward for lung transplantation. (Murphy *et al.*, 2008c) A larger sample size would be required to thoroughly evaluate possible inter-relationships between specific microbial infections and epithelial ceramide accumulation. Appropriately designed animal or cellular studies may also be of value in this area. Yu *et al.* have recently shown that in S9 immortalised human airway epithelial cells and the lungs of wild type mice there is a significant increase in acid sphingomyelinase and total ceramide levels 6 hours after infection with *P. aeruginosa*. (Yu *et al.*, 2009) This increase did not occur in the CF bronchial epithelial cell line IB3-1 or the lungs of CFTR knock out mice. (Yu *et al.*, 2009)

At a translational level my findings add weight to the suggestion that the accumulation of ceramide is a potential target for pharmacotherapy. Amitriptyline, along with a number of other agents, degrades acid sphingomyelinase, thereby reducing ceramide production, and has been shown to almost normalise ceramide levels and susceptibility to *P. aeruginosa* infection in CFTR-deficient mice. (Becker et al., 2010b, Teichgraber et al., 2008) The efficacy of amitriptyline in humans with CF is yet to be objectively evaluated however, along with potential safety issues relating to side effects and possible negative consequences for epithelial defence to infection if ceramide production is over-inhibited. (Becker et al., 2010b, Pier, 2008) This emphasises the need

for a multi-disciplinary approach to further study, and the importance of appropriate translational studies in patients.

8. Chapter 8. Reticular basement membrane thickness in advanced cystic fibrosis lung disease

Elements of the work described in this chapter have been previously reported in the form of a presentation at a medical and scientific meeting. (Brodlie *et al.*, 2009e)

8.1. Abstract

Background: Cystic fibrosis lung disease is characterised by airflow obstruction, neutrophilic inflammation and chronic endobronchial infection. Apparent thickening of the sub-epithelial reticular basement membrane due to collagen matrix deposition is a characteristic feature of airway remodelling seen in people with asthma. Airway remodelling has also been implicated in the pathogenesis of cystic fibrosis lung disease and basement membrane thickening has been reported in endobronchial biopsies from children with cystic fibrosis. Changes in airway dimension have been described in explanted cystic fibrosis lungs but basement membrane thickness has not been thoroughly investigated in end-stage disease with divergent findings in limited studies to date. The primary objective of this study was to quantify RBM thickness in the airways of people with CF requiring lung transplantation. In the context of asthma, eosinophilic inflammation has been implicated in the pathogenesis of airway remodelling *via* the maintenance and progression of aberrant airway tissue injury and repair. I therefore also investigated any relationship between number of airway mucosa eosinophils and basement membrane thickness. Finally, it has been suggested that basement membrane thickness may increase with age and I therefore investigated any correlation between basement membrane thickness and age at time of transplant.

Methods: Blocks of intermediate-large airway were dissected from the explanted lungs of 16 people with cystic fibrosis, median age 28 years [14.7 - 57.7], and fixed in 10% formalin and embedded in paraffin before 5μm sections were stained with haematoxylin and eosin. Basement membrane thickness was evaluated objectively by measuring the distance between two demarcating lines in 5 high-power fields using image analysis software. Comparison was made with basement membrane thickness measured in an earlier study of 22 healthy subjects and previously published normal measurements.

Results: Median basement membrane thickness in the cystic fibrosis airways was 9.58μm [range 7.15 - 13.56]. This compares to 7.7μm [5.2 - 9.6] in healthy subjects in the earlier study by Ward *et al.* (2002) (P=0.0008). Other published normal measurements for basement membrane thickness in formalin fixed tissue range from 2.9 to 6.7μm. There was no apparent relationship between basement membrane thickness and number of mucosal eosinophils or age at time of transplant.

Conclusion: Reticular basement membrane thickness is significantly increased in advanced cystic fibrosis lung disease. This agrees with results in children and provides evidence of airway remodelling in advanced disease. In this dataset basement membrane thickness appears to be independent of the number of mucosal eosinophils and age.

8.2. Introduction

As outlined earlier in this thesis, lung disease accounts for over 95% of morbidity and mortality in people with CF. (O'Sullivan and Freedman, 2009) CF lung disease is characterised by airflow obstruction, neutrophilic inflammation and chronic endobronchial infection. (Davies *et al.*, 2007) Children with CF are born with airways of essentially normal structure however. (Konstan and Berger, 1997)

Airway remodelling, in the form of architectural changes in the airway wall, is well recognised in people with asthma and chronic obstructive pulmonary disease (COPD). (Jeffery, 2001, Davies, 2009) One specific feature of airway remodelling seen in adults and children with asthma is apparent thickening of the sub-epithelial reticular basement membrane (RBM) due to collagen matrix deposition. (Ward et al., 2002, Payne et al., 2003) It has also been postulated that airway remodelling may be implicated in the pathogenesis of CF lung disease and RBM thickening has been reported in endobronchial biopsies from children with CF. (Hilliard *et al.*, 2007) Clinical evidence of structural airway remodelling at an early stage in CF lung disease includes the observation that airway function is demonstrably abnormal in infants newly diagnosed clinically with CF. (Ranganathan *et al.*, 2001) Furthermore, their lung function does not improve despite intensive treatment, and the airway obstruction would appear to be 'fixed'. (Ranganathan *et al.*, 2004)

However, in an earlier study Durieu *et al.* examined RBM thickness in a mixture of endobronchial and lobectomy specimens from a total of 9 children and adults with a spectrum of severity of CF lung disease. (Durieu *et al.*, 1998) Durieu *et al*. assessed the basal lamina thickness of the basement membrane qualitatively using immunostaining for murine laminin in fresh frozen sections and transmission electron microscopy. In contrast to the findings of Hilliard *et al.*, they found the RBM to be thinned in the CF specimens compared to 3 healthy, non-smoking volunteers. (Hilliard et al., 2007, Durieu et al., 1998) A dense, fibrous, acellular sub-epithelial deposit was observed however. (Durieu *et al.*, 1998) Wojnarowski *et al.* found the RBM to be thickened in association with a metaplastic epithelium in endobronchial biopsies taken from children during an acute exacerbation of CF. (Wojnarowski *et al.*, 1999) In this study the biopsies were fixed in paraformaldehyde and embedded in paraffin prior to

haematoxylin and eosin staining of 2μm sections. Biopsies from clinically stable children with CF were found to contain an intact respiratory epithelium with normal RBM appearances. (Wojnarowski *et al.*, 1999) Changes in airway dimension have been described in explanted CF lungs but RBM thickness has not been specifically investigated in end-stage disease to the best of my knowledge. (Tiddens *et al.*, 2000)

Eosinophilic inflammation has been implicated in the pathogenesis of airway remodelling in asthma *via* the maintenance and progression of aberrant airway tissue injury and repair. (Holgate and Polosa, 2008) The biological activity of eosinophils is largely mediated by the release of stored granules containing potent cytotoxic proteins such as eosinophilic cationic protein, eosinophil peroxidase and major basic protein. (Stone *et al.*, 2010) In addition, eosinophils produce oxygen radicals, lipid mediators and a wide range of pro-inflmmatory cytokines and chemokines. (Hamid and Tulic, 2009) A number of cytokines produced by eosinophils are associated with remodelling and fibrosis, most notably TGF-β but also IL-6, IL-11, IL-13, IL-17 and IL-25. (Hamid and Tulic, 2009, Minshall et al., 1997) Interestingly, Hilliard *et al.* found a positive correlation between RBM thickness and total TGF-β_1 concentration in bronchoalveolar lavage fluid in chidren with CF. (Hilliard *et al.*, 2007)

In summary, there are contradictory reports in the literature regarding RBM thickness in CF lung disease and no descriptions of RBM thickness in end-stage disease.

8.3. Aims

The objectives of the work described in this chapter were to

1. Quantify RBM thickness in the lower airways of people with advanced CF lung disease requiring transplantation
2. Investigate any relationship between RBM thickness and number of mucosal eosinophils
3. Investigate any relationship between RBM thickness and age at time of lung transplantation

8.4. Hypothesis

I hypothesised that RBM thickness is increased in advanced CF lung disease and that this is linked to mucosal eosinophils and age at time of lung transplantation.

8.5. Results

8.5.1. *Reticular basement membrane thickness in advanced cystic fibrosis lung disease*

Clinical details of the patients with CF that I quantified RBM thickness in are provided in Table 18. Measurements were performed on 5μm sections stained with haematoxylin and eosin taken from formalin-fixed, paraffin-embeeded blocks as described in Chapter 2. Figure 87 displays an example of how I quantified RBM thickness by measuring the distance between two demarcating lines in each high-power section using Image ProPlus software (MediaCybernetics).

Comparison was made with data from a group of healthy volunteers enrolled in a previous study. (Ward *et al.*, 2002) In this study endobronchial biopsies were fixed in ice-cold acetone and embedded in glycol methacrylate. RBM thickness was then quantified using the same image analysis technique that I employed in 2μm sections immunostained for collagen type I.

The median RBM thickness was significantly increased in the CF group (9.58μm) compared to healthy volunteers (7.7μm, P=0.0002) in the previous study. (Ward *et al.*, 2002) Figure 88 shows the mean RBM thickness for each individual in the study along with other reported ranges for RBM thickness in healthy volunteers in formalin-fixed haematoxylin and eosin stained tissue, namely 2.9-6.7μm. (Lundgren et al., 1988, Wilson and Li, 1997)

Age*	CFTR Variant[$]	Sputum microbiology
14	Unknown	*Pseudomonas aeruginosa* *Candida parapsilosis*
26	p.Phe508del p.Phe508del	*P. aeruginosa* *Scediosporium aspiospermum*
23	p.Phe508del p.Phe508del	*P. aeruginosa Stenotrophomonas maltophilia*
25	Unknown	*P. aeruginosa*
28	p.Phe508del p.Phe508del	*P. aeruginosa* *Aspergillus fumigatus*
57	p.Arg117His p.Arg553X	*P. aeruginosa*
32	Unknown	*P. aeruginosa* *Burkholderia vietnamiensis*
23	Unknown	*Scedosporium* sp. *Alcaligines* sp. *Staphylococcus aureus*
39	p.Phe508del p.Phe508del	*P. aeruginosa*
27	p.Phe508del p.Phe508del	*P. aeruginosa*
40	Unknown	*P. aeruginosa*
46	Unknown	*P. aeruginosa*
22	p.Phe508del p.Phe508del	*P. aeruginosa*
20	p.Phe508del p.Phe508del	*P. aeruginosa*
29	p.Phe508del c.317+10kbC>T	*P. aeruginosa,* *S. maltophilia*
28	Unknown	*P. aeruginosa* Methicillin-Resistant *S. aureus*

Abbreviations: *Age at time of transplantation (years). [$]Cystic fibrosis transmembrane conductance regulator genotype (where known).

Table 18. Clinical details of people with cystic fibrosis used to measure reticular basement membrane thickness

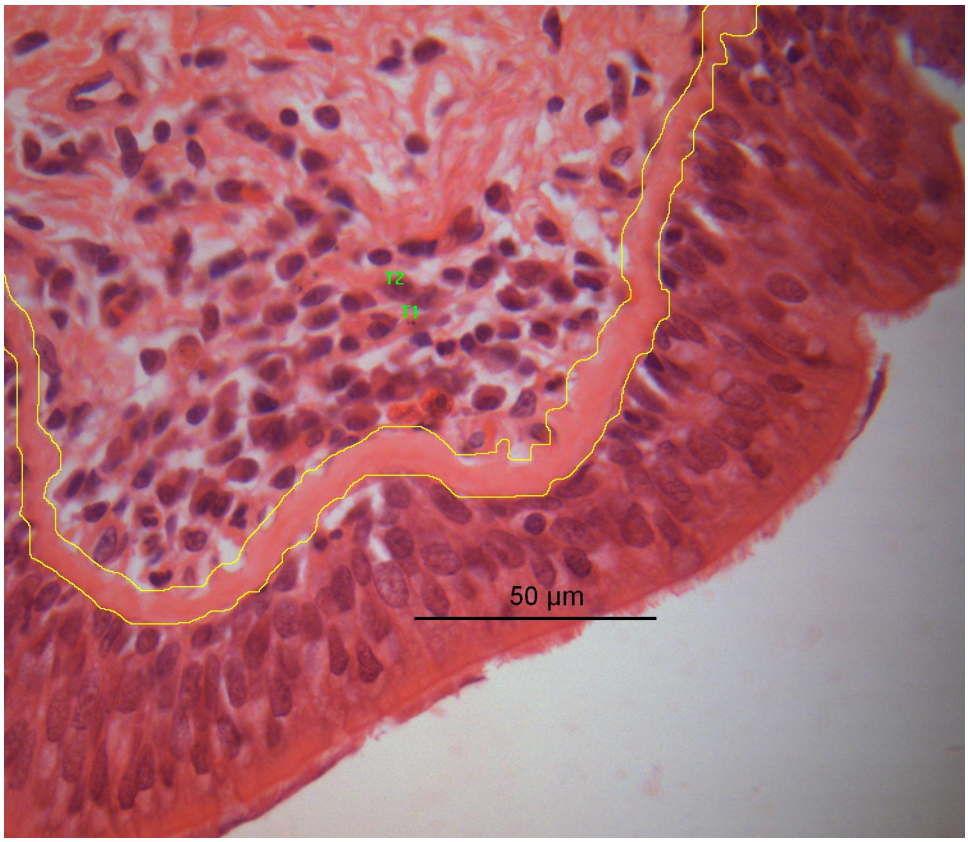


Figure 87. Example of measurement of reticular basemement membrane thickness in a haematoxylin and eosin stained section of lower airway from a person with advanced cystic fibrosis lung disease

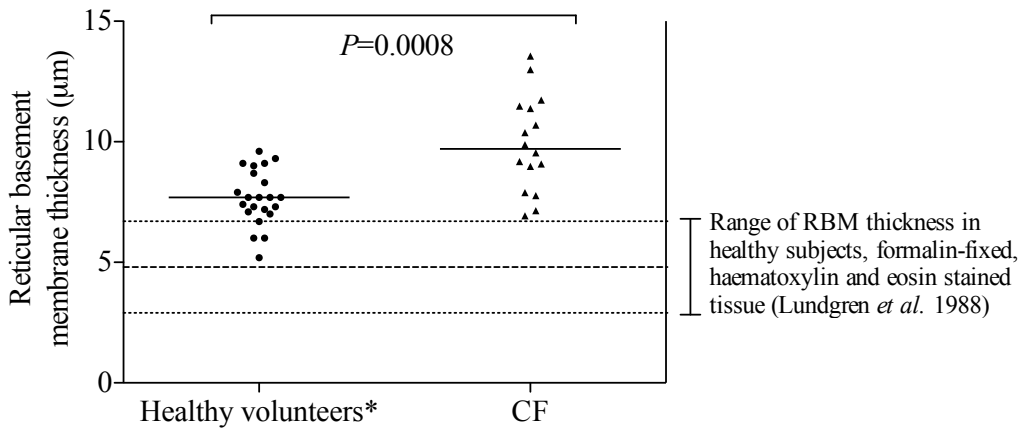


Figure 88. Reticular basement membrane thickness in healthy volunteers and people with advanced cystic fibrosis lung disease

Each symbol represents an individual patient (mean of 5 measurements). Horizontal bars indicate medians.
Abbreviations: CF, cystic fibrosis, *Data from Ward *et al.* (2002), endobronchial biopsies fixed in ice-cold acetone, embedded in glycol methacrylate. RBM thickness quantified using image analysis in 2μm sections immunostained for collagen type I. Mann-whitney test

8.5.2. *Number of mucosal eosinophils and lower airway reticular basement membrane thickness in people with advanced cystic fibrosis lung disease*

As outlined in the introduction of this chapter, eosinophilic inflammation has been implicated in the pathogenesis of airway remodelling in asthma *via* the maintenance and progression of aberrant airway tissue injury and repair.

I therefore quantified the number of mucosal eosinophils in a sub-group of 12 of the people with CF that I measured RBM thickness in. I did this by using the tinctorial stain Sirius red (Figure 89) and counting the number of mucosal eosinophils per mm of basmement membrane in 5 high-power fields in each patient. (Carvalho *et al.*, 2003) The number of eosinophils plotted against RBM thickness is shown in Figure 90. There is no clear relationship between RBM thickness and number of eosinophils in this dataset. However there is a suggestion of a negative correlation from the limited number of data points in Figure 90 if one considers the patient with the highest number of eosinophils as an outlier (R^2=0.274, P>0.05).

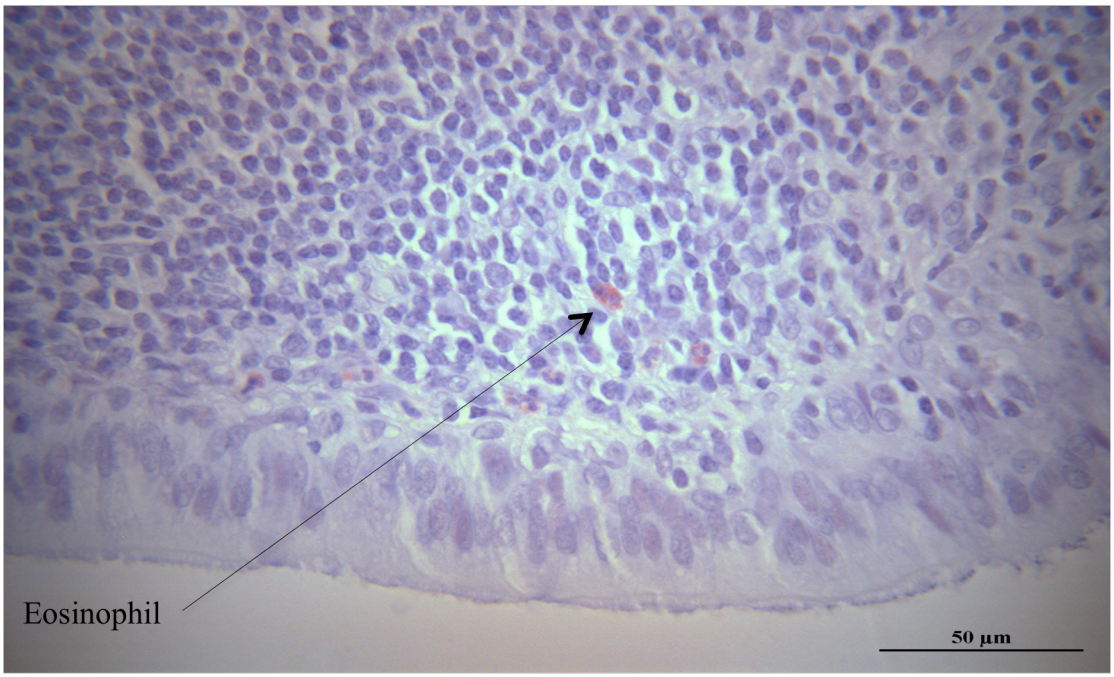


Figure 89. Example of Sirius red staining in the lower airway mucosa of a person with advanced cystic fibrosis lung disease

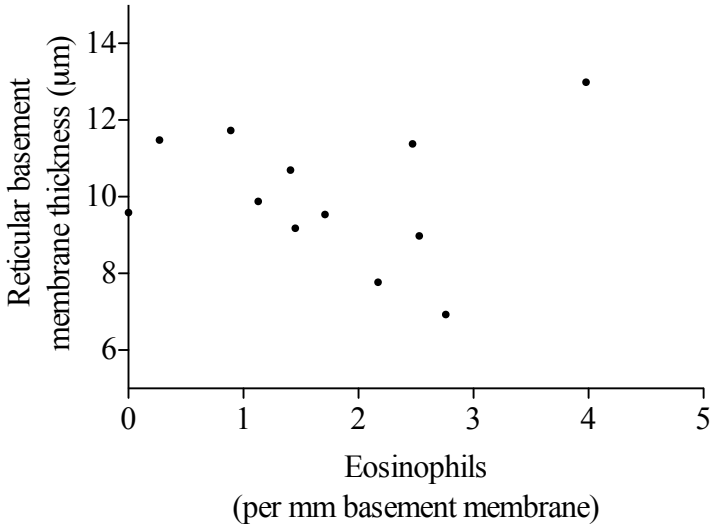


Figure 90. Scatter plot of reticular basement membrane thickness against number of lower airway mucosal eosinophils in people with advanced cystic fibrosis lung disease

8.5.3. *Age and reticular basement membrane thickness in advanced cystic fibrosis lung disease*

It has been suggested that RBM thickness changes with age although there is no definitive longitudinal or cross-sectional study published in the literature to confirm this. (Liesker *et al.*, 2009) I therefore plotted the RBM thickness results for the people with advanced CF lung disease versus age at time of transplant (Figure 91). There is no apparent relationship in this dataset.

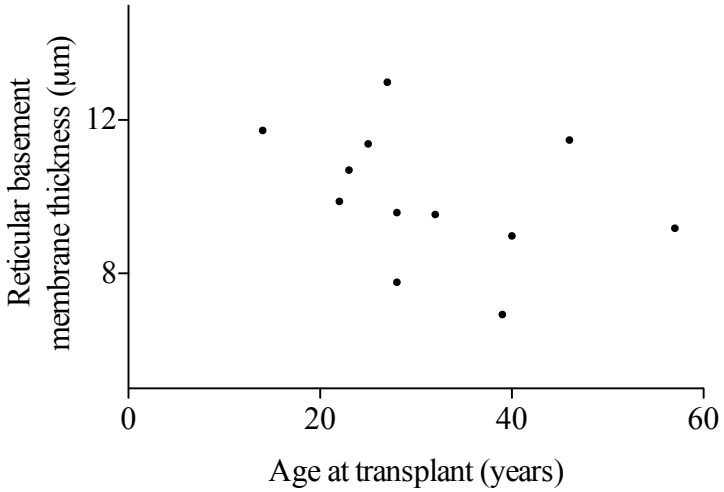


Figure 91. Scatter plot of reticular basement membrane thickness against age at transplant

8.6. Discussion

The finding of increased RBM thickness in advanced CF lung disease provides evidence of structural airway remodelling. My results agree with those published by Hilliard *et al.* in children and are opposite to the findings of Durieu *et al.* in a mixture of adults and children. (Hilliard et al., 2007, Durieu et al., 1998) The data presented in this chapter represents the largest sample size in the published literature regarding RBM thickness in advanced CF lung disease.

A number of sources of variation are important when considering the measurement of RBM thickness. Tissue acquisition (biopsy, explant or *post mortem*), processing, preservation (paraffin wax or plastic resin embedding or snap-freezing) and visualisation (tinctorial or immunostaining, light or electron microscopy) methods all impact on measurements of RBM thickness and other indices of airway remodelling. (Bergeron et al., 2007, Jeffery et al., 2003, Liesker et al., 2009, Wilson and Li, 1997) Comparisons between different publications should therefore be made with caution and inconsistencies may even exist between control and disease tissue in some studies. The fact that the CF tissue used in this study was fixed in formalin and embedded in paraffin means that my measurements are likely to be an underestimate compared to those made with the acetone and glycol methacrylate technique for the healthy volunteer samples in the previous study by Ward *et al*. (2002).

I found no apparent relationship in my relatively limited dataset between age and RBM thickness in end-stage CF lung disease (Figure 91). Similarly, Payne *et al.* found no association between RBM thickness and duration of symptoms in a cohort of children with severe asthma. (Payne *et al.*, 2003) One potential explanation is that once established in an inflammatory airway milieu, RBM thickness reaches a maximal level in individuals. Suitably powered longitudinal studies involving repeat biopsies of children and young adults with CF at different time points would be required to answer this question. Such studies would however present ethical and practical issues. As mentioned earlier there is also a paucity of good quality data in the literature regarding the natural history of RBM thickness in healthy individuals.

At an anecdotal level Wojnarowski *et al.* describe RBM thickening in endobronchial biopsies from children with CF during an acute exacerbation but not in other clinically

stable children. (Wojnarowski *et al.*, 1999) This finding would argue against a fixed maximal RBM thickness state, at least in early CF lung disease, because presumably the stable children had previously experienced acute exacerbations. The situation in advanced CF lung disease, where the spiral of inflammation and infection is profound and may be almost permanent, is likely to be different however.

In the context of asthma, the precise clinical significance of RBM thickening has been debated in the literature. In theoretical models increased RBM thickness has been shown to lead to a greater propensity for luminal collapse and obstruction. (Wiggs et al., 1997) However, it is possible that associated features of airway remodelling such as airway smooth muscle hypertrophy are of greater importance. (Bush, 2008) RBM thickening has been shown to correlate with several other parameters of airway remodelling however. (James *et al.*, 2002) Equally the involvement of eosinophilic inflammation as a primary event in airway remodelling in asthma has been challenged by indirect evidence from pre-school children, where wheezing is largely viral-induced, that corticosteroids are ineffective, the inflammation is principally neutrophilic and airway structural remodelling still occurs. (Panickar et al., 2009, Bush, 2008, Saglani et al., 2007) Eosinophilic inflammation and remodelling may be best regarded as parallel processes but the progression of each is independent. (Bush, 2008) The relationship between RBM thickness and numbers of inflammatory cells other than eosinophils in the airway mucosa, most notably neutrophils in the context of CF, is an important area for future research. (Downey et al., 2009)

In summary the data presented in this chapter provides evidence to support the hypothesis that RBM thickness is raised in advanced CF lung disease. To investigate RBM thickness more thoroughly in advanced CF lung disease it would be necessary to confirm findings in a larger cohort with contemporaneous, age-matched controls and to use alternative techniques such as immunostaining for collagen sub-types and/or electron microsocopy. More generally, the phenomenon of airway remodelling and its pathogenesis in CF lung disease is a relatively neglected area that warrants further translational research and may yield novel therapeutic strategies. (Dupuit *et al.*, 1995)

9. Chapter 9. Discussion and future work

9.1. Introduction

Clinically CF lung disease is characterised by retention of mucopurulent secretions and chronic endobronchial infection with specific organisms including *P. aeruginosa*. (O'Sullivan and Freedman, 2009) Histologically the airway pathology is dominated by intense neutrophilic inflammation. (Downey *et al.*, 2009) This results in progressive bronchiectasis, airway obstruction and ultimately respiratory failure. (Davies *et al.*, 2007) Lung transplantation is the only life-preserving intervention available for advanced CF lung disease. (Meachery *et al.*, 2008)

It is known that CF results from variants in the gene that encodes for the CFTR protein. CFTR is a complex and multifaceted protein that along with its principal function as an epithelial chloride channel amongst other properties also regulates sodium transport. (Sheppard and Welsh, 1999) The precise mechanisms involved in the translation of defective CFTR function in to the 'clinical syndrome' of CF are yet to be fully elucidated however and occupy thousands of researchers worldwide. (Wine, 2010)

9.2. Background and summary of results

9.2.1. *Development of a method to culture primary bronchial epithelial cells from the lungs of people with cystic fibrosis removed at the time of transplantation*

Valid experimental models are vital to advance research in to the pathogenesis of CF lung disease and identify novel therapeutic targets. Despite a huge amount of work and effort recapitulation of the CF lung disease phenotype in murine models has proved impossible to date. (Scholte *et al.*, 2004) Preliminary results of the recently developed porcine model describe an extreme gastrointestinal and lung phenotype with severe morbidity, frequent early mortality and significantly overall reduced survival. (Stoltz *et al.*, 2010) In terms of cellular approaches airway epithelial cells may be cultured *in vitro*, differentiated to form a ciliated multi-layered epithelium and co-cultured with other cell types or pathogens of interest. Immortalised cell lines frequently exhibit phenotypic instability and may not truly replicate the behaviour of cells *in vivo* however. Primary cells used at a low passage level are more likely to provide a superior model but are typically a scarce, expensive and time-consuming resource.

Through the work described in this thesis I have established a programme to culture PBECs from explanted CF lungs at the largest lung transplant centre in the UK. This involved developing a culture method that included tailoring of patient-specific anti-microbials that yielded a favourable success rate of around two-thirds. The PBECs were characterised in terms of morphology, cytokeratin immunohistochemistry, electrophysiology and cytokine production. Prolonged culture was possible at ALI with generation of a raised TER and mucus production, but not true ciliogenesis. Importantly low passage cells were cryopreserved and successfully reanimated for submerged culture.

The routine culture of PBECs from explanted CF lungs represents an important resource for CF research that has not previously been realised outside of a small number of large North American centres. The number of cells obtained is substantially greater than that from alternative methods such as bronchial or nasal brushings and clearly does not involve any additional invasive procedures for the donor. In addition, the procurement of fixed and snap-frozen tissue and airway lavage from explanted lungs is another

valuable resource that allowed much of the other work described in this thesis to be performed. The importance of this work has been recognised by invitations to speak at the European CF Society Basic Science Conference and the EuroCare CF 'Workshop on Epithelial Cells from Lung: Production, Cultivation and Characterization' in 2008 and led to the publication of a peer-reviewed paper. (Brodlie *et al.*, 2008a) (Brodlie et al., 2010e)

9.2.2. *The role of interleukin-17 in cystic fibrosis lung disease*

IL-17 is known to be pivotally involved in the accumulation and associated activity of neutrophils in the bronchoalveolar space and is important in pulmonary host defense. (Linden et al., 2005, Aujla et al., 2007) This is achieved indirectly via the local release of neutrophil-mobilising factors, including CXC chemokines, from cells resident in the lung. (Linden *et al.*, 2005) IL-17 also increases the expression of the mucin genes MUC5AC and MUC5B by bronchial epithelial cells *in vitro*. (Chen *et al.*, 2003) IL-17 is therefore linked to neutrophilic inflammation and mucus excess, which are two of the key features of CF lung disease.

A great deal of work in the literature has focussed on T_H-17 lymphocytes, a recently described novel $CD4^+$ T_H subset, as the principal source of IL-17 in biological systems. This is clearly of huge interest and relevance because IL-17 in this context represents a strategic link between acquired and innate immunity. (Glader *et al.*, 2010) However, γδ T cells and natural killer T cells have also recently been identified as producing IL-17. (Michel et al., 2007, Roark et al., 2008) In addition cells from the myeloid lineage have also been shown to produce IL-17, including mast cells in human rheumatoid arthritis synovium and neutrophils in human alcoholic liver disease and rodent models of lipopolysaccharide-induced airway inflammation, renal iscahemia-reperfusion injury and p-ANCA positive vasculitis. (Hueber et al., 2010, Lemmers et al., 2009, Ferretti et al., 2003, Li et al., 2010, Hoshino et al., 2008)

In the work described in this thesis I have shown using immunohistochemistry that expression of IL-17 is raised in the lower airway epithelium of people with advanced CF lung disease compared to those with PH. Furthermore, levels of IL-17 were raised in airway lavage fluid from explanted CF lungs compared to bronchoscopic BAL from

healthy volunteers. Another important finding was that IL-17 was consistently immunolocalised to mucosal neutrophils that stained positively for the transcription factor RORγ. This is the first description of neutrophils as a potential source of IL-17 in the human airway. In proof-of-principle experiments I went on to show that stimulation of PBECs from people with CF with IL-17 led to an increase in production of the pro-neutrophilic cytokines IL-8, IL-6 and GMCSF. IL-6 is known to be critical in the differentiation of T_H-17 cells and along with the suggestion that neutrophils themselves are a potential source of IL-17 in the airway a novel paradigm emerges of a positive feedback loop of neutrophilic inflammation. Figure 92 illustrates the potential positive feedback loop of neutrophilic inflammation involving IL-17 in a CF airway.

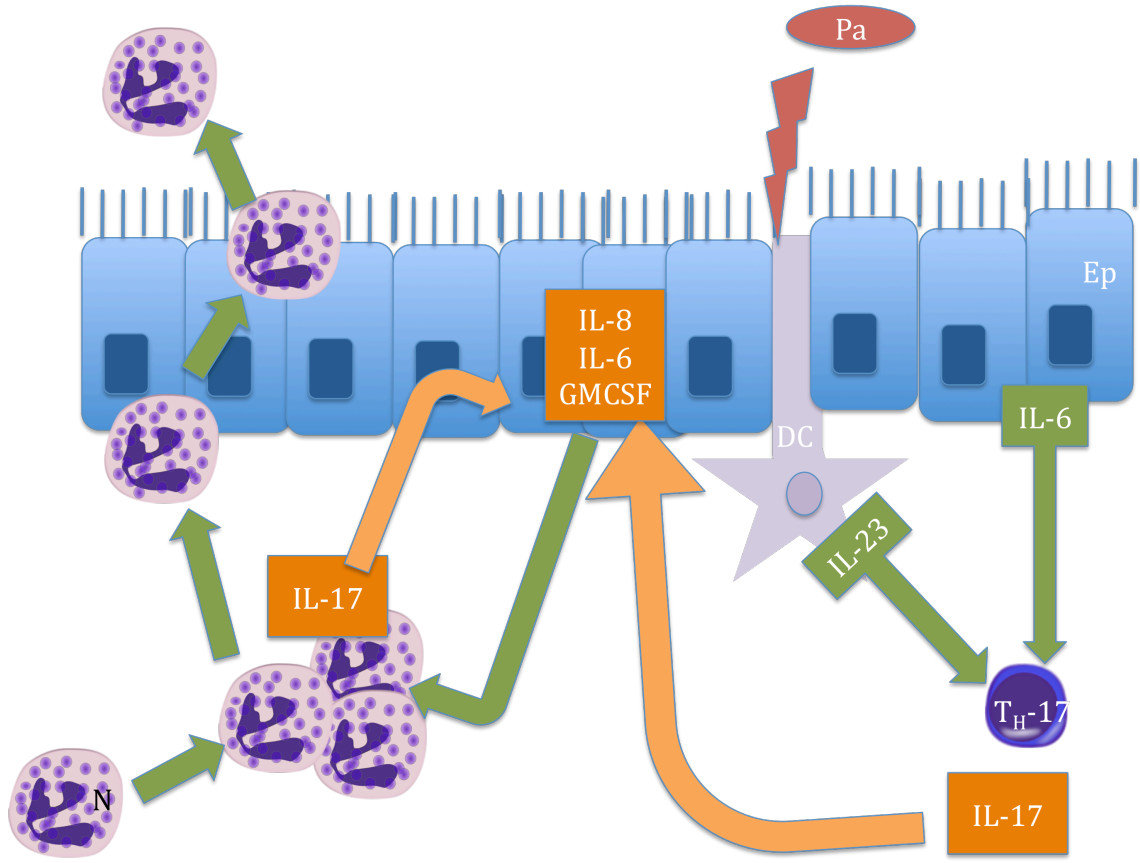


Figure 92. Illustrative diagram of potential positive feedback loop of neutrophilic inflammation involving interleukin-17 in cystic fibrosis airway

Abbreviations: Ep. Epithelium, IL. Interleukin, DC dendritic cell, N neutrophil, Pa *Pseudonas aeruginosa.*

9.2.3. *Ceramide and cystic fibrosis lung disease*

The sphingolipid ceramide is a ubiquitous component of cell membranes. In addition ceramide-enriched membrane domains are important in interactions with pathogens and intracellularly sphingolipids are key second messengers in several signalling cascades, most notably with regard to cell fate. (Uhlig and Gulbins, 2008)

Teichgraber *et al.* (2008) recently reported that CFTR-deficient mice accumulate ceramide in airway epithelial cells and that this led to chronic inflammation, death of epithelial cells and deposition of DNA in the airways and susceptibility to *P. aeruginosa* infection. Importantly all of these features could be reversed by systemic, and in a later paper nebulised, treatment with the acid sphingomyelinase inhibitor amitriptyline. (Becker et al., 2010b, Teichgraber et al., 2008) Accumulation of ceramide was demonstrated qualitatively in nasal epithelial cells from people with CF and in a limited number of lower airway sections. (Teichgraber *et al.*, 2008)

In direct contrast researchers from Canada have reported low levels of ceramide in the organs and plasma of a different CFTR-deficient mouse that could be corrected by treatment with fenretinide. (Guilbault *et al.*, 2008a) The same group also found reduced levels of ceramide in the plasma of people with CF. (Guilbault *et al.*, 2008b)

Proposed explanations for this divergence in the literature include variations between strains of CFTR-deficient mice, the effects of high-fat animal feeds and different techniques used to measure ceramide. (Pier, 2008) A definitive explanation is yet to be established. Ceramide truly represents a family of around 50 molecularly distinct species. (Novgorodov and Gudz, 2009) There is evidence that the relative levels of individual ceramide species is important in cellular homeostasis and this is likely to be true in the context of ceramide in CF. (Wooten-Blanks et al., 2007, Brodlie et al., 2010b) In this context, different measurement techniques will clearly impact on the interpretation of results. It is certain however that further translational studies are warranted and in particular relating to the lower airway in humans with CF.

In the work described in this thesis I have demonstrated that ceramide is increased in the lower airway epithelium of people with advanced CF lung disease. This was shown in comparison to PH and unused lung donors using immunohistochemistry with two different ceramide antibodies. Across all disease groups, staining for ceramide was increased in people colonised with *P. aeruginosa* and was correlated with the neutrophil markers neutrophil elastase and myeloperoxidase. Furthermore, levels of the ceramide species C16:0, C18:0 and C20:0, but not C22:0, were increased in lung homogenates of CF lungs compared to PH measured using the independent technique of HPLC-MS. These findings provide further evidence to support the 'ceramide accumulation hypothesis' suggested by the Gulbins group and add weight to the argument that ceramide may represent a potential therapeutic target in CF lung disease.

9.2.4. *Reticular basement membrane thickness in cystic fibrosis lung disease*

Thickening of the sub-epithelial RBM is a recognised feature of airway wall remodelling in asthma. (Ward et al., 2002, Payne et al., 2003) Eosinophilic inflammation has been implicated in its pathogenesis *via* the maintenance and progression of aberrant airway tissue injury and repair. (Holgate and Polosa, 2008) Airway remodelling has also been implicated in the pathogenesis of CF lung disease and RBM thickening has been described in endobronchial biopsies from children with CF. (Hilliard *et al.*, 2007) Other researchers, in studies involving only small numbers of patients, have described a thinned RBM in children and adults with CF or thickening only in association with an infective exacerbation. (Durieu et al., 1998, Wojnarowski et al., 1999) Tissue sampling, fixation, staining and measurement techniques are all likely to impact on the results of such studies. (Jeffery *et al.*, 2003) To the best of my knowledge RBM thickness has not been investigated in advanced CF lung disease.

In the work described in this thesis I have shown that RBM thickness is increased in advanced CF lung disease compared to measurements in endobronchial biopsies from healthy volunteers. There was no apparent correlation between number of mucosal eosinophils or age at time of transplantation and RBM thickness in this dataset.

9.2.5. *Experimental controls*

As has been outlined in this thesis a non-suppurative disease comparator (pulmonary hypertension) was used for the IL-17 work and in the case of the ceramide chapter sections from unused lung donors (previously healthy and objectively free of significant lung fibrosis or inflammation) were also used. These control groups were chosen largely due to logistical considerations and the availability of tissue for research use in our transplant centre. The ideal control group for many of the experiments would have been tissue and samples from healthy volunteers however there are obvious limitations in the availability of such tissue and constraints with in the scope of a PhD project.

9.3. Potential future work

9.3.1. *Development of a method to culture primary bronchial epithelial cells from the lungs of people with cystic fibrosis removed at the time of transplantation*

PBECs harvested from explanted CF lungs are by definition only representative of advanced CF lung disease. As a paediatrician I am acutely aware of the spectrum of severity and progressive nature of CF lung disease and therefore primary cellular models that reflect less advanced stages of disease are also vitally important. This may be achieved by opportunistic brushing of the lower airway at the time of flexible bronchoscopy or other general anaesthetic for a clinical indication. (McNamara *et al.*, 2008) An alternative approach is to sample nasal epithelial cells from the upper airway, which is clearly a more accessible method with the potential for repeated sampling. A related and important question however is: how representative of the lower airway are nasal epithelial cells and indeed are cells from different anatomical locations in the lower airway comparable? In the context of advanced CF lung disease the resource of explanted lungs represents a potentially useful tool to investigate these questions. A greater understanding of disease processes at the early stages of CF lung disease would allow the development of interventions with the potential to maintain lung function in the normal range.

There is also potential to use pieces of fresh intact large airway from explanted CF lungs to perform electrophysiology experiments in mini-Ussing chambers or real-time cilia or mucus studies. (Derichs, 2009, N'Dow et al., 2005)

Some of the greatest challenges in the continuation of the culturing of PBECs from explanted CF lungs will arguably relate to logistics and infrastructure. To date the programme has relied on a permanent on-call commitment from myself and funding for this form of core resource is often difficult to obtain outside of North America.

9.3.2. *The role of interleukin-17 in cystic fibrosis lung disease*

There are a number of potential future avenues for research leading from this work. In relation to CF lung disease specifically, larger scale BAL and tissue-based studies are warranted across the spectrum of severity of disease. Of interest it has been reported that in children with earlier stages of CF lung disease the inflammatory infiltrate of the endobronchial mucosa is dominated by lymphocytes with neutrophils only predominating in the airway lumen. (Tan *et al.*, 2009) This is in contrast to my findings in advanced CF lung disease of intense neutrophilic inflammation of the airway mucosa, for example in Figure 60. Using dual staining $CD4^+$ $IL\text{-}17^+$ presumed T_H-17 cells have also been demonstrated in the airway mucosa of endobronchial biopsies from children with CF and non-CF bronchiectasis. (Tan *et al.*, 2009) It is plausible therefore that T_H-17 cells may be more important in the pathogenesis of the early stages of CF lung disease than in advanced disease, although this is pure conjecture.

The suggestion from my work that neutrophils themselves may be a source of IL-17 is potentially very significant but requires further investigation. Taken with the evidence that I found that IL-17 leads to the increased production of pro-neutrophilic mediators from CF PBECs the possibility of a novel vicious cycle of inflammation emerges in advanced CF lung disease. This could also be of relevance to other neutrophilic lung pathologies such as non-CF bronchiectasis, asthma or bronchiolitis.

At a more fundamental level *ex vivo* experiments using isolated neutrophils are required to investigate IL-17 transcription and translation. Investigation of relevant factors involved in the production of IL-17 by neutrophils should also be performed. The most accessible source of neutrophils for such work would be those isolated from peripheral blood, however activated neutrophils from the lung compartment are likely to be harder to obtain but physiologically more relevant. (Sabroe et al., 2004, Zemans et al., 2009)

I believe that a much greater understanding of IL-17 receptor biology and signalling pathways is required before serious consideration is made of the potential for antagonism. (Gaffen, 2009) However, at least at a superficial level, IL-17 is an attractive therapeutic target given the major role of neutrophilic inflammation in the pathogenesis of CF lung disease. As with any immunomodulatory strategy a balance would have to be struck between the benefits of reducing the harmful effects of

neutrophilic inflammation versus potential harm from compromising innate defenses against infection. (Sabroe and Whyte, 2007) A stark parallel is provided if one considers children with autosomal dominant hyper-immunoglobulin E syndrome (HIES). Children with HIES have very high levels of IgE and reduced neutrophil chemotaxis resulting in amongst other features, problems with *S. aureus* infection of the skin and lungs. (Grimbacher *et al.*, 1999) The molecular basis of HIES has recently been shown to involve mutations in STAT3 and defective IL-17 signalling. (Holland *et al.*, 2007)

9.3.3. *Ceramide and cystic fibrosis lung disease*

The potential role of ceramide in the pathogenesis of CF lung disease remains topical but highly controversial. The genuine complexities of studying lipids in biological systems have made this field a particularly difficult one. (Becker *et al.*, 2010c) In my opinion a larger scale study is required to provide a definitive answer about levels of individual ceramide species in the human CF lower airway epithelium. This could potentially be achieved using a modern imaging modality such as matrix-assisted laser desorption and ionization mass spectrometry. (Fuchs *et al.*, 2010)

The hypothesis put forward by Teichgraber *et al.* involving impaired acidification of intracellular vesicles in the presence of defective CFTR to explain the accumulation of ceramide has been challenged. (Teichgraber et al., 2008, Haggie and Verkman, 2009b) One potential explanation for the divergent findings however relates to different methods used to measure vesicular pH. (Becker *et al.*, 2010a) A method that involves the endocytosis of a pH-sensitive marker will clearly only measure the pH of endocytotic vesicles and it is suggested that CFTR may not be involved in the regulation of this subset of vesicles. Clearly a great deal of careful *in vivo* laboratory work is required in this complex area to resolve this issue in the future.

From a clinical perspective the most attractive aspect of this area of work is the potential to antagonise the accumulation of ceramide with acid sphingomylinase inhibitors. The Gulbins group have demonstrated that acid sphingomyelinase inhibitors, including amitriptyline, amlodipine and fluoxetine, delivered *via* nebuliser can normalise ceramide levels, pulmonary inflammation and susceptibility to *P. aeruginosa* in CFTR-deficient mice. (Becker *et al.*, 2010b) Clinical evidence of the use of

amitriptyline in people with CF is largely anecdotal aside from a single n=4 placebo-controlled, double-blinded crossover trial and linked n=19 14 day phase II trial. (Becker et al., 2010b, Riethmuller et al., 2009) In this study amitriptyline was well tolerated orally and its use was associated with a modest improvement in lung function. (Riethmuller *et al.*, 2009) Clearly further trials are indicated with particular caution indicated with regard to potential toxic effects of tricyclic antidepressants and any deleterious impact on epithelial physiology and host defense of overinhibition of ceramide biosynthesis given its key homeostatic role. (Pier, 2008, Brodlie et al., 2010b)

9.3.4. *Reticular basement membrane thickness in cystic fibrosis lung disease*

The topic of airway remodelling in CF lung disease has received relatively little attention in the literature to date. This is despite histological evidence of structural airway remodelling in children with CF and demonstrably abnormal airway function in infants newly diagnosed clinically with CF. (Ranganathan et al., 2001, Hilliard et al., 2007) This abnormal lung function does not improve despite intensive treatment, and the airway obstruction would therefore appear to be 'fixed'. (Ranganathan *et al.*, 2004)

In contrast, aberrant epithelial wound repair and airway remodelling in other lung diseases, such as asthma, pulmonary fibrosis and obliterative bronchiolitis, has been the topic of intense study in recent years. (Gardner *et al.*, 2010) Interestingly polymorphisms in TGF-β_1 have been shown to be modifier genes for CF lung disease. (Collaco and Cutting, 2008) Increased concentrations of TGF-β_1 have been detected in BAL from children with CF and levels found to correlate with neutrophil markers, reduced lung function, RBM thickness and disease activity. (Harris et al., 2009, Hilliard et al., 2007) Little is known about the potential mechanisms behind these observational findings and further research in this general area is clearly warranted. The potential role of TGF-β_1 driven epithelial-mesenchymal transition in CF lung disease has also not been investigated. (Ward et al., 2005, Borthwick et al., 2009) Markers of epithelial remodelling have been linked to the relative expression and cellular distribution of CFTR in nasal polyps from wild type and p.Phe508del homozygous individuals suggesting that airway remodelling may play a role in the regulation of CFTR expression. (Dupuit *et al.*, 1995)

9.3.5. *Summary*

The work described in this thesis has led to the establishment of an *ex vivo* PBEC culture model to study CF lung disease. Using primary lung tissue and the PBEC model I have provided insights in to the possible roles of IL-17, ceramide and airway remodelling in the pathogenesis of CF lung disease, thus contributing to the scientific literature and suggesting potential therapeutic targets and areas for future translational research.

9.4. Reflection

Over the course of this PhD I have gained experience in many of the key core skills and techniques used in translational respiratory research.

These have included:

- processing and standardising of BAL
- sampling and fixation of lung tissue and associated sectioning, tinctorial staining and immunohistochemistry
- image analysis techniques for semi-quantitative histology/immunohistochemistry
- primary airway epithelial cell culture and associated laboratory techniques
- ELISA and associated techniques
- principles of HPLC-MS
- principles of electrophysiology
- compliance with the Human Tissue Act
- involvement in a randomised-control trial
- ethics application procedure
- informed consent, good clinical practice training
- setting-up of collaborations
- writing of grant applications and scientific papers
- MRC fellowship interview
- oral and poster presentation at local, national and international meetings
- review of scientific papers
- organisation and self-discipline required for a successful research project

I have been fortunate to have received excellent supervision and to have enjoyed access to and involvement in an active translational respiratory research programme in Newcastle. This has included my involvement in a number of other areas of research in addition to the work formally presented in this thesis. This has included the consenting and organisation of samples from lung transplant patients undergoing surveillance bronchoscopies, consenting and procurement of explanted lungs for a tissue bank, mitochondrial studies of paediatric cardiac tissue and investigation of aspiration as a source of lung injury in CF. Over the course of my PhD I have written several

clinically-based case reports and an editorial, in keeping with my planned career path as a paediatric respiratory clinician. This experience has established a firm platform from which I hope to build an academic clinical career and in the short-term to pursue some of the research areas outlined in section 9.3.

10. References

Abrahams, N. A., Meziane, M., Ramalingam, P., Mehta, A., DeCamp, M. and Farver, C. F. (2004) 'Incidence of primary neoplasms in explanted lungs: long-term follow-up from 214 lung transplant patients', *Transplant Proc,* 36, (9), pp. 2808-11.

Aggarwal, S., Ghilardi, N., Xie, M. H., de Sauvage, F. J. and Gurney, A. L. (2003) 'Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17', *J Biol Chem,* 278, (3), pp. 1910-4.

Aggarwal, S. and Gurney, A. L. (2002) 'IL-17: prototype member of an emerging cytokine family', *J Leukoc Biol,* 71, (1), pp. 1-8.

Alcorn, J. F., Crowe, C. R. and Kolls, J. K. (2010) 'TH17 cells in asthma and COPD', *Annu Rev Physiol,* 72, pp. 495-516.

Ammit, A. J., Hastie, A. T., Edsall, L. C., Hoffman, R. K., Amrani, Y., Krymskaya, V. P., Kane, S. A., Peters, S. P., Penn, R. B., Spiegel, S. and Panettieri, R. A., Jr. (2001) 'Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma', *Faseb J,* 15, (7), pp. 1212-4.

Andersen, D. H. (1938) 'Cystic fibrosis of the pancreas and its relation to celiac disease', *Am J Dis Child,* 56, (-), pp. 344.

Anon. (2006) 'Effective implementation of the Human Tissue Act', *The Lancet,* 368, (9539), pp. 891.

Araya, J., Cambier, S., Markovics, J. A., Wolters, P., Jablons, D., Hill, A., Finkbeiner, W., Jones, K., Broaddus, V. C., Sheppard, D., Barzcak, A., Xiao, Y., Erle, D. J. and Nishimura, S. L. (2007) 'Squamous metaplasia amplifies pathologic epithelial-mesenchymal interactions in COPD patients', *J Clin Invest,* 117, (11), pp. 3551-62.

Armstrong, D. S., Grimwood, K., Carzino, R., Carlin, J. B., Olinsky, A. and Phelan, P. D. (1995) 'Lower respiratory infection and inflammation in infants with newly diagnosed cystic fibrosis', *Bmj,* 310, (6994), pp. 1571-2.

Armstrong, D. S., Hook, S. M., Jamsen, K. M., Nixon, G. M., Carzino, R., Carlin, J. B., Robertson, C. F. and Grimwood, K. (2005) 'Lower airway inflammation in

infants with cystic fibrosis detected by newborn screening', *Pediatr Pulmonol,* 40, (6), pp. 500-10.

Asquith, D. L. and McInnes, I. B. (2007) 'Emerging cytokine targets in rheumatoid arthritis', *Curr Opin Rheumatol,* 19, (3), pp. 246-51.

Aujla, S. J., Chan, Y. R., Zheng, M., Fei, M., Askew, D. J., Pociask, D. A., Reinhart, T. A., McAllister, F., Edeal, J., Gaus, K., Husain, S., Kreindler, J. L., Dubin, P. J., Pilewski, J. M., Myerburg, M. M., Mason, C. A., Iwakura, Y. and Kolls, J. K. (2008) 'IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia', *Nat Med,* 14, (3), pp. 275-81.

Aujla, S. J., Dubin, P. J. and Kolls, J. K. (2007) 'Interleukin-17 in pulmonary host defense', *Exp Lung Res,* 33, (10), pp. 507-18.

Auld, D. S., Thorne, N., Maguire, W. F. and Inglese, J. (2009) 'Mechanism of PTC124 activity in cell-based luciferase assays of nonsense codon suppression', *Proc Natl Acad Sci U S A,* 106, (9), pp. 3585-90.

Bain, B. J. (2002) *Blood cells a practical guide.* Blackwell science: Edinburgh.

Balfour-Lynn, I. M. (1999) 'The protease-antiprotease battle in the cystic fibrosis lung', *J R Soc Med,* 92 Suppl 37, pp. 23-30.

Balough, K., McCubbin, M., Weinberger, M., Smits, W., Ahrens, R. and Fick, R. (1995) 'The relationship between infection and inflammation in the early stages of lung disease from cystic fibrosis', *Pediatr Pulmonol,* 20, (2), pp. 63-70.

Bals, R., Beisswenger, C., Blouquit, S. and Chinet, T. (2004) 'Isolation and air-liquid interface culture of human large airway and bronchiolar epithelial cells', *J Cyst Fibros,* 3 Suppl 2, pp. 49-51.

Bamias, G. and Cominelli, F. (2007) 'Immunopathogenesis of inflammatory bowel disease: current concepts', *Curr Opin Gastroenterol,* 23, (4), pp. 365-9.

Barreda, D. R., Hanington, P. C. and Belosevic, M. (2004) 'Regulation of myeloid development and function by colony stimulating factors', *Dev Comp Immunol,* 28, (5), pp. 509-54.

Becker, K. A., Grassme, H., Zhang, Y. and Gulbins, E. (2010a) 'Ceramide in Pseudomonas aeruginosa infections and cystic fibrosis', *Cell Physiol Biochem,* 26, (1), pp. 57-66.

Becker, K. A., Riethmuller, J., Luth, A., Doring, G., Kleuser, B. and Gulbins, E. (2010b) 'Acid sphingomyelinase inhibitors normalize pulmonary ceramide and inflammation in cystic fibrosis', *Am J Respir Cell Mol Biol,* 42, (6), pp. 716-24.

Becker, K. A., Riethmuller, J., Zhang, Y. and Gulbins, E. (2010c) 'The role of sphingolipids and ceramide in pulmonary inflammation in cystic fibrosis', *Open Respir Med J,* 4, pp. 39-47.

Becker, M. N., Sauer, M. S., Muhlebach, M. S., Hirsh, A. J., Wu, Q., Verghese, M. W. and Randell, S. H. (2004) 'Cytokine secretion by cystic fibrosis airway epithelial cells', *Am J Respir Crit Care Med,* 169, (5), pp. 645-53.

Ben-Porath, I. and Weinberg, R. A. (2005) 'The signals and pathways activating cellular senescence', *Int J Biochem Cell Biol,* 37, (5), pp. 961-76.

Bergeron, C., Tulic, M. K. and Hamid, Q. (2007) 'Tools used to measure airway remodelling in research', *Eur Respir J,* 29, (3), pp. 596-604.

Bettelli, E., Oukka, M. and Kuchroo, V. K. (2007) 'T(H)-17 cells in the circle of immunity and autoimmunity', *Nat Immunol,* 8, (4), pp. 345-50.

Bland, J. M. and Altman, D. G. (1986) 'Statistical methods for assessing agreement between two methods of clinical measurement', *Lancet,* 1, (8476), pp. 307-10.

Blouquit, S., Regnier, A., Dannhoffer, L., Fermanian, C., Naline, E., Boucher, R. and Chinet, T. (2006) 'Ion and fluid transport properties of small airways in cystic fibrosis', *Am J Respir Crit Care Med,* 174, (3), pp. 299-305.

Bonfield, T. L., Konstan, M. W., Burfeind, P., Panuska, J. R., Hilliard, J. B. and Berger, M. (1995) 'Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis', *Am J Respir Cell Mol Biol,* 13, (3), pp. 257-61.

Borthwick, D. W., West, J. D., Keighren, M. A., Flockhart, J. H., Innes, B. A. and Dorin, J. R. (1999) 'Murine submucosal glands are clonally derived and show a cystic fibrosis gene-dependent distribution pattern', *Am J Respir Cell Mol Biol,* 20, (6), pp. 1181-9.

Borthwick, L. A., McGaw, J., Conner, G., Taylor, C. J., Gerke, V., Mehta, A., Robson, L. and Muimo, R. (2007) 'The formation of the cAMP/protein kinase A-dependent annexin 2-S100A10 complex with cystic fibrosis conductance regulator protein (CFTR) regulates CFTR channel function', *Mol Biol Cell,* 18, (9), pp. 3388-97.

Borthwick, L. A., Parker, S. M., Brougham, K. A., Johnson, G. E., Gorowiec, M. R., Ward, C., Lordan, J. L., Corris, P. A., Kirby, J. A. and Fisher, A. J. (2009) 'Epithelial to mesenchymal transition (EMT) and airway remodelling after human lung transplantation', *Thorax,* 64, (9), pp. 770-7.

Boucher, R. C. (2007a) 'Airway surface dehydration in cystic fibrosis: pathogenesis and therapy', *Annu Rev Med,* 58, pp. 157-70.

Boucher, R. C. (2007b) 'Evidence for airway surface dehydration as the initiating event in CF airway disease', *J Intern Med,* 261, (1), pp. 5-16.

Bradford, M. M. (1976) 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding', *Anal Biochem,* 72, pp. 248-54.

Bridges, M. A., Walker, D. C. and Davidson, A. G. (1991) 'Cystic fibrosis and control nasal epithelial cells harvested by a brushing procedure', *In Vitro Cell Dev Biol,* 27A, (9), pp. 684-6.

Brodlie, M., Eger, K., Hilkens, C. M. and Ward, C. (2009a) 'Airway epithelial cells as guardians of immune homeostasis?', *Thorax,* 64, (11), pp. 1009.

Brodlie, M., Johnson, G. E., Perry, J., Hinchcliffe, M., Fisher, A., Corris, P. A., Pearson, J. P., McKean, M. C., Lordan, J. L. and Ward, C. (2008a) 'The culture of primary bronchial epithelial cells from the lungs of people with cystic fibrosis removed at the time of transplantation – A model to study cystic fibrosis lung disease*', Paper presented at the New Frontiers in Basic Science of Cystic Fibrosis. European Cystic Fibrosis Society meeting.* April, 2008, in Douro, Portugal.

Brodlie, M. and McKean, M. C. (2009) 'Exhaled nitric oxide in the diagnosis of childhood asthma', *Bmj,* 339, pp. b5418.

Brodlie, M., McKean MC, Johnson G, Fisher AJ, Corris PA, Lordan J and Ward, C. (2009b) 'Ceramide is a potential biomarker of epithelial injury which is raised in the airway of people with cystic fibrosis*', Paper presented at the New Frontiers In Basic Science of Cystic Fibrosis European Cystic Fibrosis Society meeting.* April 2009, in Tavira, Portugal.

Brodlie, M., McKean MC, Johnson G, Fisher AJ, Corris PA, Lordan J and Ward, C. (2009c) 'The cytokine interleukin-17 is raised in the airway of people with cystic fibrosis', *Eur Respir J,* 34, (Supplement 53), pp. 59s-60s.

Brodlie, M., McKean MC, Johnson G, Fisher AJ, Corris PA, Lordan J and Ward, C. (2010a) 'Ceramide is increased and associated with neutrophilic inflammation in the lower airway epithelium of people with cystic fibrosis*', Paper presented at 8th ERS Lung Science Conference.* March 2010, in Estoril, Portugal, March 2010.

Brodlie, M., McKean MC, Johnson GE, Fisher AJ, Corris PA, Lordan JL and Ward, C. (2009d) 'Ceramide is increased and associated with neutrophilic inflammation in the lower airway epithelium of people with cystic fibrosis', *Thorax,* 64, (Suppl IV), pp. A70-A71.

Brodlie, M., McKean MC, Johnson GE, Fisher AJ, Corris PA, Lordan JL and Ward, C. (2009e) 'Reticular basement membrane thickening in end-stage cystic fibrosis lung disease', *Thorax,* 64, (Suppl 4), pp. A110.

Brodlie, M., McKean MC, Johnson GE, Gray J, Fisher AJ, Corris PA, Lordan J and Ward, C. (2010b) 'Ceramide is increased in the lower airway epithelium of people with advanced cystic fibrosis lung disease', *Am J Respir Crit Care Med,* 182, (3), pp. 369-375.

Brodlie, M., McKean, M. C., Johnson, G. E., Anderson, A. E., Hilkens, C. M., Fisher, A. J., Corris, P. A., Lordan, J. L. and Ward, C. (2010c) 'Raised interleukin-17 is immuno-localised to neutrophils in cystic fibrosis lung disease', *Eur Respir J.*

Brodlie, M., McKean, M. C., Johnson, G. E., Perry, J. D., Nicholson, A., Verdon, B., Gray, M. A., Dark, J. H., Pearson, J. P., Fisher, A. J., Corris, P. A., Lordan, J. and Ward, C. (2010e) 'Primary bronchial epithelial cell culture from explanted cystic fibrosis lungs', *Exp Lung Res,* 36, (2), pp. 101-10.

Brodlie, M., McKean, M. C., Perry, J., Nicholson, A., Johnson, G. E., Pearson, J. P., Fisher, A., Corris, P. A., Lordan, J. L. and Ward, C. (2008b) 'The culture of primary bronchial epithelial cells from the lungs of people with cystic fibrosis removed at the time of transplantation: A model to study cystic fibrosis lung disease', *Thorax,* 63, pp. A37-A39.

Brodlie, M., McKean, M. C., Perry, J., Nicholson, A., Johnson, G. E., Pearson, J. P., Fisher, A., Corris, P. A., Ward, C. and Lordan, J. L. (2009f) *Communications for the Spring Meeting of the Medical Research Society, held in conjunction with the Academy of Medical Sciences.* London:Medical Research Society.

Bronsveld, I., Mekus, F., Bijman, J., Ballmann, M., de Jonge, H. R., Laabs, U., Halley, D. J., Ellemunter, H., Mastella, G., Thomas, S., Veeze, H. J. and Tummler, B. (2001) 'Chloride conductance and genetic background modulate the cystic fibrosis phenotype of Delta F508 homozygous twins and siblings', *J Clin Invest,* 108, (11), pp. 1705-15.

Buchanan, P. J., Ernst, R. K., Elborn, J. S. and Schock, B. (2009) 'Role of CFTR, Pseudomonas aeruginosa and Toll-like receptors in cystic fibrosis lung inflammation', *Biochem Soc Trans,* 37, (Pt 4), pp. 863-7.

Bullens, D. M., Truyen, E., Coteur, L., Dilissen, E., Hellings, P. W., Dupont, L. J. and Ceuppens, J. L. (2006) 'IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx?', *Respir Res,* 7, pp. 135.

Bush, A. (2008) 'How early do airway inflammation and remodeling occur?', *Allergol Int,* 57, (1), pp. 11-9.

Bush, A. and Davies, J. (2006) 'Rebuttal: You are wrong, Dr. Mallory ...', *Pediatr Pulmonol,* 41, pp. 1017-1020.

Campodonico, V. L., Gadjeva, M., Paradis-Bleau, C., Uluer, A. and Pier, G. B. (2008) 'Airway epithelial control of Pseudomonas aeruginosa infection in cystic fibrosis', *Trends Mol Med,* 14, (3), pp. 120-33.

Cardell, L. O., Agusti, C., Takeyama, K., Stjarne, P. and Nadel, J. A. (1999) 'LTB(4)-induced nasal gland serous cell secretion mediated by neutrophil elastase', *Am J Respir Crit Care Med,* 160, (2), pp. 411-4.

Carvalho, A. T., Elia, C. C., de Souza, H. S., Elias, P. R., Pontes, E. L., Lukashok, H. P., de Freitas, F. C. and Lapa e Silva, J. R. (2003) 'Immunohistochemical study of intestinal eosinophils in inflammatory bowel disease', *J Clin Gastroenterol,* 36, (2), pp. 120-5.

Carvalho-Oliveira, I., Scholte, B. J. and Penque, D. (2007) 'What have we learned from mouse models for cystic fibrosis?', *Expert Rev Mol Diagn,* 7, (4), pp. 407-17.

Chakraborty, J. B. and Mann, D. A. (2010) 'NF-kappaB signalling: embracing complexity to achieve translation', *J Hepatol,* 52, (2), pp. 285-91.

Chen, Y., Thai, P., Zhao, Y. H., Ho, Y. S., DeSouza, M. M. and Wu, R. (2003) 'Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop', *J Biol Chem,* 278, (19), pp. 17036-43.

Cheung, J. C. and Deber, C. M. (2008) 'Misfolding of the cystic fibrosis transmembrane conductance regulator and disease', *Biochemistry,* 47, (6), pp. 1465-73.

Chilvers, M. A. and O'Callaghan, C. (2000) 'Local mucociliary defence mechanisms', *Paediatr Respir Rev,* 1, (1), pp. 27-34.

Choe, M. M., Sporn, P. H. and Swartz, M. A. (2003) 'An in vitro airway wall model of remodeling', *Am J Physiol Lung Cell Mol Physiol,* 285, (2), pp. L427-33.

Choe, M. M., Tomei, A. A. and Swartz, M. A. (2006) 'Physiological 3D tissue model of the airway wall and mucosa', *Nat Protoc,* 1, (1), pp. 357-62.

Claus, R. A., Bunck, A. C., Bockmeyer, C. L., Brunkhorst, F. M., Losche, W., Kinscherf, R. and Deigner, H. P. (2005) 'Role of increased sphingomyelinase

activity in apoptosis and organ failure of patients with severe sepsis', *Faseb J,* 19, (12), pp. 1719-21.

Collaco, J. M. and Cutting, G. R. (2008) 'Update on gene modifiers in cystic fibrosis', *Curr Opin Pulm Med,* 14, (6), pp. 559-66.

Colledge, W. H., Abella, B. S., Southern, K. W., Ratcliff, R., Jiang, C., Cheng, S. H., MacVinish, L. J., Anderson, J. R., Cuthbert, A. W. and Evans, M. J. (1995) 'Generation and characterization of a delta F508 cystic fibrosis mouse model', *Nat Genet,* 10, (4), pp. 445-52.

Corris, P. A. (2008) 'Lung transplantation for cystic fibrosis', *Curr Opin Organ Transplant,* 13, (5), pp. 484-8.

Cosmi, L., De Palma, R., Santarlasci, V., Maggi, L., Capone, M., Frosali, F., Rodolico, G., Querci, V., Abbate, G., Angeli, R., Berrino, L., Fambrini, M., Caproni, M., Tonelli, F., Lazzeri, E., Parronchi, P., Liotta, F., Maggi, E., Romagnani, S. and Annunziato, F. (2008) 'Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor', *J Exp Med,* 205, (8), pp. 1903-16.

Cowart, L. A., Szulc, Z., Bielawska, A. and Hannun, Y. A. (2002) 'Structural determinants of sphingolipid recognition by commercially available anti-ceramide antibodies', *J Lipid Res,* 43, (12), pp. 2042-8.

Crystal, R. G., Randell, S. H., Engelhardt, J. F., Voynow, J. and Sunday, M. E. (2008) 'Airway epithelial cells: current concepts and challenges', *Proc Am Thorac Soc,* 5, (7), pp. 772-7.

Curtis, J. L., Freeman, C. M. and Hogg, J. C. (2007) 'The immunopathogenesis of chronic obstructive pulmonary disease: insights from recent research', *Proc Am Thorac Soc,* 4, (7), pp. 512-21.

Davidson, D. J., Dorin, J. R., McLachlan, G., Ranaldi, V., Lamb, D., Doherty, C., Govan, J. and Porteous, D. J. (1995) 'Lung disease in the cystic fibrosis mouse exposed to bacterial pathogens', *Nat Genet,* 9, (4), pp. 351-7.

Davidson, D. J. and Rolfe, M. (2001) 'Mouse models of cystic fibrosis', *Trends Genet,* 17, (10), pp. S29-37.

Davies, D. E. (2009) 'The role of the epithelium in airway remodeling in asthma', *Proc Am Thorac Soc,* 6, (8), pp. 678-82.

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. and Greenberg, E. P. (1998) 'The involvement of cell-to-cell signals in the development of a bacterial biofilm', *Science,* 280, (5361), pp. 295-8.

Davies, J. C., Alton, E. W. and Bush, A. (2007) 'Cystic fibrosis', *BMJ,* 335, (7632), pp. 1255-9.

Davis, P. B. (2006) 'Cystic fibrosis since 1938', *Am J Respir Crit Care Med,* 173, (5), pp. 475-82.

de Boer, O. J., van der Meer, J. J., Teeling, P., van der Loos, C. M., Idu, M. M., van Maldegem, F., Aten, J. and van der Wal, A. C. (2010) 'Differential expression of interleukin-17 family cytokines in intact and complicated human atherosclerotic plaques', *J Pathol,* 220, (4), pp. 499-508.

de Jong, E., Suddason, T. and Lord, G. M. (2010) 'Translational mini-review series on Th17 cells: development of mouse and human T helper 17 cells', *Clin Exp Immunol,* 159, (2), pp. 148-58.

de Jong, P. M., van Sterkenburg, M. A., Kempenaar, J. A., Dijkman, J. H. and Ponec, M. (1993) 'Serial culturing of human bronchial epithelial cells derived from biopsies', *In Vitro Cell Dev Biol Anim,* 29A, (5), pp. 379-87.

De Soyza, A., Silipo, A., Lanzetta, R., Govan, J. R. and Molinaro, A. (2008) 'Chemical and biological features of Burkholderia cepacia complex lipopolysaccharides', *Innate Immun,* 14, (3), pp. 127-44.

Delaney, S. J., Alton, E. W., Smith, S. N., Lunn, D. P., Farley, R., Lovelock, P. K., Thomson, S. A., Hume, D. A., Lamb, D., Porteous, D. J., Dorin, J. R. and Wainwright, B. J. (1996) 'Cystic fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations', *Embo J,* 15, (5), pp. 955-63.

Derichs, N. Z., I. Hyde, R. Langer, F. Simon, A. Ballmann, M. (2009) 'Human lung explant tissue as a new ex vivo model for preclinical optimisation of CFTR pharmacotherapy*', New Frontiers in Basic Science of Cystic Fibrosis.* Tavira, Portugal, 15-19 April 2009. Human lung explant tissue as a new ex vivo model for preclinical optimisation of CFTR pharmacotherapy: European Cystic Fibrosis Society, pp.

Di, A., Brown, M. E., Deriy, L. V., Li, C., Szeto, F. L., Chen, Y., Huang, P., Tong, J., Naren, A. P., Bindokas, V., Palfrey, H. C. and Nelson, D. J. (2006) 'CFTR regulates phagosome acidification in macrophages and alters bactericidal activity', *Nat Cell Biol,* 8, (9), pp. 933-44.

Di Sant'Agnese, P. A. (1956) 'Fibrocytic disease of the pancreas, a generalized disease of exocrine glands', *JAMA,* 160, (1), pp. 186.

Di Sant'Agnese, P. A., R. C. Darling, G.A. Perera and E. Shea. (1953) 'Abnormal electrolyte composition of sweat in cystic fibrosis of the pancreas. Clinical significance and relationship to disease', *Pediatrics,* 12, (1), pp. 549-563.

Dickinson, P., Smith, S. N., Webb, S., Kilanowski, F. M., Campbell, I. J., Taylor, M. S., Porteous, D. J., Willemsen, R., de Jonge, H. R., Farley, R., Alton, E. W. and Dorin, J. R. (2002) 'The severe G480C cystic fibrosis mutation, when replicated in the mouse, demonstrates mistrafficking, normal survival and organ-specific bioelectrics', *Hum Mol Genet,* 11, (3), pp. 243-51.

Dodge, J. (2004) 'Storing CF Tissues', *J Cyst Fibros,* 3, (3), pp. 141.

Dodge, J. A., Lewis, P. A., Stanton, M. and Wilsher, J. (2007) 'Cystic fibrosis mortality and survival in the UK: 1947-2003', *Eur Respir J,* 29, (3), pp. 522-6.

Doherty, G. M., Christie, S. N., Skibinski, G., Puddicombe, S. M., Warke, T. J., de Courcey, F., Cross, A. L., Lyons, J. D., Ennis, M., Shields, M. D. and Heaney, L. G. (2003) 'Non-bronchoscopic sampling and culture of bronchial epithelial cells in children', *Clin Exp Allergy,* 33, (9), pp. 1221-5.

Donaldson, S. H., Bennett, W. D., Zeman, K. L., Knowles, M. R., Tarran, R. and Boucher, R. C. (2006) 'Mucus clearance and lung function in cystic fibrosis with hypertonic saline', *N Engl J Med,* 354, (3), pp. 241-50.

Dorin, J. R., Dickinson, P., Emslie, E., Clarke, A. R., Dobbie, L., Hooper, M. L., Halford, S., Wainwright, B. J. and Porteous, D. J. (1992) 'Successful targeting of the mouse cystic fibrosis transmembrane conductance regulator gene in embryonal stem cells', *Transgenic Res,* 1, (2), pp. 101-5.

Doring, G., Elborn, J. S., Johannesson, M., de Jonge, H., Griese, M., Smyth, A. and Heijerman, H. (2007) 'Clinical trials in cystic fibrosis', *J Cyst Fibros,* 6, (2), pp. 85-99.

Downey, D. G., Bell, S. C. and Elborn, J. S. (2009) 'Neutrophils in cystic fibrosis', *Thorax,* 64, (1), pp. 81-8.

Downing, M. and Pollitt, R. (2008) 'Newborn bloodspot screening in the UK--past, present and future', *Ann Clin Biochem,* 45, (Pt 1), pp. 11-7.

Du, M., Jones, J. R., Lanier, J., Keeling, K. M., Lindsey, J. R., Tousson, A., Bebok, Z., Whitsett, J. A., Dey, C. R., Colledge, W. H., Evans, M. J., Sorscher, E. J. and Bedwell, D. M. (2002) 'Aminoglycoside suppression of a premature stop mutation in a Cftr-/- mouse carrying a human CFTR-G542X transgene', *J Mol Med,* 80, (9), pp. 595-604.

Du, M., Liu, X., Welch, E. M., Hirawat, S., Peltz, S. W. and Bedwell, D. M. (2008) 'PTC124 is an orally bioavailable compound that promotes suppression of the human CFTR-G542X nonsense allele in a CF mouse model', *Proc Natl Acad Sci U S A,* 105, (6), pp. 2064-9.

Dubin, P. J., McAllister, F. and Kolls, J. K. (2007) 'Is cystic fibrosis a TH17 disease?', *Inflamm Res,* 56, (6), pp. 221-7.

Dufresne, V., Knoop, C., Van Muylem, A., Malfroot, A., Lamotte, M., Opdekamp, C., Deboeck, G., Cassart, M., Stallenberg, B., Casimir, G., Duchateau, J. and Estenne, M. (2009) 'Effect of systemic inflammation on inspiratory and limb muscle strength and bulk in cystic fibrosis', *Am J Respir Crit Care Med,* 180, (2), pp. 153-8.

Dupuit, F., Kalin, N., Brezillon, S., Hinnrasky, J., Tummler, B. and Puchelle, E. (1995) 'CFTR and differentiation markers expression in non-CF and delta F 508 homozygous CF nasal epithelium', *J Clin Invest,* 96, (3), pp. 1601-11.

Durieu, I., Peyrol, S., Gindre, D., Bellon, G., Durand, D. V. and Pacheco, Y. (1998) 'Subepithelial fibrosis and degradation of the bronchial extracellular matrix in cystic fibrosis', *Am J Respir Crit Care Med,* 158, (2), pp. 580-8.

Eastham, K. M., Fall, A. J., Mitchell, L. and Spencer, D. A. (2004) 'The need to redefine non-cystic fibrosis bronchiectasis in childhood', *Thorax,* 59, (4), pp. 324-7.

Elizur, A., Cannon, C. L. and Ferkol, T. W. (2008) 'Airway inflammation in cystic fibrosis', *Chest,* 133, (2), pp. 489-95.

Elkins, M. R., Robinson, M., Rose, B. R., Harbour, C., Moriarty, C. P., Marks, G. B., Belousova, E. G., Xuan, W. and Bye, P. T. (2006) 'A controlled trial of long-term inhaled hypertonic saline in patients with cystic fibrosis', *N Engl J Med,* 354, (3), pp. 229-40.

Farber, S. (1944) 'Panceatic function and disease in early life v. pathologic changes associated with pancreatic insufficiency early in life', *Arch Pathol Lab Med,* 37, (1), pp. 328.

Farrell, P. M., Kosorok, M. R., Laxova, A., Shen, G., Koscik, R. E., Bruns, W. T., Splaingard, M. and Mischler, E. H. (1997) 'Nutritional benefits of neonatal screening for cystic fibrosis. Wisconsin Cystic Fibrosis Neonatal Screening Study Group', *N Engl J Med,* 337, (14), pp. 963-9.

Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H. and Prince, A. (1998) 'Role of flagella in pathogenesis of Pseudomonas aeruginosa pulmonary infection', *Infect Immun,* 66, (1), pp. 43-51.

Ferretti, S., Bonneau, O., Dubois, G. R., Jones, C. E. and Trifilieff, A. (2003) 'IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger', *J Immunol,* 170, (4), pp. 2106-12.

Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) 'A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues', *J Biol Chem,* 226, pp. 497-509.

Forrest, I. A., Murphy, D. M., Ward, C., Jones, D., Johnson, G. E., Archer, L., Gould, F. K., Cawston, T. E., Lordan, J. L. and Corris, P. A. (2005) 'Primary airway epithelial cell culture from lung transplant recipients', *Eur Respir J,* 26, (6), pp. 1080-5.

Fuchs, B., Suss, R. and Schiller, J. (2010) 'An update of MALDI-TOF mass spectrometry in lipid research', *Prog Lipid Res*.

Fulcher M. L., S. G., K. A. Burns, J. R. Yankaskas and S. H. Randell. (2005) 'Well-Differentiated Human Airway Epithelial Cell Cultures', in J., P.(ed), *Methods In Molecular Medicine.* Vol. 107 Humana Press Inc: Totowa, pp. 183-206.

Fulcher, M. L., Gabriel, S., Burns, K. A., Yankaskas, J. R. and Randell, S. H. (2005) 'Well-Differentiated Human Airway Epithelial Cell Cultures', *Methods In Molecular Medicine,* 107, pp. 183-206.

Fulcher, M. L., Gabriel, S. E., Olsen, J. C., Tatreau, J. R., Gentzsch, M., Livanos, E., Saavedra, M. T., Salmon, P. and Randell, S. H. (2009) 'Novel human bronchial epithelial cell lines for cystic fibrosis research', *Am J Physiol Lung Cell Mol Physiol,* 296, (1), pp. L82-91.

Gabay, C. (2006) 'Interleukin-6 and chronic inflammation', *Arthritis Res Ther,* 8 Suppl 2, pp. S3.

Gaffen, S. L. (2009) 'Structure and signalling in the IL-17 receptor family', *Nat Rev Immunol,* 9, (8), pp. 556-67.

Gaggar, A., Li, Y., Weathington, N., Winkler, M., Kong, M., Jackson, P., Blalock, J. E. and Clancy, J. P. (2007) 'Matrix metalloprotease-9 dysregulation in lower airway secretions of cystic fibrosis patients', *Am J Physiol Lung Cell Mol Physiol,* 293, (1), pp. L96-L104.

Garcia-Medina, R., Dunne, W. M., Singh, P. K. and Brody, S. L. (2005) 'Pseudomonas aeruginosa acquires biofilm-like properties within airway epithelial cells', *Infect Immun,* 73, (12), pp. 8298-305.

Gardner, A., Borthwick, L. A. and Fisher, A. J. (2010) 'Lung epithelial wound healing in health and disease', *Expert review of respiratory medicine,* In press.

Glader, P., Smith, M. E., Malmhall, C., Balder, B., Sjostrand, M., Qvarfordt, I. and Linden, A. (2010) 'IL-17-producing T helper cells and Th17 Cytokines in human airways exposed to endotoxin', *Eur Respir J*, pp. Feb 25. [Epub ahead of print].

Goldman, M. J., Anderson, G. M., Stolzenberg, E. D., Kari, U. P., Zasloff, M. and Wilson, J. M. (1997) 'Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis', *Cell,* 88, (4), pp. 553-60.

Govan, J. R. and Deretic, V. (1996) 'Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia', *Microbiol Rev,* 60, (3), pp. 539-74.

Grassme, H., Jendrossek, V., Riehle, A., von Kurthy, G., Berger, J., Schwarz, H., Weller, M., Kolesnick, R. and Gulbins, E. (2003) 'Host defense against Pseudomonas aeruginosa requires ceramide-rich membrane rafts', *Nat Med,* 9, (3), pp. 322-30.

Grassme, H., Riehle, A., Wilker, B. and Gulbins, E. (2005) 'Rhinoviruses infect human epithelial cells via ceramide-enriched membrane platforms', *J Biol Chem,* 280, (28), pp. 26256-62.

Gray, M. A., Winpenny, J. P., Verdon, B., McAlroy, H. and Argent, B. E. (1995) 'Chloride channels and cystic fibrosis of the pancreas', *Biosci Rep,* 15, (6), pp. 531-41.

Griese, M., Kappler, M., Gaggar, A. and Hartl, D. (2008) 'Inhibition of airway proteases in cystic fibrosis lung disease', *Eur Respir J,* 32, (3), pp. 783-95.

Grimbacher, B., Holland, S. M., Gallin, J. I., Greenberg, F., Hill, S. C., Malech, H. L., Miller, J. A., O'Connell, A. C. and Puck, J. M. (1999) 'Hyper-IgE syndrome with recurrent infections - An autosomal dominant multisystem disorder', *New England Journal of Medicine,* 340, (9), pp. 692-702.

Grubb, B. R. and Boucher, R. C. (1999) 'Pathophysiology of gene-targeted mouse models for cystic fibrosis', *Physiol Rev,* 79, (1 Suppl), pp. S193-214.

Grubb, B. R., Paradiso, A. M. and Boucher, R. C. (1994) 'Anomalies in ion transport in CF mouse tracheal epithelium', *Am J Physiol,* 267, (1 Pt 1), pp. C293-300.

Gruenert, D. C., Finkbeiner, W. E. and Widdicombe, J. H. (1995) 'Culture and transformation of human airway epithelial cells', *Am J Physiol,* 268, (3 Pt 1), pp. L347-60.

Gruenert, D. C., Willems, M., Cassiman, J. J. and Frizzell, R. A. (2004) 'Established cell lines used in cystic fibrosis research', *J Cyst Fibros,* 3 Suppl 2, pp. 191-6.

Guilbault, C., De Sanctis, J. B., Wojewodka, G., Saeed, Z., Lachance, C., Skinner, T. A., Vilela, R. M., Kubow, S., Lands, L. C., Hajduch, M., Matouk, E. and Radzioch, D. (2008a) 'Fenretinide corrects newly found ceramide deficiency in cystic fibrosis', *Am J Respir Cell Mol Biol,* 38, (1), pp. 47-56.

Guilbault, C., Saeed, Z., Downey, G. P. and Radzioch, D. (2007) 'Cystic fibrosis mouse models', *Am J Respir Cell Mol Biol,* 36, (1), pp. 1-7.

Guilbault, C., Wojewodka, G., Saeed, Z., Hajduch, M., Matouk, E., De Sanctis, J. B. and Radzioch, D. (2008b) 'Cystic Fibrosis Fatty Acid Imbalance is Linked to Ceramide Deficiency and Corrected by Fenretinide', *Am J Respir Cell Mol Biol,* 41, (1), pp. 100-6.

Gulbins, E., Dreschers, S., Wilker, B. and Grassme, H. (2004) 'Ceramide, membrane rafts and infections', *J Mol Med,* 82, (6), pp. 357-63.

Haggie, P. M. and Verkman, A. S. (2009a) 'Defective Organellar Acidification as a Cause of Cystic Fibrosis Lung Disease - Re-Examination of a Recurring Hypothesis', *Am J Physiol Lung Cell Mol Physiol,* 296, (6), pp. L859-67.

Haggie, P. M. and Verkman, A. S. (2009b) 'Unimpaired lysosomal acidification in respiratory epithelial cells in cystic fibrosis', *J Biol Chem,* 284, (12), pp. 7681-6.

Hamai, H., Keyserman, F., Quittell, L. M. and Worgall, T. S. (2009) 'Defective CFTR increases synthesis and mass of sphingolipids that modulate membrane composition and lipid signaling', *J Lipid Res,* 50, (6), pp. 1101-8.

Hamid, Q. and Tulic, M. (2009) 'Immunobiology of asthma', *Annu Rev Physiol,* 71, pp. 489-507.

Hamutcu, R., Rowland, J. M., Horn, M. V., Kaminsky, C., MacLaughlin, E. F., Starnes, V. A. and Woo, M. S. (2002) 'Clinical findings and lung pathology in children with cystic fibrosis', *Am J Respir Crit Care Med,* 165, (8), pp. 1172-5.

Hannun, Y. A. and Obeid, L. M. (2002) 'The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind', *J Biol Chem,* 277, (29), pp. 25847-50.

Hannun, Y. A. and Obeid, L. M. (2008) 'Principles of bioactive lipid signalling: lessons from sphingolipids', *Nat Rev Mol Cell Biol,* 9, (2), pp. 139-50.

Harris, W. T., Muhlebach, M. S., Oster, R. A., Knowles, M. R. and Noah, T. L. (2009) 'Transforming growth factor-beta(1) in bronchoalveolar lavage fluid from children with cystic fibrosis', *Pediatr Pulmonol,* 44, (11), pp. 1057-64.

Hartzell, C., Putzier, I. and Arreola, J. (2005) 'Calcium-activated chloride channels', *Annu Rev Physiol,* 67, (1), pp. 719-58.

Haslett, C. (1999) 'Granulocyte apoptosis and its role in the resolution and control of lung inflammation', *Am J Respir Crit Care Med,* 160, (5 Pt 2), pp. S5-11.

Haslett, C., Savill, J. S. and Meagher, L. (1989) 'The neutrophil', *Curr Opin Immunol,* 2, (1), pp. 10-8.

Hasty, P., O'Neal, W. K., Liu, K. Q., Morris, A. P., Bebok, Z., Shumyatsky, G. B., Jilling, T., Sorscher, E. J., Bradley, A. and Beaudet, A. L. (1995) 'Severe phenotype in mice with termination mutation in exon 2 of cystic fibrosis gene', *Somat Cell Mol Genet,* 21, (3), pp. 177-87.

Haus, J. M., Kashyap, S. R., Kasumov, T., Zhang, R., Kelly, K. R., Defronzo, R. A. and Kirwan, J. P. (2009) 'Plasma ceramides are elevated in obese subjects with type 2 diabetes and correlate with the severity of insulin resistance', *Diabetes,* 58, (2), pp. 337-43.

Hays, S. R. and Fahy, J. V. (2006) 'Characterizing mucous cell remodeling in cystic fibrosis: relationship to neutrophils', *Am J Respir Crit Care Med,* 174, (9), pp. 1018-24.

Hilliard, T. N., Regamey, N., Shute, J. K., Nicholson, A. G., Alton, E. W., Bush, A. and Davies, J. C. (2007) 'Airway remodelling in children with cystic fibrosis', *Thorax,* 62, (12), pp. 1074-80.

Holgate, S. T. (2008) 'Pathogenesis of asthma', *Clin Exp Allergy,* 38, (6), pp. 872-97.

Holgate, S. T. and Polosa, R. (2008) 'Treatment strategies for allergy and asthma', *Nat Rev Immunol,* 8, (3), pp. 218-30.

Holland, S. M., DeLeo, F. R., Elloumi, H. Z., Hsu, A. P., Uzel, G., Brodsky, N., Freeman, A. F., Demidowich, A., Davis, J., Turner, M. L., Anderson, V. L., Darnell, D. N., Welch, P. A., Kuhns, D. B., Frucht, D. M., Malech, H. L., Gallin, J. I., Kobayashi, S. D., Whitney, A. R., Voyich, J. M., Musser, J. M., Woellner, C., Schaffer, A. A., Puck, J. M. and Grimbacher, B. (2007) 'STAT3 mutations in the hyper-IgE syndrome', *N Engl J Med,* 357, (16), pp. 1608-19.

Holm, S. (1979) 'A simple sequentially rejective multiple test procedure', *Scandinavian Journal of Statistics,* 6, (2), pp. 65-70.

Holt, P. G. (2005) 'Pulmonary dendritic cells in local immunity to inert and pathogenic antigens in the respiratory tract', *Proc Am Thorac Soc,* 2, (2), pp. 116-20.

Hoshino, A., Nagao, T., Nagi-Miura, N., Ohno, N., Yasuhara, M., Yamamoto, K., Nakayama, T. and Suzuki, K. (2008) 'MPO-ANCA induces IL-17 production by

activated neutrophils in vitro via classical complement pathway-dependent manner', *J Autoimmun,* 31, (1), pp. 79-89.

Hoshino, H., Laan, M., Sjostrand, M., Lotvall, J., Skoogh, B. E. and Linden, A. (2000) 'Increased elastase and myeloperoxidase activity associated with neutrophil recruitment by IL-17 in airways in vivo', *J Allergy Clin Immunol,* 105, (1 Pt 1), pp. 143-9.

Hubeau, C., Lorenzato, M., Couetil, J. P., Hubert, D., Dusser, D., Puchelle, E. and Gaillard, D. (2001a) 'Quantitative analysis of inflammatory cells infiltrating the cystic fibrosis airway mucosa', *Clin Exp Immunol,* 124, (1), pp. 69-76.

Hubeau, C., Puchelle, E. and Gaillard, D. (2001b) 'Distinct pattern of immune cell population in the lung of human fetuses with cystic fibrosis', *J Allergy Clin Immunol,* 108, (4), pp. 524-9.

Hueber, A. J., Asquith, D. L., Miller, A. M., Reilly, J., Kerr, S., Leipe, J., Melendez, A. J. and McInnes, I. B. (2010) 'Mast cells express IL-17A in rheumatoid arthritis synovium', *J Immunol,* 184, (7), pp. 3336-40.

Hwang, T. C. and Sheppard, D. N. (2009) 'Gating of the CFTR Cl- channel by ATP-driven nucleotide-binding domain dimerisation', *J Physiol,* 587, (Pt 10), pp. 2151-61.

Illek, B., Lei, D., Fischer, H. and Gruenert, D. C. (2010) 'Sensitivity of chloride efflux vs. transepithelial measurements in mixed CF and normal airway epithelial cell populations', *Cell Physiol Biochem,* 26, (6), pp. 983-90.

Imundo, L., Barasch, J., Prince, A. and Al-Awqati, Q. (1995) 'Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface', *Proc Natl Acad Sci U S A,* 92, (7), pp. 3019-23.

Ivanov, II, McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J. and Littman, D. R. (2006) 'The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells', *Cell,* 126, (6), pp. 1121-33.

Jackson, A., Foley, L., Daly, L., Fitzpatrick, P., Harrington, M., Zhou, S. and Kelleher, C. (2010) 'Delayed cystic fibrosis presentation in children in the absence of newborn screening', *Ir Med J,* 103, (4), pp. 113-6.

Jaffar, Z. H., Stanciu, L., Pandit, A., Lordan, J., Holgate, S. T. and Roberts, K. (1999) 'Essential role for both CD80 and CD86 costimulation, but not CD40 interactions, in allergen-induced Th2 cytokine production from asthmatic

bronchial tissue: role for alphabeta, but not gammadelta, T cells', *J Immunol,* 163, (11), pp. 6283-91.

James, A. L., Maxwell, P. S., Pearce-Pinto, G., Elliot, J. G. and Carroll, N. G. (2002) 'The relationship of reticular basement membrane thickness to airway wall remodeling in asthma', *Am J Respir Crit Care Med,* 166, (12 Pt 1), pp. 1590-5.

Jeffery, P., Holgate, S. and Wenzel, S. (2003) 'Methods for the assessment of endobronchial biopsies in clinical research: application to studies of pathogenesis and the effects of treatment', *Am J Respir Crit Care Med,* 168, (6 Pt 2), pp. S1-17.

Jeffery, P. K. (2001) 'Remodeling in asthma and chronic obstructive lung disease', *Am J Respir Crit Care Med,* 164, (10 Pt 2), pp. S28-38.

Jones, A. P. and Wallis, C. E. (2003) 'Recombinant human deoxyribonuclease for cystic fibrosis', *Cochrane Database Syst Rev*, (3), pp. CD001127.

Joseph, T., Look, D. and Ferkol, T. (2005) 'NF-kappaB activation and sustained IL-8 gene expression in primary cultures of cystic fibrosis airway epithelial cells stimulated with Pseudomonas aeruginosa', *Am J Physiol Lung Cell Mol Physiol,* 288, (3), pp. L471-9.

Jurkuvenaite, A., Chen, L., Bartoszewski, R., Goldstein, R., Bebok, Z., Matalon, S. and Collawn, J. F. (2009) 'Functional Stability of Rescued {Delta}F508 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in Airway Epithelial Cells', *Am J Respir Cell Mol Biol*.

Kabra, S. K., Kabra, M., Lodha, R. and Shastri, S. (2007) 'Cystic fibrosis in India', *Pediatr Pulmonol,* 42, (12), pp. 1087-94.

Kabra, S. K., Kabra, M., Shastri, S. and Lodha, R. (2006) 'Diagnosing and managing cystic fibrosis in the developing world', *Paediatr Respir Rev,* 7 Suppl 1, pp. S147-50.

Kai, J., Ulph, F., Cullinan, T. and Qureshi, N. (2009) 'Communication of carrier status information following universal newborn screening for sickle cell disorders and cystic fibrosis: qualitative study of experience and practice', *Health Technol Assess,* 13, (57), pp. 1-82, iii.

Karp, P. H., Moninger, T. O., Weber, S. P., Nesselhauf, T. S., Launspach, J. L., Zabner, J. and Welsh, M. J. (2002) 'An In Vitro Model of Differentiated Human Airway Epithelia', in Wise, C.(ed), *Epithelial Cell Culture Protocols.* Vol. 188 Humana Press: New York City, pp. 115-37.

Kerem, B., Rommens, J. M., Buchanan, J. A., Markiewicz, D., Cox, T. K., Chakravarti, A., Buchwald, M. and Tsui, L. C. (1989) 'Identification of the cystic fibrosis gene: genetic analysis', *Science,* 245, (4922), pp. 1073-80.

Kerem, E., Hirawat, S., Armoni, S., Yaakov, Y., Shoseyov, D., Cohen, M., Nissim-Rafinia, M., Blau, H., Rivlin, J., Aviram, M., Elfring, G. L., Northcutt, V. J., Miller, L. L., Kerem, B. and Wilschanski, M. (2008) 'Effectiveness of PTC124 treatment of cystic fibrosis caused by nonsense mutations: a prospective phase II trial', *Lancet,* 372, (9640), pp. 719-27.

Kettle, A. J., Chan, T., Osberg, I., Senthilmohan, R., Chapman, A. L., Mocatta, T. J. and Wagener, J. S. (2004) 'Myeloperoxidase and protein oxidation in the airways of young children with cystic fibrosis', *Am J Respir Crit Care Med,* 170, (12), pp. 1317-23.

Khan, T. Z., Wagener, J. S., Bost, T., Martinez, J., Accurso, F. J. and Riches, D. W. (1995) 'Early pulmonary inflammation in infants with cystic fibrosis', *Am J Respir Crit Care Med,* 151, (4), pp. 1075-82.

Kiernan, J. (2008) *Histological and histochemical methods: theory and practice.* Bloxham: London.

Konstan, M. W. and Berger, M. (1997) 'Current understanding of the inflammatory process in cystic fibrosis: onset and etiology', *Pediatr Pulmonol,* 24, (2), pp. 137-42; discussion 159-61.

Korkmaz, B., Moreau, T. and Gauthier, F. (2008) 'Neutrophil elastase, proteinase 3 and cathepsin G: physicochemical properties, activity and physiopathological functions', *Biochimie,* 90, (2), pp. 227-42.

Kreindler, J. L., Bertrand, C. A., Lee, R. J., Karasic, T., Aujla, S., Pilewski, J. M., Frizzell, R. A. and Kolls, J. K. (2009) 'Interleukin-17A induces bicarbonate secretion in normal human bronchial epithelial cells', *Am J Physiol Lung Cell Mol Physiol,* 296, (2), pp. L257-66.

Kroesen, B. J., Jacobs, S., Pettus, B. J., Sietsma, H., Kok, J. W., Hannun, Y. A. and de Leij, L. F. (2003) 'BcR-induced apoptosis involves differential regulation of C16 and C24-ceramide formation and sphingolipid-dependent activation of the proteasome', *J Biol Chem,* 278, (17), pp. 14723-31.

Kunzelmann, K., Scheidt, K., Scharf, B., Ousingsawat, J., Schreiber, R., Wainwright, B. and McMorran, B. (2006) 'Flagellin of Pseudomonas aeruginosa inhibits Na+ transport in airway epithelia', *Faseb J,* 20, (3), pp. 545-6.

Laan, M., Palmberg, L., Larsson, K. and Linden, A. (2002) 'Free, soluble interleukin-17 protein during severe inflammation in human airways', *Eur Respir J,* 19, (3), pp. 534-7.

Lane, C., Burgess, S., Kicic, A., Knight, D. and Stick, S. (2005) 'The use of non-bronchoscopic brushings to study the paediatric airway', *Respir Res,* 6, pp. 53.

Laurence, A. and O'Shea, J. J. (2007) 'T(H)-17 differentiation: of mice and men', *Nat Immunol,* 8, (9), pp. 903-5.

Le Gouvello, S., Bastuji-Garin, S., Aloulou, N., Mansour, H., Chaumette, M. T., Berrehar, F., Seikour, A., Charachon, A., Karoui, M., Leroy, K., Farcet, J. P. and Sobhani, I. (2008) 'High prevalence of Foxp3 and IL17 in MMR-proficient colorectal carcinomas', *Gut,* 57, (6), pp. 772-9.

Lechner, J. F., Haugen, A., Autrup, H., McClendon, I. A., Trump, B. F. and Harris, C. C. (1981) 'Clonal growth of epithelial cells from normal adult human bronchus', *Cancer Res,* 41, (6), pp. 2294-304.

Lemmers, A., Moreno, C., Gustot, T., Marechal, R., Degre, D., Demetter, P., de Nadai, P., Geerts, A., Quertinmont, E., Vercruysse, V., Le Moine, O. and Deviere, J. (2009) 'The interleukin-17 pathway is involved in human alcoholic liver disease', *Hepatology,* 49, (2), pp. 646-57.

Li, L., Huang, L., Vergis, A. L., Ye, H., Bajwa, A., Narayan, V., Strieter, R. M., Rosin, D. L. and Okusa, M. D. (2010) 'IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury', *J Clin Invest,* 120, (1), pp. 331-42.

Liesker, J. J., Ten Hacken, N. H., Zeinstra-Smith, M., Rutgers, S. R., Postma, D. S. and Timens, W. (2009) 'Reticular basement membrane in asthma and COPD: similar thickness, yet different composition', *Int J Chron Obstruct Pulmon Dis,* 4, pp. 127-35.

Linden, A., Laan, M. and Anderson, G. P. (2005) 'Neutrophils, interleukin-17A and lung disease', *Eur Respir J,* 25, (1), pp. 159-72.

Lindner, K., Uhlig, U. and Uhlig, S. (2005) 'Ceramide alters endothelial cell permeability by a nonapoptotic mechanism', *Br J Pharmacol,* 145, (1), pp. 132-40.

Lorenz, J., Zahlten, J., Pollok, I., Lippmann, J., Scharf, S., N'Guessan P, D., Opitz, B., Flieger, A., Suttorp, N., Hippenstiel, S. and Schmeck, B. (2010) 'Legionella pneumophila induced i{kappa}b{zeta}-dependent expression of il-6 in lung epithelium', *Eur Respir J.*

Lundberg, A. S., Randell, S. H., Stewart, S. A., Elenbaas, B., Hartwell, K. A., Brooks, M. W., Fleming, M. D., Olsen, J. C., Miller, S. W., Weinberg, R. A. and Hahn, W. C. (2002) 'Immortalization and transformation of primary human airway epithelial cells by gene transfer', *Oncogene,* 21, (29), pp. 4577-86.

Lundgren, R., Soderberg, M., Horstedt, P. and Stenling, R. (1988) 'Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids', *Eur Respir J,* 1, (10), pp. 883-9.

Ma, T., Vetrivel, L., Yang, H., Pedemonte, N., Zegarra-Moran, O., Galietta, L. J. and Verkman, A. S. (2002) 'High-affinity activators of cystic fibrosis transmembrane conductance regulator (CFTR) chloride conductance identified by high-throughput screening', *J Biol Chem,* 277, (40), pp. 37235-41.

Major, E. H. and Elborn, J. S. (2009) 'Inflammation, Hyperinflammation & Cystic Fibrosis Lung Disease - A Paradigm Shift?', *Curr Respir Med Rev,* 5, (3), pp. 136-148.

Mall, M., Gonska, T., Thomas, J., Schreiber, R., Seydewitz, H. H., Kuehr, J., Brandis, M. and Kunzelmann, K. (2003) 'Modulation of Ca2+-activated Cl- secretion by basolateral K+ channels in human normal and cystic fibrosis airway epithelia', *Pediatr Res,* 53, (4), pp. 608-18.

Mall, M., Grubb, B. R., Harkema, J. R., O'Neal, W. K. and Boucher, R. C. (2004) 'Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice', *Nat Med,* 10, (5), pp. 487-93.

Mall, M. A., Harkema, J. R., Trojanek, J. B., Treis, D., Livraghi, A., Schubert, S., Zhou, Z., Kreda, S. M., Tilley, S. L., Hudson, E. J., O'Neal, W. K. and Boucher, R. C. (2008) 'Development of chronic bronchitis and emphysema in beta-epithelial Na+ channel-overexpressing mice', *Am J Respir Crit Care Med,* 177, (7), pp. 730-42.

Mallory, G. B., Jr. (2006) 'Pitfalls in non-therapeutic research in children', *Pediatr Pulmonol,* 41, (11), pp. 1014-6; discussion 1017-20.

Manes, S., del Real, G. and Martinez, A. C. (2003) 'Pathogens: raft hijackers', *Nat Rev Immunol,* 3, (7), pp. 557-68.

Mann, H. B. and Whitney, D. R. (1947) 'On a test whether one of two random variables is statistically larger than the other', *The Annals of Mathematical Statistics,* 18, pp. 50-60.

Marcus, R., Peritz, E. and Gabriel, K. R. (1976) 'On closed testing procedures with special reference to ordered analysis of variance', *Biometrika,* 63, pp. 655-660.

Martin, T. R. and Frevert, C. W. (2005) 'Innate immunity in the lungs', *Proc Am Thorac Soc,* 2, (5), pp. 403-11.

Matsui, H., Grubb, B. R., Tarran, R., Randell, S. H., Gatzy, J. T., Davis, C. W. and Boucher, R. C. (1998) 'Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease', *Cell,* 95, (7), pp. 1005-15.

Matsui, H., Wagner, V. E., Hill, D. B., Schwab, U. E., Rogers, T. D., Button, B., Taylor, R. M., 2nd, Superfine, R., Rubinstein, M., Iglewski, B. H. and Boucher, R. C. (2006) 'A physical linkage between cystic fibrosis airway surface dehydration and Pseudomonas aeruginosa biofilms', *Proc Natl Acad Sci U S A,* 103, (48), pp. 18131-6.

Maxwell, A. I., Morrison, G. M. and Dorin, J. R. (2003) 'Rapid sequence divergence in mammalian beta-defensins by adaptive evolution', *Mol Immunol,* 40, (7), pp. 413-21.

McAllister, F., Henry, A., Kreindler, J. L., Dubin, P. J., Ulrich, L., Steele, C., Finder, J. D., Pilewski, J. M., Carreno, B. M., Goldman, S. J., Pirhonen, J. and Kolls, J. K. (2005) 'Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-alpha and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis', *J Immunol,* 175, (1), pp. 404-12.

McDougall, C. M., Blaylock, M. G., Douglas, J. G., Brooker, R. J., Helms, P. J. and Walsh, G. M. (2008) 'Nasal epithelial cells as surrogates for bronchial epithelial cells in airway inflammation studies', *Am J Respir Cell Mol Biol,* 39, (5), pp. 560-8.

McFarland, H. F. and Martin, R. (2007) 'Multiple sclerosis: a complicated picture of autoimmunity', *Nat Immunol,* 8, (9), pp. 913-9.

McGeachy, M. J. and Anderton, S. M. (2005) 'Cytokines in the induction and resolution of experimental autoimmune encephalomyelitis', *Cytokine,* 32, (2), pp. 81-4.

McNamara, P. S., Kicic, A., Sutanto, E. N., Stevens, P. T. and Stick, S. M. (2008) 'Comparison of techniques for obtaining lower airway epithelial cells from children', *Eur Respir J,* 32, (3), pp. 763-8.

McNamara, P. S., Ritson, P., Selby, A., Hart, C. A. and Smyth, R. L. (2003) 'Bronchoalveolar lavage cellularity in infants with severe respiratory syncytial virus bronchiolitis', *Arch Dis Child,* 88, (10), pp. 922-6.

Meachery, G., De Soyza, A., Nicholson, A., Parry, G., Hasan, A., Tocewicz, K., Pillay, T., Clark, S., Lordan, J. L., Schueler, S., Fisher, A. J., Dark, J. H., Gould, F. K. and Corris, P. A. (2008) 'Outcomes of Lung transplantation for Cystic Fibrosis in a large United Kingdom cohort', *Thorax,* 63, (8), pp. 725-731.

Mehta, A. (2005) 'CFTR: more than just a chloride channel', *Pediatr Pulmonol,* 39, (4), pp. 292-8.

Meyerholz, D. K., Griffin, M. A., Castilow, E. M. and Varga, S. M. (2009) 'Comparison of histochemical methods for murine eosinophil detection in an RSV vaccine-enhanced inflammation model', *Toxicol Pathol,* 37, (2), pp. 249-55.

Meyerholz, D. K., Stoltz, D. A., Pezzulo, A. A. and Welsh, M. J. (2010) 'Pathology of gastrointestinal organs in a porcine model of cystic fibrosis', *Am J Pathol,* 176, (3), pp. 1377-89.

Michel, M. L., Keller, A. C., Paget, C., Fujio, M., Trottein, F., Savage, P. B., Wong, C. H., Schneider, E., Dy, M. and Leite-de-Moraes, M. C. (2007) 'Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia', *J Exp Med,* 204, (5), pp. 995-1001.

Minshall, E. M., Leung, D. Y., Martin, R. J., Song, Y. L., Cameron, L., Ernst, P. and Hamid, Q. (1997) 'Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma', *Am J Respir Cell Mol Biol,* 17, (3), pp. 326-33.

Moran, O. and Zegarra-Moran, O. (2008) 'On the measurement of the functional properties of the CFTR', *J Cyst Fibros,* 7, (6), pp. 483-94.

Mosler, K., Coraux, C., Fragaki, K., Zahm, J. M., Bajolet, O., Bessaci-Kabouya, K., Puchelle, E., Abely, M. and Mauran, P. (2008) 'Feasibility of nasal epithelial brushing for the study of airway epithelial functions in CF infants', *J Cyst Fibros,* 7, (1), pp. 44-53.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. (1986) 'Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins', *J Immunol,* 136, (7), pp. 2348-57.

Muhlebach, M. S., Stewart, P. W., Leigh, M. W. and Noah, T. L. (1999) 'Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients', *Am J Respir Crit Care Med,* 160, (1), pp. 186-91.

Murphy, D. M. (2008) *The potential role of the airway epithelium and novel therapeutic strategies in post-transplant obliterative bronchiolitis.* thesis. Newcastle University.

Murphy, D. M., Forrest, I. A., Corris, P. A., Johnson, G. E., Small, T., Jones, D., Fisher, A. J., Egan, J. J., Cawston, T. E., Lordan, J. L. and Ward, C. (2008a) 'Azithromycin attenuates effects of lipopolysaccharide on lung allograft bronchial epithelial cells', *J Heart Lung Transplant,* 27, (11), pp. 1210-6.

Murphy, D. M., Forrest, I. A., Corris, P. A., Johnson, G. E., Small, T., Jones, D., Fisher, A. J., Egan, J. J., Cawston, T. E., Ward, C. and Lordan, J. L. (2008b) 'Simvastatin attenuates release of neutrophilic and remodeling factors from primary bronchial epithelial cells derived from stable lung transplant recipients', *Am J Physiol Lung Cell Mol Physiol,* 294, (3), pp. L592-9.

Murphy, T. F., Brauer, A. L., Eschberger, K., Lobbins, P., Grove, L., Cai, X. and Sethi, S. (2008c) 'Pseudomonas aeruginosa in chronic obstructive pulmonary disease', *Am J Respir Crit Care Med,* 177, (8), pp. 853-60.

N'Dow, J., Jordan, N., Robson, C. N., Neal, D. E. and Pearson, J. P. (2005) 'The bladder does not appear to have a dynamic secreted continuous mucous gel layer', *J Urol,* 173, (6), pp. 2025-31.

NC3Rs (2010) *National Centre for the Replacement Refinement and Reduction of Animals in Research*. Available at: http://www.nc3rs.org.uk/ (Accessed: August).

Nembrini, C., Marsland, B. J. and Kopf, M. (2009) 'IL-17-producing T cells in lung immunity and inflammation', *J Allergy Clin Immunol,* 123, (5), pp. 986-94; quiz 995-6.

Niessen, F., Schaffner, F., Furlan-Freguia, C., Pawlinski, R., Bhattacharjee, G., Chun, J., Derian, C. K., Andrade-Gordon, P., Rosen, H. and Ruf, W. (2008) 'Dendritic cell PAR1-S1P3 signalling couples coagulation and inflammation', *Nature,* 452, (7187), pp. 654-8.

Nieuwenhuis, E. E., Matsumoto, T., Exley, M., Schleipman, R. A., Glickman, J., Bailey, D. T., Corazza, N., Colgan, S. P., Onderdonk, A. B. and Blumberg, R. S. (2002) 'CD1d-dependent macrophage-mediated clearance of Pseudomonas aeruginosa from lung', *Nat Med,* 8, (6), pp. 588-93.

Noe, J., Petrusca, D., Rush, N., Deng, P., Vandemark, M., Berdyshev, E., Gu, Y., Smith, P., Schweitzer, K., Pilewsky, J., Natarajan, V., Xu, Z., Obhukov, A. G. and Petrache, I. (2009) 'CFTR Regulation of Intracellular pH and Ceramides is

Required for Lung Endothelial Cell Apoptosis', *Am J Respir Cell Mol Biol,* 41, pp. 314-323.

Novgorodov, S. A. and Gudz, T. I. (2009) 'Ceramide and mitochondria in ischemia/reperfusion', *J Cardiovasc Pharmacol,* 53, (3), pp. 198-208.

O'Neal, W. K., Hasty, P., McCray, P. B., Jr., Casey, B., Rivera-Perez, J., Welsh, M. J., Beaudet, A. L. and Bradley, A. (1993) 'A severe phenotype in mice with a duplication of exon 3 in the cystic fibrosis locus', *Hum Mol Genet,* 2, (10), pp. 1561-9.

O'Sullivan, B. P. and Freedman, S. D. (2009) 'Cystic fibrosis', *Lancet,* 373, (9678), pp. 1891-904.

Oskeritzian, C. A., Milstien, S. and Spiegel, S. (2007) 'Sphingosine-1-phosphate in allergic responses, asthma and anaphylaxis', *Pharmacol Ther,* 115, (3), pp. 390-9.

Ostedgaard, L. S., Baldursson, O. and Welsh, M. J. (2001) 'Regulation of the cystic fibrosis transmembrane conductance regulator Cl- channel by its R domain', *J Biol Chem,* 276, (11), pp. 7689-92.

Pack, R. J., Al-Ugaily, L. H., Morris, G. and Widdicombe, J. G. (1980) 'The distribution and structure of cells in the tracheal epithelium of the mouse', *Cell Tissue Res,* 208, (1), pp. 65-84.

Panickar, J., Lakhanpaul, M., Lambert, P. C., Kenia, P., Stephenson, T., Smyth, A. and Grigg, J. (2009) 'Oral prednisolone for preschool children with acute virus-induced wheezing', *N Engl J Med,* 360, (4), pp. 329-38.

Parker, J., Sarlang, S., Thavagnanam, S., Williamson, G., O'Donoghue, D., Villenave, R., Power, U., Shields, M., Heaney, L. and Skibinski, G. (2010) 'A 3-D well-differentiated model of pediatric bronchial epithelium demonstrates unstimulated morphological differences between asthmatic and nonasthmatic cells', *Pediatr Res,* 67, (1), pp. 17-22.

Parmar, J. S. and Nasser, S. (2005) 'Antibiotic allergy in cystic fibrosis', *Thorax,* 60, (6), pp. 517-20.

Payne, D. N., Rogers, A. V., Adelroth, E., Bandi, V., Guntupalli, K. K., Bush, A. and Jeffery, P. K. (2003) 'Early thickening of the reticular basement membrane in children with difficult asthma', *Am J Respir Crit Care Med,* 167, (1), pp. 78-82.

Pedemonte, N., Lukacs, G. L., Du, K., Caci, E., Zegarra-Moran, O., Galietta, L. J. and Verkman, A. S. (2005) 'Small-molecule correctors of defective DeltaF508-

CFTR cellular processing identified by high-throughput screening', *J Clin Invest,* 115, (9), pp. 2564-71.

Pena, J., Fu, Z., Schwarzer, C. and Machen, T. E. (2009) 'Pseudomonas aeruginosa Inhibition of Flagellin-activated NF-kappaB and interleukin-8 by human airway epithelial cells', *Infect Immun,* 77, (7), pp. 2857-65.

Penque, D., Mendes, F., Beck, S., Farinha, C., Pacheco, P., Nogueira, P., Lavinha, J., Malho, R. and Amaral, M. D. (2000) 'Cystic fibrosis F508del patients have apically localized CFTR in a reduced number of airway cells', *Lab Invest,* 80, (6), pp. 857-68.

Petrache, I., Medler, T. R., Richter, A. T., Kamocki, K., Chukwueke, U., Zhen, L., Gu, Y., Adamowicz, J., Schweitzer, K. S., Hubbard, W. C., Berdyshev, E. V., Lungarella, G. and Tuder, R. M. (2008) 'Superoxide dismutase protects against apoptosis and alveolar enlargement induced by ceramide', *Am J Physiol Lung Cell Mol Physiol,* 295, (1), pp. L44-53.

Petrache, I., Natarajan, V., Zhen, L., Medler, T. R., Richter, A. T., Cho, C., Hubbard, W. C., Berdyshev, E. V. and Tuder, R. M. (2005) 'Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice', *Nat Med,* 11, (5), pp. 491-8.

Pettus, B. J., Bielawska, A., Kroesen, B. J., Moeller, P. D., Szulc, Z. M., Hannun, Y. A. and Busman, M. (2003) 'Observation of different ceramide species from crude cellular extracts by normal-phase high-performance liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry', *Rapid Commun Mass Spectrom,* 17, (11), pp. 1203-11.

Pettus, B. J., Chalfant, C. E. and Hannun, Y. A. (2002) 'Ceramide in apoptosis: an overview and current perspectives', *Biochim Biophys Acta,* 1585, (2-3), pp. 114-25.

Pfeffer, K. D., Huecksteadt, T. P. and Hoidal, J. R. (1993) 'Expression and regulation of tumor necrosis factor in macrophages from cystic fibrosis patients', *Am J Respir Cell Mol Biol,* 9, (5), pp. 511-9.

Pier, G. B. (2007) 'Pseudomonas aeruginosa lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity', *Int J Med Microbiol,* 297, (5), pp. 277-95.

Pier, G. B. (2008) 'Dropping acid to help cystic fibrosis', *Nat Med,* 14, (4), pp. 367-9.

Pier, G. B., Grout, M. and Zaidi, T. S. (1997) 'Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of Pseudomonas aeruginosa from the lung', *Proc Natl Acad Sci U S A,* 94, (22), pp. 12088-93.

Pier, G. B., Grout, M., Zaidi, T. S., Olsen, J. C., Johnson, L. G., Yankaskas, J. R. and Goldberg, J. B. (1996) 'Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections', *Science,* 271, (5245), pp. 64-7.

Pilewski, J. M. and Frizzell, R. A. (1999) 'Role of CFTR in airway disease', *Physiol Rev,* 79, (1 Suppl), pp. S215-55.

Plotkowski, M. C., Costa, A. O., Morandi, V., Barbosa, H. S., Nader, H. B., de Bentzmann, S. and Puchelle, E. (2001) 'Role of heparan sulphate proteoglycans as potential receptors for non-piliated Pseudomonas aeruginosa adherence to non-polarised airway epithelial cells', *J Med Microbiol,* 50, (2), pp. 183-90.

Plotkowski, M. C., de Bentzmann, S., Pereira, S. H., Zahm, J. M., Bajolet-Laudinat, O., Roger, P. and Puchelle, E. (1999) 'Pseudomonas aeruginosa internalization by human epithelial respiratory cells depends on cell differentiation, polarity, and junctional complex integrity', *Am J Respir Cell Mol Biol,* 20, (5), pp. 880-90.

Poschet, J. F., Skidmore, J., Boucher, J. C., Firoved, A. M., Van Dyke, R. W. and Deretic, V. (2002) 'Hyperacidification of cellubrevin endocytic compartments and defective endosomal recycling in cystic fibrosis respiratory epithelial cells', *J Biol Chem,* 277, (16), pp. 13959-65.

Prause, O., Bozinovski, S., Anderson, G. P. and Linden, A. (2004) 'Increased matrix metalloproteinase-9 concentration and activity after stimulation with interleukin-17 in mouse airways', *Thorax,* 59, (4), pp. 313-7.

Prince, A. (2006) 'Flagellar activation of epithelial signaling', *Am J Respir Cell Mol Biol,* 34, (5), pp. 548-51.

Proesmans, M., Vermeulen, F. and De Boeck, K. (2008) 'What's new in cystic fibrosis? From treating symptoms to correction of the basic defect', *Eur J Pediatr*.

Pyne, S. and Pyne, N. J. (2000) 'Sphingosine 1-phosphate signalling in mammalian cells', *Biochem J,* 349, (Pt 2), pp. 385-402.

Quinton, P. M. (2008) 'Cystic fibrosis: impaired bicarbonate secretion and mucoviscidosis', *Lancet,* 372, (9636), pp. 415-7.

Rakonczay, Z., Jr., Hegyi, P., Hasegawa, M., Inoue, M., You, J., Iida, A., Ignath, I., Alton, E. W., Griesenbach, U., Ovari, G., Vag, J., Da Paula, A. C., Crawford, R. M., Varga, G., Amaral, M. D., Mehta, A., Lonovics, J., Argent, B. E. and Gray,

M. A. (2008) 'CFTR gene transfer to human cystic fibrosis pancreatic duct cells using a Sendai virus vector', *J Cell Physiol,* 214, (2), pp. 442-55.

Randell, S. H., Walstad, L., Schwab, U. E., Grubb, B. R. and Yankaskas, J. R. (2001) 'Isolation and culture of airway epithelial cells from chronically infected human lungs', *In Vitro Cell Dev Biol Anim,* 37, (8), pp. 480-9.

Ranganathan, S. C., Dezateux, C., Bush, A., Carr, S. B., Castle, R. A., Madge, S., Price, J., Stroobant, J., Wade, A., Wallis, C. and Stocks, J. (2001) 'Airway function in infants newly diagnosed with cystic fibrosis', *Lancet,* 358, (9297), pp. 1964-5.

Ranganathan, S. C., Stocks, J., Dezateux, C., Bush, A., Wade, A., Carr, S., Castle, R., Dinwiddie, R., Hoo, A. F., Lum, S., Price, J., Stroobant, J. and Wallis, C. (2004) 'The evolution of airway function in early childhood following clinical diagnosis of cystic fibrosis', *Am J Respir Crit Care Med,* 169, (8), pp. 928-33.

Rao, S. and Grigg, J. (2006) 'New insights into pulmonary inflammation in cystic fibrosis', *Arch Dis Child,* 91, (9), pp. 786-8.

Raoust, E., Balloy, V., Garcia-Verdugo, I., Touqui, L., Ramphal, R. and Chignard, M. (2009) 'Pseudomonas aeruginosa LPS or flagellin are sufficient to activate TLR-dependent signaling in murine alveolar macrophages and airway epithelial cells', *PLoS One,* 4, (10), pp. e7259.

Ratcliff, R., Evans, M. J., Cuthbert, A. W., MacVinish, L. J., Foster, D., Anderson, J. R. and Colledge, W. H. (1993) 'Production of a severe cystic fibrosis mutation in mice by gene targeting', *Nat Genet,* 4, (1), pp. 35-41.

Ratjen, F. (2006) 'Restoring airway surface liquid in cystic fibrosis', *N Engl J Med,* 354, (3), pp. 291-3.

Ratjen, F. (2009) 'Update in cystic fibrosis 2008', *Am J Respir Crit Care Med,* 179, (6), pp. 445-8.

Reisin, I. L., Prat, A. G., Abraham, E. H., Amara, J. F., Gregory, R. J., Ausiello, D. A. and Cantiello, H. F. (1994) 'The cystic fibrosis transmembrane conductance regulator is a dual ATP and chloride channel', *J Biol Chem,* 269, (32), pp. 20584-91.

Ribeiro, C. M., Hurd, H., Wu, Y., Martino, M. E., Jones, L., Brighton, B., Boucher, R. C. and O'Neal, W. K. (2009) 'Azithromycin treatment alters gene expression in inflammatory, lipid metabolism, and cell cycle pathways in well-differentiated human airway epithelia', *PLoS One,* 4, (6), pp. e5806.

Riethmuller, J., Anthonysamy, J., Serra, E., Schwab, M., Doring, G. and Gulbins, E. (2009) 'Therapeutic efficacy and safety of amitriptyline in patients with cystic fibrosis', *Cell Physiol Biochem,* 24, (1-2), pp. 65-72.

Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L. and et al. (1989) 'Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA', *Science,* 245, (4922), pp. 1066-73.

Roark, C. L., Simonian, P. L., Fontenot, A. P., Born, W. K. and O'Brien, R. L. (2008) 'gammadelta T cells: an important source of IL-17', *Curr Opin Immunol,* 20, (3), pp. 353-7.

Robertson, A. G., Griffin, S. M., Murphy, D. M., Pearson, J. P., Forrest, I. A., Dark, J. H., Corris, P. A. and Ward, C. (2009) 'Targeting allograft injury and inflammation in the management of post-lung transplant bronchiolitis obliterans syndrome', *Am J Transplant,* 9, (6), pp. 1272-8.

Rogers, C. S., Abraham, W. M., Brogden, K. A., Engelhardt, J. F., Fisher, J. T., McCray, P. B., Jr., McLennan, G., Meyerholz, D. K., Namati, E., Ostedgaard, L. S., Prather, R. S., Sabater, J. R., Stoltz, D. A., Zabner, J. and Welsh, M. J. (2008a) 'The porcine lung as a potential model for cystic fibrosis', *Am J Physiol Lung Cell Mol Physiol,* 295, (2), pp. L240-63.

Rogers, C. S., Hao, Y., Rokhlina, T., Samuel, M., Stoltz, D. A., Li, Y., Petroff, E., Vermeer, D. W., Kabel, A. C., Yan, Z., Spate, L., Wax, D., Murphy, C. N., Rieke, A., Whitworth, K., Linville, M. L., Korte, S. W., Engelhardt, J. F., Welsh, M. J. and Prather, R. S. (2008b) 'Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer', *J Clin Invest,* 118, (4), pp. 1571-1577.

Rogers, C. S., Stoltz, D. A., Meyerholz, D. K., Ostedgaard, L. S., Rokhlina, T., Taft, P. J., Rogan, M. P., Pezzulo, A. A., Karp, P. H., Itani, O. A., Kabel, A. C., Wohlford-Lenane, C. L., Davis, G. J., Hanfland, R. A., Smith, T. L., Samuel, M., Wax, D., Murphy, C. N., Rieke, A., Whitworth, K., Uc, A., Starner, T. D., Brogden, K. A., Shilyansky, J., McCray, P. B., Jr., Zabner, J., Prather, R. S. and Welsh, M. J. (2008c) 'Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs', *Science,* 321, (5897), pp. 1837-41.

Rommens, J. M., Iannuzzi, M. C., Kerem, B., Drumm, M. L., Melmer, G., Dean, M., Rozmahel, R., Cole, J. L., Kennedy, D., Hidaka, N. and et al. (1989)

'Identification of the cystic fibrosis gene: chromosome walking and jumping', *Science,* 245, (4922), pp. 1059-65.

Roos, D., van Bruggen, R. and Meischl, C. (2003) 'Oxidative killing of microbes by neutrophils', *Microbes Infect,* 5, (14), pp. 1307-15.

Rosenfeld, M., Gibson, R. L., McNamara, S., Emerson, J., Burns, J. L., Castile, R., Hiatt, P., McCoy, K., Wilson, C. B., Inglis, A., Smith, A., Martin, T. R. and Ramsey, B. W. (2001) 'Early pulmonary infection, inflammation, and clinical outcomes in infants with cystic fibrosis', *Pediatr Pulmonol,* 32, (5), pp. 356-66.

Rosenfeld, M., Ramsey, B. W. and Gibson, R. L. (2003) 'Pseudomonas acquisition in young patients with cystic fibrosis: pathophysiology, diagnosis, and management', *Curr Opin Pulm Med,* 9, (6), pp. 492-7.

Roussel, L. and Rousseau, S. (2009) 'IL-17 primes airway epithelial cells lacking functional Cystic Fibrosis Transmembrane conductance Regulator (CFTR) to increase NOD1 responses', *Biochem Biophys Res Commun,* 391, pp. 505-509.

Rouvier, E., Luciani, M. F., Mattei, M. G., Denizot, F. and Golstein, P. (1993) 'CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene', *J Immunol,* 150, (12), pp. 5445-56.

Rowe, S. M., Miller, S. and Sorscher, E. J. (2005) 'Cystic fibrosis', *N Engl J Med,* 352, (19), pp. 1992-2001.

Rowntree, R. K. and Harris, A. (2003) 'The phenotypic consequences of CFTR mutations', *Ann Hum Genet,* 67, (Pt 5), pp. 471-85.

Rozmahel, R., Wilschanski, M., Matin, A., Plyte, S., Oliver, M., Auerbach, W., Moore, A., Forstner, J., Durie, P., Nadeau, J., Bear, C. and Tsui, L. C. (1996) 'Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor', *Nat Genet,* 12, (3), pp. 280-7.

Rubin, B. K. (2007) 'Mucus structure and properties in cystic fibrosis', *Paediatr Respir Rev,* 8, (1), pp. 4-7.

Rubin, B. K. (2009) 'Mucus, phlegm, and sputum in cystic fibrosis', *Respir Care,* 54, (6), pp. 726-32; discussion 732.

Sabroe, I., Prince, L. R., Dower, S. K., Walmsley, S. R., Chilvers, E. R. and Whyte, M. K. B. (2004) 'What can we learn from highly purified neutrophils?', *Biochem Soc Trans,* 32, pp. 468-469.

Sabroe, I. and Whyte, M. K. (2007) 'Incapacitating the immune system in cystic fibrosis', *Nat Med,* 13, (12), pp. 1417-8.

Sagel, S. D., Gibson, R. L., Emerson, J., McNamara, S., Burns, J. L., Wagener, J. S. and Ramsey, B. W. (2009) 'Impact of Pseudomonas and Staphylococcus infection on inflammation and clinical status in young children with cystic fibrosis', *J Pediatr,* 154, (2), pp. 183-8.

Sagel, S. D., Sontag, M. K., Wagener, J. S., Kapsner, R. K., Osberg, I. and Accurso, F. J. (2002) 'Induced sputum inflammatory measures correlate with lung function in children with cystic fibrosis', *J Pediatr,* 141, (6), pp. 811-7.

Saglani, S., Payne, D. N., Zhu, J., Wang, Z., Nicholson, A. G., Bush, A. and Jeffery, P. K. (2007) 'Early detection of airway wall remodeling and eosinophilic inflammation in preschool wheezers', *Am J Respir Crit Care Med,* 176, (9), pp. 858-64.

Schmeck, B., N'Guessan, P. D., Ollomang, M., Lorenz, J., Zahlten, J., Opitz, B., Flieger, A., Suttorp, N. and Hippenstiel, S. (2007) 'Legionella pneumophila-induced NF-kappaB- and MAPK-dependent cytokine release by lung epithelial cells', *Eur Respir J,* 29, (1), pp. 25-33.

Scholte, B. J., Davidson, D. J., Wilke, M. and De Jonge, H. R. (2004) 'Animal models of cystic fibrosis', *J Cyst Fibros,* 3, (Suppl 2), pp. 183-90.

Schuchman, E. H. (2007) 'The pathogenesis and treatment of acid sphingomyelinase-deficient Niemann-Pick disease', *J Inherit Metab Dis,* 30, (5), pp. 654-63.

Schuster, A., Ueki, I. and Nadel, J. A. (1992) 'Neutrophil elastase stimulates tracheal submucosal gland secretion that is inhibited by ICI 200,355', *Am J Physiol,* 262, (1 Pt 1), pp. L86-91.

Schwiebert, E. M., Egan, M. E., Hwang, T. H., Fulmer, S. B., Allen, S. S., Cutting, G. R. and Guggino, W. B. (1995) 'CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP', *Cell,* 81, (7), pp. 1063-73.

Sha, Q., Truong-Tran, A. Q., Plitt, J. R., Beck, L. A. and Schleimer, R. P. (2004) 'Activation of airway epithelial cells by toll-like receptor agonists', *Am J Respir Cell Mol Biol,* 31, (3), pp. 358-64.

Sheppard, D. N. and Welsh, M. J. (1999) 'Structure and function of the CFTR chloride channel', *Physiol Rev,* 79, (1 Suppl), pp. S23-45.

Simons, K. and Ikonen, E. (1997) 'Functional rafts in cell membranes', *Nature,* 387, (6633), pp. 569-72.

Singer, S. J. and Nicolson, G. L. (1972) 'The fluid mosaic model of the structure of cell membranes', *Science,* 175, (23), pp. 720-31.

Smirnova, M. G., Guo, L., Birchall, J. P. and Pearson, J. P. (2003) 'LPS up-regulates mucin and cytokine mRNA expression and stimulates mucin and cytokine secretion in goblet cells', *Cell Immunol,* 221, (1), pp. 42-9.

Smith, J. J., Travis, S. M., Greenberg, E. P. and Welsh, M. J. (1996) 'Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid', *Cell,* 85, (2), pp. 229-36.

Smyth, R. L. (2009) 'The airway epithelium in health and disease: "calm on the surface, paddling furiously underneath"', *Thorax,* 64, (4), pp. 277-8.

Snouwaert, J. N., Brigman, K. K., Latour, A. M., Malouf, N. N., Boucher, R. C., Smithies, O. and Koller, B. H. (1992) 'An animal model for cystic fibrosis made by gene targeting', *Science,* 257, (5073), pp. 1083-8.

Sonderegger, I., Iezzi, G., Maier, R., Schmitz, N., Kurrer, M. and Kopf, M. (2008) 'GM-CSF mediates autoimmunity by enhancing IL-6-dependent Th17 cell development and survival', *J Exp Med,* 205, (10), pp. 2281-94.

Song, C., Luo, L., Lei, Z., Li, B., Liang, Z., Liu, G., Li, D., Zhang, G., Huang, B. and Feng, Z. H. (2008) 'IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma', *J Immunol,* 181, (9), pp. 6117-24.

Southern, K. W., Merelle, M. M., Dankert-Roelse, J. E. and Nagelkerke, A. D. (2009) 'Newborn screening for cystic fibrosis', *Cochrane Database Syst Rev*, (1), pp. CD001402.

Spencer, D. A., Venkataraman, M., Higgins, S., Stevenson, K. and Weller, P. H. (1994) 'Cystic fibrosis in children from ethnic minorities in the West Midlands', *Respir Med,* 88, (9), pp. 671-5.

Stafler, P., Davies, J. C., Balfour-Lynn, I. M., Rosenthal, M. and Bush, A. (2009) 'Cystic fibrosis newborn screening: The case for invasive surveillance after diagnosis', *Thorax,* 64 (Suppl IV), pp. A49-A50.

Standiford, T. J., Kunkel, S. L. and Strieter, R. M. (1993) 'Interleukin-8: a major mediator of acute pulmonary inflammation', *Reg Immunol,* 5, (3-4), pp. 134-41.

Stoltz, D. A., Meyerholz, D. K., Pezzulo, A. A., Ramachandran, S., Rogan, M. P., Davis, G. J., Hanfland, R. A., Wohlford-Lenane, C., Dohrn, C. L., Bartlett, J. A., Nelson, G. A. t., Chang, E. H., Taft, P. J., Ludwig, P. S., Estin, M., Hornick, E. E., Launspach, J. L., Samuel, M., Rokhlina, T., Karp, P. H., Ostedgaard, L. S., Uc, A., Starner, T. D., Horswill, A. R., Brogden, K. A., Prather, R. S., Richter,

S. S., Shilyansky, J., McCray, P. B., Jr., Zabner, J. and Welsh, M. J. (2010) 'Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth', *Sci Transl Med,* 2, (29), pp. 29ra31.

Stone, K. D., Prussin, C. and Metcalfe, D. D. (2010) 'IgE, mast cells, basophils, and eosinophils', *J Allergy Clin Immunol,* 125, (2 Suppl 2), pp. S73-80.

Stutts, M. J., Canessa, C. M., Olsen, J. C., Hamrick, M., Cohn, J. A., Rossier, B. C. and Boucher, R. C. (1995) 'CFTR as a cAMP-dependent regulator of sodium channels', *Science,* 269, (5225), pp. 847-50.

Sun, X., Yan, Z., Yi, Y., Li, Z., Lei, D., Rogers, C. S., Chen, J., Zhang, Y., Welsh, M. J., Leno, G. H. and Engelhardt, J. F. (2008) 'Adeno-associated virus-targeted disruption of the CFTR gene in cloned ferrets', *J Clin Invest,* 118, (4), pp. 1578-1583.

Tan, H., Regamey, N., Hilliard, T., Alton, E., Bush, A., Lloyd, C. M. and Davies, J. C. (2009) 'Exploring the phenotype of the lymphocytic infiltrate in the CF airway: Do IL-17+ cells play a role in disease pathogenesis?', *Thorax,* 64 Suppl 4, pp. A110.

Tarran, R., Donaldson, S. and Boucher, R. C. (2007) 'Rationale for hypertonic saline therapy for cystic fibrosis lung disease', *Semin Respir Crit Care Med,* 28, (3), pp. 295-302.

Tarran, R., Loewen, M. E., Paradiso, A. M., Olsen, J. C., Gray, M. A., Argent, B. E., Boucher, R. C. and Gabriel, S. E. (2002) 'Regulation of murine airway surface liquid volume by CFTR and Ca2+-activated Cl- conductances', *J Gen Physiol,* 120, (3), pp. 407-18.

Taylor, C., Allen, A., Dettmar, P. W. and Pearson, J. P. (2004) 'Two rheologically different gastric mucus secretions with different putative functions', *Biochim Biophys Acta,* 1674, (2), pp. 131-8.

Teichgraber, V., Ulrich, M., Endlich, N., Riethmuller, J., Wilker, B., De Oliveira-Munding, C. C., van Heeckeren, A. M., Barr, M. L., von Kurthy, G., Schmid, K. W., Weller, M., Tummler, B., Lang, F., Grassme, H., Doring, G. and Gulbins, E. (2008) 'Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis', *Nat Med,* 14, (4), pp. 382-91.

Thomas, G. R., Costelloe, E. A., Lunn, D. P., Stacey, K. J., Delaney, S. J., Passey, R., McGlinn, E. C., McMorran, B. J., Ahadizadeh, A., Geczy, C. L., Wainwright, B. J. and Hume, D. A. (2000) 'G551D cystic fibrosis mice exhibit abnormal

regulation of inflammation in lungs and macrophages', *J Immunol,* 164, (7), pp. 3870-7.

Tiddens, H. A., Koopman, L. P., Lambert, R. K., Elliott, W. M., Hop, W. C., van der Mark, T. W., de Boer, W. J. and de Jongste, J. C. (2000) 'Cartilaginous airway wall dimensions and airway resistance in cystic fibrosis lungs', *Eur Respir J,* 15, (4), pp. 735-42.

Tirouvanziam, R., de Bentzmann, S., Hubeau, C., Hinnrasky, J., Jacquot, J., Peault, B. and Puchelle, E. (2000) 'Inflammation and infection in naive human cystic fibrosis airway grafts', *Am J Respir Cell Mol Biol,* 23, (2), pp. 121-7.

Trouet, C. (2004) 'New European guidelines for the use of stored human biological materials in biomedical research', *J Med Ethics,* 30, (1), pp. 99-103.

Trouet, C., Dreezen, I., Dierickx, K., Cassiman, J. J., Dequeker, E. and Nys, H. (2004) 'The use of cells from cystic fibrosis patients in research. A model consent form', *J Cyst Fibros,* 3, (3), pp. 197-200.

Tsushima, K., King, L. S., Aggarwal, N. R., De Gorordo, A., D'Alessio, F. R. and Kubo, K. (2009) 'Acute lung injury review', *Intern Med,* 48, (9), pp. 621-30.

Uhlig, S. and Gulbins, E. (2008) 'Sphingolipids in the lungs', *Am J Respir Crit Care Med,* 178, (11), pp. 1100-14.

Ulrich, M. and Doring, G. (2004) 'Three-dimensional human airway epithelial cell cultures', *J Cyst Fibros,* 3 Suppl 2, pp. 55-7.

Unutmaz, D. (2009) 'RORC2: the master of human Th17 cell programming', *Eur J Immunol,* 39, (6), pp. 1452-5.

van den Berg, A., Kuiper, M., Snoek, M., Timens, W., Postma, D. S., Jansen, H. M. and Lutter, R. (2005) 'Interleukin-17 induces hyperresponsive interleukin-8 and interleukin-6 production to tumor necrosis factor-alpha in structural lung cells', *Am J Respir Cell Mol Biol,* 33, (1), pp. 97-104.

van Doorninck, J. H., French, P. J., Verbeek, E., Peters, R. H., Morreau, H., Bijman, J. and Scholte, B. J. (1995) 'A mouse model for the cystic fibrosis delta F508 mutation', *Embo J,* 14, (18), pp. 4403-11.

Van Goor, F., Hadida, S., Grootenhuis, P. D., Burton, B., Cao, D., Neuberger, T., Turnbull, A., Singh, A., Joubran, J., Hazlewood, A., Zhou, J., McCartney, J., Arumugam, V., Decker, C., Yang, J., Young, C., Olson, E. R., Wine, J. J., Frizzell, R. A., Ashlock, M. and Negulescu, P. (2009) 'Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770', *Proc Natl Acad Sci U S A,* 106, (44), pp. 18825-30.

Van Goor, F., Straley, K. S., Cao, D., Gonzalez, J., Hadida, S., Hazlewood, A., Joubran, J., Knapp, T., Makings, L. R., Miller, M., Neuberger, T., Olson, E., Panchenko, V., Rader, J., Singh, A., Stack, J. H., Tung, R., Grootenhuis, P. D. and Negulescu, P. (2006) 'Rescue of DeltaF508-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules', *Am J Physiol Lung Cell Mol Physiol,* 290, (6), pp. L1117-30.

Verhaeghe, C., Delbecque, K., de Leval, L., Oury, C. and Bours, V. (2007) 'Early inflammation in the airways of a cystic fibrosis foetus', *J Cyst Fibros,* 6, (4), pp. 304-8.

Verkman, A. S. and Galietta, L. J. (2009) 'Chloride channels as drug targets', *Nat Rev Drug Discov,* 8, (2), pp. 153-71.

Vermaelen, K. Y., Carro-Muino, I., Lambrecht, B. N. and Pauwels, R. A. (2001) 'Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes', *J Exp Med,* 193, (1), pp. 51-60.

Vielhaber, G., Brade, L., Lindner, B., Pfeiffer, S., Wepf, R., Hintze, U., Wittern, K. P. and Brade, H. (2001) 'Mouse anti-ceramide antiserum: a specific tool for the detection of endogenous ceramide', *Glycobiology,* 11, (6), pp. 451-7.

Vijay-Kumar, M. and Gewirtz, A. T. (2009) 'Flagellin: key target of mucosal innate immunity', *Mucosal Immunol,* 2, (3), pp. 197-205.

Volpe, E., Servant, N., Zollinger, R., Bogiatzi, S. I., Hupe, P., Barillot, E. and Soumelis, V. (2008) 'A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses', *Nat Immunol,* 9, (6), pp. 650-7.

Walters, E. H., Reid, D., Soltani, A. and Ward, C. (2008) 'Angiogenesis: a potentially critical part of remodelling in chronic airway diseases?', *Pharmacol Ther,* 118, (1), pp. 128-37.

Wang, H., Su, Z. and Schwarze, J. (2009) 'Healthy but not RSV-infected lung epithelial cells profoundly inhibit T cell activation', *Thorax,* 64, (4), pp. 283-90.

Ward, C., Eger, K., Diboll, J., Jones, D., Haniffa, M. A., Fisher, A., Brodlie, M., Lordan, J. L., Corris, P. A. and Hilkens, C. M. (2009) 'Bronchial epithelial cells cultured from clinically stable lung allograft patients promote the development of macrophages from monocytes rather than dendritic cells', *Thorax,* 64, (5), pp. 430-5.

Ward, C., Forrest, I. A., Murphy, D. M., Johnson, G. E., Robertson, H., Cawston, T. E., Fisher, A. J., Dark, J. H., Lordan, J. L., Kirby, J. A. and Corris, P. A. (2005)

'Phenotype of airway epithelial cells suggests epithelial to mesenchymal cell transition in clinically stable lung transplant recipients', *Thorax,* 60, (10), pp. 865-71.

Ward, C., Johns, D. P., Bish, R., Pais, M., Reid, D. W., Ingram, C., Feltis, B. and Walters, E. H. (2001) 'Reduced airway distensibility, fixed airflow limitation, and airway wall remodeling in asthma', *Am J Respir Crit Care Med,* 164, (9), pp. 1718-21.

Ward, C., Pais, M., Bish, R., Reid, D., Feltis, B., Johns, D. and Walters, E. H. (2002) 'Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma', *Thorax,* 57, (4), pp. 309-16.

Watt, A. P., Brown, V., Courtney, J., Kelly, M., Garske, L., Elborn, J. S. and Ennis, M. (2004) 'Neutrophil apoptosis, proinflammatory mediators and cell counts in bronchiectasis', *Thorax,* 59, (3), pp. 231-6.

Welch, E. M., Barton, E. R., Zhuo, J., Tomizawa, Y., Friesen, W. J., Trifillis, P., Paushkin, S., Patel, M., Trotta, C. R., Hwang, S., Wilde, R. G., Karp, G., Takasugi, J., Chen, G., Jones, S., Ren, H., Moon, Y. C., Corson, D., Turpoff, A. A., Campbell, J. A., Conn, M. M., Khan, A., Almstead, N. G., Hedrick, J., Mollin, A., Risher, N., Weetall, M., Yeh, S., Branstrom, A. A., Colacino, J. M., Babiak, J., Ju, W. D., Hirawat, S., Northcutt, V. J., Miller, L. L., Spatrick, P., He, F., Kawana, M., Feng, H., Jacobson, A., Peltz, S. W. and Sweeney, H. L. (2007) 'PTC124 targets genetic disorders caused by nonsense mutations', *Nature,* 447, (7140), pp. 87-91.

Widdicombe, J. H. (1990) 'Use of cultured airway epithelial cells in studies of ion transport', *Am J Physiol,* 258, (2 Pt 1), pp. L13-8.

Wiggs, B. R., Hrousis, C. A., Drazen, J. M. and Kamm, R. D. (1997) 'On the mechanism of mucosal folding in normal and asthmatic airways', *J Appl Physiol,* 83, (6), pp. 1814-21.

Wilcoxon, F. (1945) 'Individual comparisons by ranking methods', *Biometrics Bulletin,* 1, (6), pp. 80-83.

Williams, T. J. (2004) 'The eosinophil enigma', *J Clin Invest,* 113, (4), pp. 507-9.

Wilschanski, M., Zielenski, J., Markiewicz, D., Tsui, L. C., Corey, M., Levison, H. and Durie, P. R. (1995) 'Correlation of sweat chloride concentration with classes of the cystic fibrosis transmembrane conductance regulator gene mutations', *J Pediatr,* 127, (5), pp. 705-10.

Wilson, J. W. and Li, X. (1997) 'The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway', *Clin Exp Allergy,* 27, (4), pp. 363-71.

Wilson, N. J., Boniface, K., Chan, J. R., McKenzie, B. S., Blumenschein, W. M., Mattson, J. D., Basham, B., Smith, K., Chen, T., Morel, F., Lecron, J. C., Kastelein, R. A., Cua, D. J., McClanahan, T. K., Bowman, E. P. and de Waal Malefyt, R. (2007) 'Development, cytokine profile and function of human interleukin 17-producing helper T cells', *Nat Immunol,* 8, (9), pp. 950-7.

Wine, J. J. (2010) 'The development of lung disease in cystic fibrosis pigs', *Sci Transl Med,* 2, (29), pp. 29ps20.

Winnie, G. B. and Cowan, R. G. (1991) 'Respiratory tract colonization with Pseudomonas aeruginosa in cystic fibrosis: correlations between anti-Pseudomonas aeruginosa antibody levels and pulmonary function', *Pediatr Pulmonol,* 10, (2), pp. 92-100.

Winpenny, J. P., Verdon, B., McAlroy, H. L., Colledge, W. H., Ratcliff, R., Evans, M. J., Gray, M. A. and Argent, B. E. (1995) 'Calcium-activated chloride conductance is not increased in pancreatic duct cells of CF mice', *Pflugers Arch,* 430, (1), pp. 26-33.

Wojnarowski, C., Frischer, T., Hofbauer, E., Grabner, C., Mosgoeller, W., Eichler, I. and Ziesche, R. (1999) 'Cytokine expression in bronchial biopsies of cystic fibrosis patients with and without acute exacerbation', *Eur Respir J,* 14, (5), pp. 1136-44.

Wong, C. K., Cao, J., Yin, Y. B. and Lam, C. W. (2010) 'Interleukin-17A activation on bronchial epithelium and basophils: a novel inflammatory mechanism', *Eur Respir J,* 35, (4), pp. 883-93.

Wooten-Blanks, L. G., Song, P., Senkal, C. E. and Ogretmen, B. (2007) 'Mechanisms of ceramide-mediated repression of the human telomerase reverse transcriptase promoter via deacetylation of Sp3 by histone deacetylase 1', *Faseb J,* 21, (12), pp. 3386-97.

Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K. C., Birrer, P., Bellon, G., Berger, J., Weiss, T., Botzenhart, K., Yankaskas, J. R., Randell, S., Boucher, R. C. and Doring, G. (2002) 'Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients', *J Clin Invest,* 109, (3), pp. 317-25.

Xu, J., Benyon, R. C., Leir, S. H., Zhang, S., Holgate, S. T. and Lackie, P. M. (2002) 'Matrix metalloproteinase-2 from bronchial epithelial cells induces the proliferation of subepithelial fibroblasts', *Clin Exp Allergy,* 32, (6), pp. 881-8.

Yamaya, M., Finkbeiner, W. E., Chun, S. Y. and Widdicombe, J. H. (1992) 'Differentiated structure and function of cultures from human tracheal epithelium', *Am J Physiol,* 262, (6 Pt 1), pp. L713-24.

Yang, L., Anderson, D. E., Baecher-Allan, C., Hastings, W. D., Bettelli, E., Oukka, M., Kuchroo, V. K. and Hafler, D. A. (2008) 'IL-21 and TGF-beta are required for differentiation of human T(H)17 cells', *Nature,* 454, pp. 350-2.

Yao, Z., Fanslow, W. C., Seldin, M. F., Rousseau, A. M., Painter, S. L., Comeau, M. R., Cohen, J. I. and Spriggs, M. K. (1995a) 'Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor', *Immunity,* 3, (6), pp. 811-21.

Yao, Z., Painter, S. L., Fanslow, W. C., Ulrich, D., Macduff, B. M., Spriggs, M. K. and Armitage, R. J. (1995b) 'Human IL-17: a novel cytokine derived from T cells', *J Immunol,* 155, (12), pp. 5483-6.

Ye, P., Garvey, P. B., Zhang, P., Nelson, S., Bagby, G., Summer, W. R., Schwarzenberger, P., Shellito, J. E. and Kolls, J. K. (2001a) 'Interleukin-17 and lung host defense against Klebsiella pneumoniae infection', *Am J Respir Cell Mol Biol,* 25, (3), pp. 335-40.

Ye, P., Rodriguez, F. H., Kanaly, S., Stocking, K. L., Schurr, J., Schwarzenberger, P., Oliver, P., Huang, W., Zhang, P., Zhang, J., Shellito, J. E., Bagby, G. J., Nelson, S., Charrier, K., Peschon, J. J. and Kolls, J. K. (2001b) 'Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense', *J Exp Med,* 194, (4), pp. 519-27.

Yu, H., Zeidan, Y. H., Wu, B. X., Jenkins, R. W., Flotte, T. R., Hannun, Y. A. and Virella-Lowell, I. (2009) 'Defective Acid Sphingomyelinase Pathway with Pseudomonas aeruginosa Infection in Cystic Fibrosis', *Am J Respir Cell Mol Biol,* 41, pp. 367-375.

Zabner, J., Karp, P., Seiler, M., Phillips, S. L., Mitchell, C. J., Saavedra, M., Welsh, M. and Klingelhutz, A. J. (2003) 'Development of cystic fibrosis and noncystic fibrosis airway cell lines', *Am J Physiol Lung Cell Mol Physiol,* 284, (5), pp. L844-54.

Zabner, J., Smith, J. J., Karp, P. H., Widdicombe, J. H. and Welsh, M. J. (1998) 'Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia in vitro', *Mol Cell,* 2, (3), pp. 397-403.

Zeiher, B. G., Eichwald, E., Zabner, J., Smith, J. J., Puga, A. P., McCray, P. B., Jr., Capecchi, M. R., Welsh, M. J. and Thomas, K. R. (1995) 'A mouse model for the delta F508 allele of cystic fibrosis', *J Clin Invest,* 96, (4), pp. 2051-64.

Zemans, R. L., Colgan, S. P. and Downey, G. P. (2009) 'Transepithelial migration of neutrophils: mechanisms and implications for acute lung injury', *Am J Respir Cell Mol Biol,* 40, (5), pp. 519-35.

Zhang, S., Smartt, H., Holgate, S. T. and Roche, W. R. (1999) 'Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma', *Lab Invest,* 79, (4), pp. 395-405.

Zou, W. and Restifo, N. P. (2010) 'T(H)17 cells in tumour immunity and immunotherapy', *Nat Rev Immunol,* 10, (4), pp. 248-56.