Specialized Pro-resolution Mediators in Cystic Fibrosis Lung Disease

AUTHOR(S)

Fiona C. Ringholz

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Specialized Pro-resolution Mediators in Cystic Fibrosis Lung Disease

Dr Fiona Ringholz MBBChir, BAcantab, MRCPCH (UK)
Department of Molecular Medicine
RCSI

A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfilment of the degree of Doctor of Philosophy

Supervisors:
Professor Brian Harvey
Dr Valerie Urbach
Professor Paul McNally

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Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree Doctor of Philosophy is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other source except where such work has been cited and acknowledged within the text.

Signed _____

Student Number ____12111074_____

Date _____

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List of Abbreviations

[Ca]i	Intracellular calcium concentration
13, 14-eMaR	13, 14-epoxy-maresin
15-d-PGJ2	15-d-Prostaglandin J2
15-LO	15 Lipoxygenase
15-LO1	15 Lipoxygenase type 1
15-LO2	15 Lipoxygenase type 2
18-HEPE	18-hydroxy-eicosapentaenoic acid
5-LO	5-Lipoxygenase
A2b	Adenosine A 2B receptor
AA	Arachidonic Acid
ABTS	2,2'-azino-bis
AhR	Aryl hydrocarbon receptor
ALOX12	12 Lipoxygenase
ALOX15	15 Lipoxygenase type 1
ALOX15B	15 Lipoxygenase type 2
ALOX5	5 Lipoxygenase
ALX/FPR2	Formyl Peptide Receptor 2
AM	Alveolar Macrophages
ANOVA	Analysis Of Variance
AREST CF	Australian Respiratory Early Surveillance Team for Cystic Fibrosis
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ASL	Airway Surface Liquid
ATP	Adenosine Tri-phosphate
BAL	Bronchoalveolar Lavage
Bcc	Burkholderia cepacia complex
BEBM	Bronchial Epithelial Cell Basal Medium
BEGM	Bronchial Epithelial Cell Growth Medium
Best-1	Bestrophin 1
BLT1	Leukotriene B4 receptor 1
Boc-2	Boc-Phe-Leu-Phe-Leu-Phe
BSA	Bovine Serum Albumin

CaCC	Calcium activated Chloride channels
cAMP	cyclic Adenosine Monophosphate
CCL22	CC Chemokine type 22
CF	Cystic Fibrosis
CFAM	Cystic Fibrosis alveolar macrophages
CFPE	Cystic Fibrosis Pulmonary Exacerbation
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFU	Colony Forming Units
CLCA family	Calcium-Activated Chloride channel family
CMKLR1	Chemokine receptor-like 1
COX	Cyclo-oxygenase
Ct	Cycle threshold
CuFi-1	Cystic Fibrosis, University of Iowa-1
CXCR1	Interleukin 8 Receptor alpha
DAG	Diacylglycerol
DCF	29,79-dichlorodihydrofluorescein diacetate
DHA	Docosahexaenoic acid
DICE	Differentiation Control Element
DiHETE	5(S), 6(R)-dihydroxy-7, 9-trans-11, 14-cis-eicosatetraenoic acid
DMEM	Dulbecco's Modified Eagle Medium
DNase	Deoxyribonuclease
DPBS	Dulbecco's Phosphate Buffered Solution
ECL	Enhanced Chemi-luminescence
ELISA	Enzyme Linked Immunosorbent Assay
ENaC	Epithelial Sodium Channel
EORs	Eicosanoid Oxidoreductases
EPA	Eicosapentaenoic acid
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FEV_1	Forced Expiratory Volume in 1 second
FITC	Fluorescein isothiocyanate
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FPRs	Formyl Peptide Receptors
FR	Fiona Ringholz

FVC	Forced Vital Capacity
GM-CSF	Granulocyte Monocyte-Colony Stimulating Factor
GPCR	G-Protein Coupled Receptor
H(p)ETE	Hydroperoxyeicosatetraenoic acid
$HCO3^-$	Bicarbonate
HETE	Hydroxyeicosatetraenoic acid
HNRNP	Heterogeneous nuclear ribonucleoprotein
HODE	Octadecadienoic acid
HPLC	High Performance Liquid Chromatography
HPV-16	Human Papillomavirus type 16
HRP	Horseradish Peroxidase
ICAM-1	Intercellular Adhesion Molecule 1
IDO1	Indoleamine 2,3-dioxygenase 1
IFN-ɔ	Interferon-ɔ
IHB	Inhibitor of kappa B
IKK	IHB kinase complex
IL1	Interleukin 1
IL10	Interleukin 10
IL-12 p35	Interleukin-12 p35
IL13	Interleukin 13
IL-1ɒ	Interleukin 1-beta
IL2	Interleukin 2
IL4	Interleukin 4
IL6	Interleukin 6
IL8	Interleukin 8
IL9	Interleukin 9
iNOS	inducible Nitric Oxide Synthetase
IP3	Inositol triphosphate
IP3R	Inositol triphosphate receptor
LA	Linoleic acid
LB agar	Lysogeny broth agar
LCI	Lung Clearance Index
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LNA	Ŭ-Linolenic acid

LPS	Lipopolysaccharide
LTA_4	Leukotriene A_4
LTA_4H	Leukotriene A_4 Hydrolase
LTB_4	Leukotriene B_4
LTE4	Leukotriene E4
LXA_4	Lipoxin A_4
M1	Classical activation phenotype in macrophages
M2	Alternative activation phenotype in macrophages
MAPK	Mitogen Activated Protein Kinase
MaR2	Maresin-2
MRSA	*Methicillin Resistant Staphylococcus aureus*
MS	Mass Spectrometry
NBD1	Nucleotide Binding Domain 1
NBD2	Nucleotide Binding Domain 2
NE	Neutrophil Elastase
NEMO	NF kappa B Essential Modulator
NFκB	Nuclear Factor kappa B
NO	Nitric Oxide
NOS2	Nitric Oxide Synthetase isoform 2
NS	Not Significant
NSAIDâs	Non-Steroidal Anti-inflammatory Drugs
NTM	Nontuberculous Mycobacteria
NuLi-1	Normal Lung, University of Iowa-1
OLCHC	Our Ladyâs Children Hospital, Crumlin
Orai1	Calcium release-activated calcium channel protein 1
P. aeruginosa	*Pseudomonas aeruginosa*
P2Y2	Purinergic receptor P2Y, G-protein coupled, 2
PBMC	Peripheral Blood Mononuclear Cells
PBS-T	PBS-Tween
PCL	Peri Ciliary Layer
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PDx	Protectin Dx
PGD2	Prostaglandin D2

PGD2S	Prostaglandin D2 Synthase
PGE2	Prostaglandin E2
PIP2	Phosphatidylinositol 4,5 bisphosphate
PKA	Protein Kinase A
PLA2	Phospholipase A2
PLD	Phospholipase D
PLUNC	Palate Lung and Nasal epithelial Clone
PPARɔ	Peroxisome proliferator-activated receptor gamma
PRINCE study	The Pro-Resolution Mediators in CF Exacerbation study
PS	Phosphatidylserine
PSDP	Presqualene Diphosphate
PSMP	Presqualene Monophosphate
PTC	Premature Termination Codon
PUFA	Poly-unsaturated Fatty Acids
qPCR	Quantitative Polymerase Chain Reaction
R	Regulatory domain
RANTES	Regulated upon Activation Normal T-cell Expressed, and presumably Secreted
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
RT	Room Temperature
RvD1	Resolvin D1
RvD2	Resolvin D2
RvD5	Resolvin D5
RvE3	Resolvin E3
S. aureus	*Staphylococcus aureus*
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SERCA2	Sarco/Endoplasmic Reticulum Calcium ATPase
SHIELD CF Study	Study of Host Immunity and Early Lung Disease in Children with CF
Socs2	Suppressor of Cytokine Synthesis 2

SPE	Solid Phase Extraction
SPLUNC1	Short Palate Lung and Nasal epithelial Clone
SPM's	Specialized Pro-Resolution Mediators
STAT-1	Signal Transducer and Activator of Transcription-1
STAT-6	Signal Transducer and Activator of Transcription-6
STIM1	Stromal Interaction Molecule 1
TAB1	TAK1 binding protein
TAK1	TGF-ƀ-activated kinase 1
TEER	Trans-Epithelial Electrical Resistance
TEMED	Tetramethylethylenediamine
TGF-ƀ	Transforming Growth Factor beta
TLR	Toll-like Receptor
TMD1	Transmembrane Membrane Spanning Domain 1
TMD2	Transmembrane Membrane Spanning Domain 2
TMEM16	Anoctamin 1
TNFR	Tumour Necrosis Factor Receptor
TNFŬ	Tumour Necrosis Factor Ŭ
TRAF6	TNF receptor-associated factor 6
TRPC6	Transient Receptor Potential 6 channel
UPR	Unfolded Protein Response

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Abstract

Specialized Pro-Resolution Mediators (SPMôs) in Paediatric Cystic Fibrosis (CF) Lung Disease

CF is caused by a mutation in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) and results in airway surface liquid (ASL) dehydration, impaired muco-ciliary clearance, chronic pulmonary infection and inflammation leading to progressive lung destruction. Specialized Pro-Resolution Mediators (SPMôs) such as Lipoxins and Resolvins are SPMôs involved in the active resolution of inflammation. Previous work showed that Lipoxin A_4 restores airway surface liquid height in CF bronchial epithelium. Our aim was to investigate the role of SPMs, including Lipoxin A_4 and Resolvin D_1 in CF airway disease.

We studied the physiological effects of RvD1 in CF using primary CF alveolar macrophages and polarized, differentiated Primary CF and NuLi-1, and CuFi-1 cell lines as a bronchial epithelial model. We collected bronchoalveolar lavage (BAL) samples from young children with CF and controls at clinical baseline (SHIELD CF) and sputum from school aged children during CF Pulmonary Exacerbation (CFPE) (PRINCE Study).

We report that Resolvin D1 restored ASL height in Primary CF bronchial epithelia and CuFi-1 cells. Resolvin D1 attenuated TNFŬ induced IL8 secretion in CuFi-1 cells. Furthermore Resolvin D1 improved phagocytic capacity and bacterial killing of *Pseudomonas aeruginosa* in primary CF alveolar macrophages. We demonstrate an imbalance in Lipoxin A_4 (LXA_4) versus Leukotriene B_4 (LTB_4) production in CF airways, despite the absence of infection, related to a deficiency of the LXA_4 synthetic enzyme 15 Lipoxygenase-2 in CF macrophages. We have demonstrated that the levels of Resolvin D2 and 15-d-Prostaglandin J_2 correlate strongly with the extent of recovery of premorbid FEV_1 during CF pulmonary exacerbation.

We have elucidated important beneficial physiological effects of SPMs in CF airway cells, shown defective class switching in children with CF compared to controls, and demonstrated a link between sputum SPM levels and lung function recovery during acute infection in CF. Our findings shed some light on the failure

to resolve inflammation in CF airways and suggest strong therapeutic potential for SPMs in CF.

Acknowledgements

Valerie Urbach, Paul McNally and Brian Harvey conceived of this project, afforded me the opportunity to contribute my efforts to it, shaped and guided the work and have equipped me with all kinds of tools that have shaped the way I think.

You have allowed me to ñtouch the edges of the mapò and taught me how to approach a question to which the world does not yet know the answer. I have greatly enjoyed my apprenticeship in translational research and the scientific method.

I took up this particular PhD because I was genuinely excited about the potential for the groupôs work one day to bear fruit in terms of the possibility to develop new therapy for a group of kids who bear significant hardship in childhood because of the impact of cystic fibrosis. Three years down the line that hope is unscathed and in fact redoubled.

My colleagues at NCRC have been a wealth of encouragement and support. In particular Gerry Higgins, Monika Hollenhorst, Paul Buchanan, Ahmad Moukachar, Coral Fustero, Rosin Millar, Donna Clarke and Tracey Mullen were lovely to work with and an inexhaustible source of guidance. Lorna, Elaine, Maureen, and Naomi made coming to work fun. Jacinta Kelly, Carlos Blanco and Pat Walsh have both encouraged and actively supported me in my research endeavours.

Specific Acknowledgements

BAL fluid, BAL cell pellets, primary bronchial epithelial cells, and source material for primary alveolar macrophages were obtained via the SHIELD CF Study set up by Drôs Paul McNally and Barry Linnane. Drôs Des Cox, Ahmad Zaid, Sheila Javadpour, Peter Greally and Basil Elnazir contributed to the SHIELD CF project including by performing bronchoscopy. Donna Clarke and Rosin Millar collected clinical data and performed BAL processing for the SHIELD CF Study. Microbiological analysis of BAL fluid in the SHIELD CF study was performed by the clinical microbiology lab at Our Ladyôs Childrenôs Hospital, Crumlin (OLCHC) and the BAL differential cell counts in the SHIELD CF Study were performed by Dr Michael McDermott, consultant histopathologist at OLCHC.

Post- doctoral researcher Dr Gerard Higgins in the Urbach lab taught me the techniques to perform primary cell culture, confocal microscopy and quantitative microbiology. Whilst conducting the early work on Resolvin D1, I taught Masterôs student Ahmad Moukachar cell culture and confocal microscopy techniques, and as such he contributed technical assistance under my direct supervision. Lab technician, Coral Fustero provided technical assistance in the primary CF bronchial epithelial cell culture. Specific contributions will be highlighted again when I present the results to which the assistance pertained.

The LC-MS/MS analysis of lipid mediators in sputum was performed by our collaborator, Dr Justine Bertrand-Michel at the Lipidomic Platform in Toulouse. The IHB antibody used in Chapter 3 was obtained from Dr Sarah Doyleôs group at the National Childrenôs Research Centre, Crumlin. The staff in the pulmonary function lab at OLCHC trained me in spirometry and performed baseline spirometry measurements (as per routine clinical care) which we obtained from the medical records.

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Publications

Original Article

Reduced 15-lipoxygenase 2 and LXA4 /LTB4 ratio in children with cystic fibrosis. F. C. Ringholz, P. J. Buchanan, D. T. Clarke, R. G. Millar, M. McDermott, B. Linnane, B. J. Harvey, P. McNally and V. Urbach. Eur Respir J, 2014 Aug; 44(2):394-404 (PMID: 24696116)

Reviews

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Physiological Impact of Abnormal LXA4 Production on Cystic Fibrosis Airway Epithelium and Therapeutic Potential. Gerard Higgins, Fiona Ringholz, Paul Buchanan, Paul McNally, and Valérie Urbach. BioMed Research International Volume 2014, Article ID 781087, 11 pages (Awaiting PMID)

Book Chapter

Modulation of airway epithelial cell calcium and airway hydration by anti-inflammatory mediators in Cystic Fibrosis. F. Ringholz, Higgins, G, Buyck, JM, Urbach, V. New Developments in Calcium Signaling Research. Nova Science Publishers, Inc, Editors: Masayoshi Yamaguchi, 2014, Chapter II, pp.23-51

Oral Presentations

Spontaneous Pro-Resolution Mediators (SPMõs) in Paediatric Cystic Fibrosis (CF) Lung Disease. Fiona Ringholz, Paul McNally, and Valerie Urbach.
The Biochemical Pharmacology Discussion Group at the New York Academy of Sciences meeting ñPharmacologic Resolution of Inflammation as a Novel Therapeutic Approachò Tuesday, October 28, 2014.

Spontaneous pro-resolution mediators (SPMõs) in paediatric cystic fibrosis (CF) lung disease. Paul McNally & Fiona Ringholz.
The 7th Annual Meeting of the Irish Epithelial Physiology Group, Kilkenny, 23-24 Oct. 2014

Specialized Pro-Resolution Mediators in Paediatric CF Lung disease. Fiona Ringholz.
Cystic Fibrosis COST ACTION, Dublin Training School, Ion Transport, Airway Liquid Dynamics & Host Pathogen Interactions in CF Lung Epithelia, Dublin 10-12 Sept. 2014

Resolvin D1 restores airway surface liquid hydration and attenuates IL8 secretion in Cystic Fibrosis (CF) bronchial epithelial cells. F.C. Ringholz, A. Moukachar, G. Higgins, P. McNally, V. Urbach.
Irish Thoracic Society Annual Scientific Meeting, Paediatric Forum & Plenary Session, November 2013, Derry **Winner of Best Oral Presentation in Paediatric Forum**

Reduced LXA4 / LTB4 ratio in Early Cystic Fibrosis BAL.
Fiona Ringholz, Paul McNally, Valerie Urbach.
Paediatric Forum, Irish Thoracic Society Annual Scientific Meeting 2012, Limerick, November 2012 **Winner of Best Oral Presentation in Paediatric Forum**

Lipid mediators of the resolution of inflammation in the Cystic Fibrosis airway.
Fiona Ringholz.
National Childrenõs Research Centre, Paediatric Clinical Research Open Day, December 6th 2011 **Joint Best Presentation Award**

International Poster Presentations

Reduced LXA4 / LTB4 ratio in Early Cystic Fibrosis BAL - Impaired epithelial LXA4 synthesis capacity. Fiona Ringholz, Paul McNally and Valerie Urbach.
4th European Workshop on Lipid Mediators, 27-28 September 2012, Pasteur Institute, Paris

Reduced LXA4 / LTB4 ratio in Early Cystic Fibrosis BAL - Airway epithelial LXA4 synthesis capacity is impaired. Fiona Ringholz, Paul McNally and Valerie Urbach.
UK Clinical Research Facility Network, Conference, 5-6 July, 2012, Dublin.

Resolvin D1 restores airway surface liquid hydration & attenuates IL8 secretion in CF bronchial epithelial cells. Fiona Ringholz, Ahmad Moukachar, Gerard Higgins, Paul McNally & Valerie Urbach.
Epithelia and Smooth Muscle Interactions in Health and Disease, meeting of the Physiological Society, Dublin, 11 - 13 December, 2013.

Dedication

ñI think I can.ò(Little blue engine)

Brendan & Olivia McFerran, my Daddy and Mammy. My parents are my ducky blanket in infinitely resourceful ways. They have insulated me from all harm and cleared a path for me in life to dream and think and explore the world. There has never been a ñglass ceilingò for me. The fact that ñI thought I couldò is no stroke of luck or accident of birth, it is a gift that my parents defended and protected with all their wits in infinitely resourceful ways. As such, this thesis represents a blank canvas and they have given me the freedom to think, the pen, and the courage with which to write it and they are the authors of any good that ever comes of it.

ñCourage doesnôt always roar. Sometimes courage is the quiet voice at the end of the day that says ñI will try again tomorrowòò (Mary Anne Radmacher)

Henning was there when this PhD was the seed of an idea, on the phone in the middle of the day when I hid in the back stairwell to vent frustration or share the excitement of a breakthrough, at the end of the day with the kids safe, happy and in good order with the dinner on the table, a glass of white wine and an ear for the daysôdevelopments. I couldnôt attempt to articulate what contribution Henning has made to this work and itôs only a minute fraction of the impact he has had on my life ï suffice it to say that he is my best friend, a giant in my life and intricately wound around the foundation stones of everything I have worked to build in the last ten years.

ñMay God bless and keep you always, May your wishes all come trueé May your heart always be joyful, And may your song always be sung.ò(Bob Dylan)

My beautiful children, Johan and Beatrice. You bring delight and happiness to my heart. You make it very easy for me to get out of bed every morning, and to bust a gut to get my daysôwork done, so as to be able to rush home in the evening just to be around you. You are both courageous, good natured little characters who have embraced life with both hands. I am literally in awe of you and will always love you to pieces.

Jannah & Wolfgang, my German family know how to live! You have shared your home, your table, your wine, gemutlich, philosophy, swimming pools, mountains and sea. Jannah in particular played a big part in making this work possible by helping me and the kids when Henning needed to travel. Jannah you were good company, held the home-front together and shared in many of the little milestones along the way.

My family Stephen, Ellen, Aimee, Eoghan, Ethy, Oonagh and Bernard. You are my home and having floated around in the world for a while Iôve never found anyone to beat the lot of ye. Thanks for everything that you are.

A few friends in particular have supported me over the lifetime of this project and have been there to remind me what life is really all about. Barry and Sheila Boland, Andrea Miklasova, Maeve Nunez, Mariya Kryvyak, Vicky Mars, Steve Koh, Fergus Simpson, Josie and Tom Usher, Karsten Barrs and the Knorr twins.

ñI see a brighter star tonight as I look at the sky,
It glistens in a teardrop as it drifts upon a sigh (1).ò

ñLike the stars, our ideals are hard to reach, but they serve for navigation in the night.ò(Oxford Handbook of Clinical Medicine)

Oisin Boland was an electric child. He is one of a few kids whose memory echoes around in my head and drives me on to try harder. And so we work on in the memory of those kids that we couldnôt help enough.

CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Cystic Fibrosis (CF)

CF is the most common life-limiting inherited disease in Caucasians, with approximately 1 in 19 people carrying one mutation in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), and 1 in 1,461 people in Ireland being affected by CF (2). CF affects many organ systems, however, lung destruction is the main cause of mortality in CF.

The most common mutation of the CFTR gene (Phe508del) results in defective chloride secretion in the lung, which reduces airway surface liquid hydration. Sodium hyperabsorption is also a feature of CF airways which further exacerbates the airway liquid dehydration. Airway surface liquid dehydration impairs muco-ciliary clearance and disrupts innate immune defence mechanisms favouring chronic bacterial colonization. Chronic bacterial bronchitis and the associated neutrophil predominant inflammatory response cause progressive bronchiectasis, lung destruction, respiratory failure and premature mortality.

1.1.1 Dorothy Andersen and the Recognition of Cystic Fibrosis

Prior to 1938, the ñcoeliac syndromeò was a recognised clinical entity, linked to features of pancreatic steatorrhoea. This ñsyndromeò encompassed a mixture of cases that today we would recognise as coeliac disease and cystic fibrosis. In 1938, Dorothy Andersen (niece of Hans Christian Andersen) was the first to extensively describe ñCystic Fibrosis of the pancreasò whilst working at Babiesô Hospital, in New York (3). She described the clinical findings of a group of children who all had cystic fibrosis of the pancreas at post mortem. A neonatal cohort (many of whom had meconium ileus), and a second group of children dying between 1 and 6 months were examined. The characteristic features which these children had in common were described; failure to gain weight with an adequate diet beginning in the neonatal period, a large abdomen at birth, hunger, absence of vomiting or diarrhoea, large stools, sometimes recognised as fatty, intolerance to fat in the diet, and chronic infection of the respiratory tract. A third group died between 6 months and 14.5 years and had additional features including; osteoporosis, exophthalmia, bronchiectasis, otitis media and sinus infection. The cause of death in all children surviving the first week of life was bronchitis, bronchiectasis or bronchopneumonia (3).

In an early report, Lloyd Dickey, writing about the pulmonary aspects of the disease, suggests that the condition may have existed in cases previously diagnosed as ñchronic, or unresolved pneumoniasò (4). He describes the treatments of the day including; pancreatic enzymes, large amounts of Vitamin A, banana powder and sulfathiazole (4).

1.1.2 Presentation & Diagnosis

CF can come to the attention of the physician; prenatally, via new-born screening, via detection in a close relative, or via symptomatic presentation in children or adults. The CF Foundation Consensus Report (1996) recommends that the diagnosis of CF should be based on the presence of one or more characteristic clinical features (described in Table 1-1), a history of CF in a sibling, or a positive new-born screening test, plus laboratory evidence of an abnormality in the CFTR gene or protein (i.e. abnormal sweat chloride or nasal potential difference OR identification of a disease-causing mutation in each copy (trans) of the CFTR gene) (5). The primary test for confirming the diagnosis of CF is the sweat chloride test. A sweat chloride (performed according to the correct procedure in an accredited lab) of Ó60mmol/L is abnormal and indicative of CF, whilst a sweat chloride Ò39mmol/L (Ò29mmol/L in infants under 6 months of age) is considered normal and makes a diagnosis of CF unlikely (5). Where the sweat chloride falls in the intermediate range, the diagnosis of CF becomes more complicated and consensus guidelines have been established to assist the physician in managing such cases (5).

Table 1-1 Phenotypic features consistent with a diagnosis of CF

Classic Symptoms and Signs of CF (5)
Chronic sino-pulmonary disease:
Persistent colonisation/infection with typical CF pathogens
Chronic cough and sputum production
Persistent chest radiograph abnormalities (e.g. bronchiectasis, atelectasis, infiltrates, hyperinflation)
Airway obstruction, manifested by wheezing and air trapping
Nasal polyps
Digital clubbing
Gastrointestinal and nutritional abnormalities:
Meconium ileus, distal intestinal obstruction syndrome, rectal prolapse
Pancreatic insufficiency, recurrent acute pancreatitis, chronic pancreatitis
Prolonged neonatal jaundice, chronic hepatic disease with evidence of focal biliary cirrhosis or multilobular cirrhosis
Failure to thrive, hypoproteinemia and oedema, fat soluble vitamin deficiencies
Salt loss syndromes, acute salt depletion, chronic metabolic alkalosis
Genital abnormalities in males, obstructive azoospermia

1.1.3 Extra-Pulmonary Manifestations

CFTR expression is highest among epithelial sites in the body and as such, epithelial sites are particularly affected by CF disease. CF affects the gastrointestinal tract, causing; fat malabsorption, meconium ileus, rectal prolapse, and distal intestinal obstruction syndrome. In the 1950ôs, improvements in the surgical management of meconium ileus led to significant advances in the life expectancy of children with CF (6). In the liver, CF gives rise to extra-hepatic manifestations including; cholelithiasis and sclerosing cholangitis, as well as, intra-hepatic manifestations, such as; neonatal cholestasis, steatosis, focal biliary cirrhosis, and multi-lobular cirrhosis. CF affects the pancreas causing pancreatic insufficiency, pancreatitis, and diabetes mellitus. In the 1980ôs, the development of acid resistant microspheres (Pancrease & Creon) significantly improved the management of pancreatic insufficiency in CF (7). Nutritional effects include malnutrition, micronutrient deficiencies, and osteopaenia. In the reproductive tract, CF causes obstructive azoospermia in men, and impaired fertility in women.

1.1.4 Prognosis

The life expectancy for individuals with CF has increased dramatically since the first description of the disease by Dorothy Andersen in 1938. In a recent CF Foundation report, more than half of 18-29 year olds with CF (52.8%) had normal ï mildly impaired lung function (FEV_1 Ó70%) and the predicted median survival was 41.1 years of age (8). 68.5% of adults with CF has at least some college education, 39.8% were married or living with a partner and 69% were either studying or in employment (8). The relationship between genotype and pulmonary disease severity is weak and therefore prognostic predictions on the basis of genotype analysis are to be avoided in individual patients with CF (5).

In order to continue to achieve gains in life expectancy and quality of life for people with CF, therapeutic innovations are called for to develop new treatments to; restore CFTR function, rehydrate the airway surface, improve muco-ciliary clearance, prevent, supress and eradicate or treat bacterial infection, disrupt biofilm formation, deal with the emergence of multi-drug resistance and antibiotic side effects, improve innate immune defence and promote repair.

1.2 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

In 1989, three back to back papers were published in Science identifying the CFTR gene and its protein product as the underlying causative gene defect in CF opening the door to the understanding the disease at its most basic level (9-11).

1.2.1 CFTR Structure

The CFTR is a protein comprising 1,480 amino acids which functions as a chloride channel in the apical membrane of exocrine epithelial cells (9-11). It is a member of the ATP-binding cassette (ABC) transporter family, which conducts anions, principally chloride and bicarbonate (Figure 1-1).

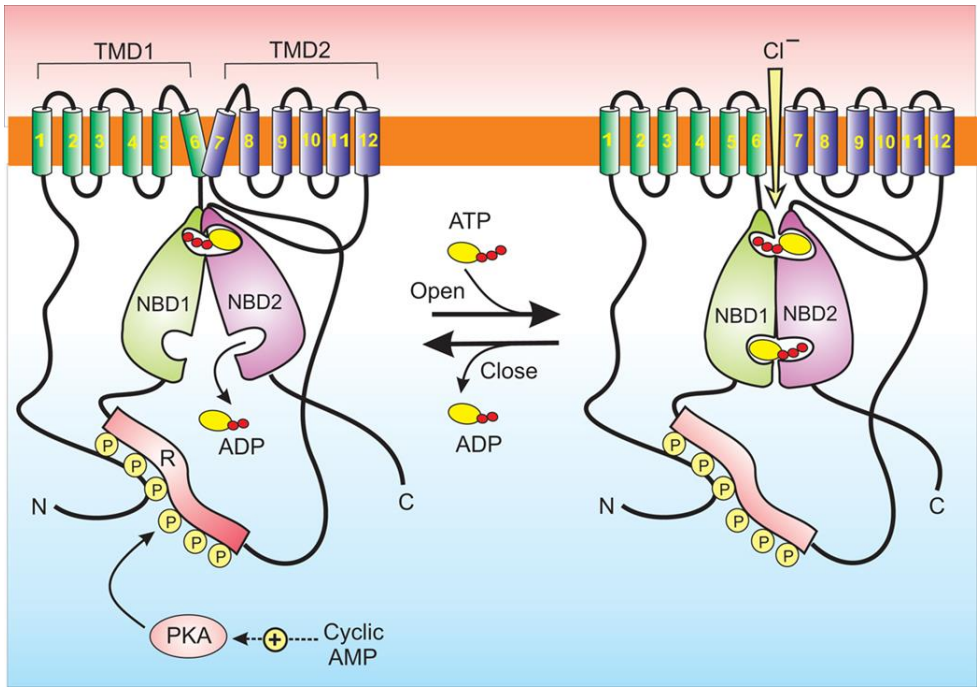

Figure 1-1 Structure of the cystic fibrosis transmembrane conductance regulator (CFTR) channel

Structurally, the CFTR comprises five domains; two transmembrane membrane spanning domains (TMD1 and TMD2), each composed of six transmembrane segments (TM 1-12) that form the channel; two nucleotide binding domains (NBD1 & NBD2) capable of ATP hydrolysis; and a regulatory domain (R) which has phosphorylation sites. Phosphorylation of the R domain by Protein Kinase A (PKA), regulated by cAMP and hydrolysis of ATP by the NBDs activate the chloride channel (10, 12, 13) (reproduced from (12)).

1.2.2 CFTR Ion Channel Function

Long before the CFTR gene and its protein product were ever identified, Darling and Di SantôAgnese recognised that sweat chloride was elevated in CF (14). Andersen had reported heat prostration in a group of children with CF admitted during a severely hot spell during August 1948 (15). In 1953, Darling and Di SantôAgnese performed ñsweat testsò by subjecting participants to thermal stress, and found abnormally high sweat sodium and chloride in children with CF (14). They concluded that this elevated sweat salt content was due to a disturbance of the sweat glands themselves (14). In 1983, experiments performed on isolated sweat ducts identified chloride as the principal affected electrolyte in CF and revealed the physiological basis for high sweat chloride values (16).

The membrane spanning domains of CFTR form an anion selective pore through which anions flow, driven by the transmembrane electrochemical gradient (17). Cycles of ATP binding and hydrolysis control channel gating and phosphorylation of the R domain by Protein Kinase A regulates channel activity (12, 13, 17, 18). In airway epithelium, CFTR functions as an apical chloride channel where it selectively transports chloride and bicarbonate between the cytoplasm and the airway lumen. Trans-epithelial chloride secretion also involves basolateral chloride entry into the cell via Na/K/2Cl co-transporters. Trans-epithelial chloride secretion plays a major role in the hydration of the airway surface liquid. In sweat duct epithelia, CFTR drives the reabsorption of salt (13, 16, 19).

1.2.3 CFTR Regulatory Function

CFTR affects the function of other ion channels and transporters. Whilst the link between dysfunctional CFTR and alterations in the function of other ion channels in airway epithelia remains an active area of debate, a number of authors have proposed mechanistic links. CFTR is reported to have a C terminal cognate binding motif which can facilitate interaction with PDZ domain containing proteins (20). Chloride conductance through CFTR contributes to the transmembrane electrochemical potential, and therefore affects the transport of other charged species (13, 21). Particularly relevant to airway surface liquid hydration, CFTR regulates sodium transport and other chloride transporters in airway epithelia.

Sodium absorption in the airways is mediated by the amiloride-sensitive epithelial Na+ Channel (ENaC) which plays a major role in regulating the hydration of the airway surface liquid (22, 23). Amiloride-sensitive sodium absorption is elevated by 2- to 3- fold in CF (24). Both direct (25-28) and indirect (21, 29) coupling between CFTR and ENaC activity (30-33), expression (34-36) and sodium absorption (37) have been reported.

CFTR also regulates the activity of other Chloride secretory pathways (38). Calcium-activated chloride conductance is reported to be amplified in CF cells, implying that CFTR normally inhibits calcium sensitive chloride conductance (39). CFTR interacts with intracellular signalling networks, including via intracellular calcium and cAMP in confined functional compartments which could regulate the activity of other chloride ion channels (40). CFTR was further reported to regulate intracellular calcium by mediating nucleotide release and activating cell surface purinoreceptors in airway epithelia (41). Ousingsawat et al. recently demonstrated that CFTR and the calcium activated chloride channel TMEM16 show both functional and molecular interaction (42, 43).

1.2.4 CFTR Mutations and CFTR Directed Therapeutic Approaches

Despite CF being recognised as an inherited condition (autosomal recessive) as early as 1944 (44), the gene encoding the CFTR was finally identified in 1989 (9-11). It is located on the long arm of chromosome 7 (7q31.2) and comprises a promoter region and 27 coding exons. Two major international collaborative projects catalogue data relating to mutations in the CFTR gene; CFTR1 (initiated by the Cystic Fibrosis Genetic Analysis Consortium in 1989 and maintained by the CF centre at Sick Kid's in Toronto), and; CFTR2 (2010, supported by the US CF Foundation (45)).

The CFTR2 project reports that more than 1800 variations in the CFTR gene have been discovered (45). Twenty three mutations have been identified by the American College of Medical Genetics as clearly CF-causing. Among the remaining 1700 plus mutations, some cause full blown CF, some cause CF some of the time, some cause a milder form of CF, and other mutations likely have no association with CF (45). Some CFTR polymorphisms modify disease severity.

CFTR mutations have been classified into 6 classes according to their effect on CFTR function (illustrated in Figure 1-2).

Class I: Defective Protein Synthesis

Class I mutations (e.g. W1282X) include nonsense and frame-shift mutations which lead to premature termination codons (PTCõs) and disruption of CFTR protein synthesis. They are associated with a severe form of disease (46). Aminoglycosides can alter ribosomal RNA conformation and reduce the accuracy of codon ï anti-codon pairing and lead to read-through of PTCõs (47, 48). Ataluren (PTC124) was tested as a drug designed to ñread-throughò PTCõs, however a phase III trial demonstrated no significant benefit of treatment over placebo (49).

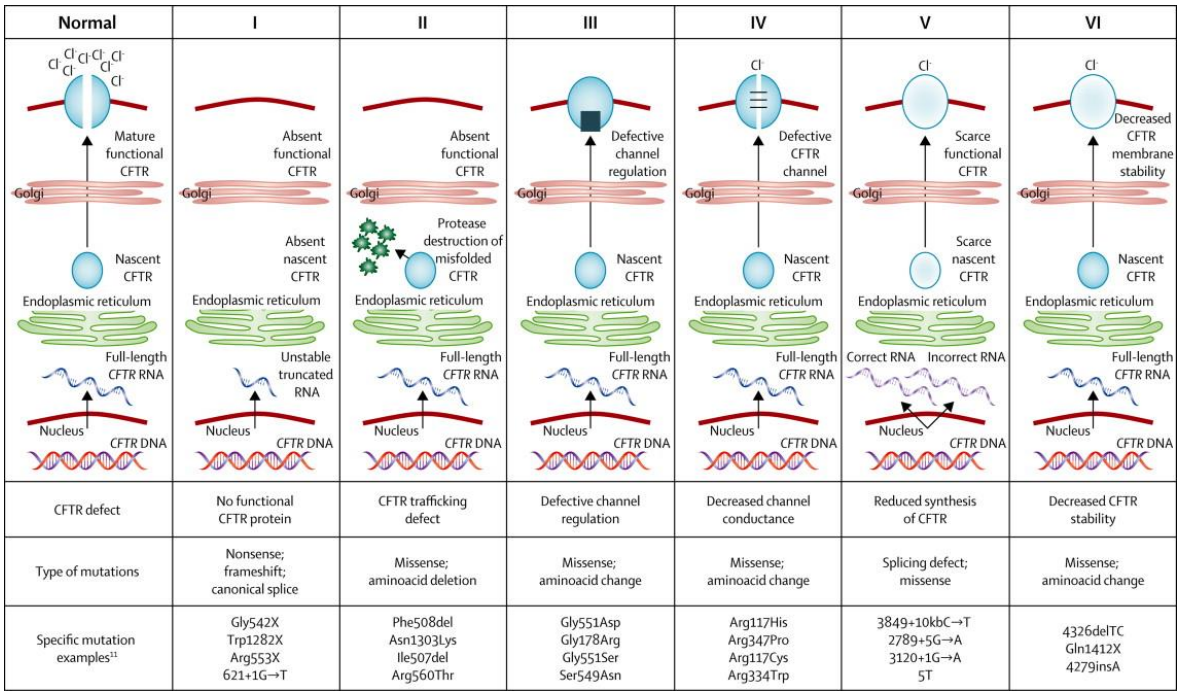

Figure 1-2 Classification of CFTR mutations

Class I: Defective Protein Synthesis; Class II: Defective Protein Processing; Class III: Defective Protein Regulation; Class IV: Impaired Conductance; Class V: Reduced CFTR Level; Class VI: Decreased CFTR stability (Reproduced from (50)).

Class II: Defective Protein Processing

The CFTR mutations which affect the majority of Caucasian patients are class II mutations including Phe508del. Phe508del is a 3 base pair deletion causing a loss of phenylalanine at position 508. After translation, CFTR is normally glycosylated and folded in the endoplasmic reticulum / Golgi apparatus in such a way that allows the protein to be trafficked to the apical membrane. In class II mutations this processing is impaired and the abnormally processed protein is degraded (50).

CFTR ñCorrectorsò

The aim of ñcorrectoròdrugs is to address the processing and trafficking defect of the F508del-CFTR protein to enable it to reach the cell surface. The corrector Lumacaftor (VX-809) alone was trialled among people homozygous for the Phe508del CFTR mutation in a Phase IIa study (51). Lumacaftor gave a small reduction in sweat chloride but didnôt significantly affect any other clinical outcome. The results of Phase III studies (called TRAFFIC and TRANSPORT) of the drug combination Ivacaftor (CFTR Potentiator discussed below) plus Lumacaftor for people homozygous for the Phe508del CFTR mutation have recently been publicised in a press release from Vertex pharmaceuticals (52). The preliminary results reported by Vertex Pharmaceuticals included a mean absolute improvement in FEV_1 of 2.6 to 4.0% and a reduction in the rate of pulmonary exacerbation. VX-661 is also in Phase II trials in combination with Ivacaftor (53).

Class III: Defective Protein Regulation

Class III mutations (e.g. G551D) lead to the production of CFTR proteins which reach the apical membrane but their regulation is defective and the channel cannot be activated by ATP or cAMP.

CFTR Potentiators

The potentiator Kalydeco (also known as Ivacaftor VX-770) is now licenced for people with CF who are 6 years and above with the G551D mutation in the CFTR gene (54, 55). It is believed to work by increasing the open probability of the chloride channel and thus improving the activity of cell surface located mutant CFTR (54, 56, 57). Clinically important improvements reported by the phase III

trials included; increases in FEV_1, weight gain, reduced sweat chloride, reduced exacerbation rate and improved scores on the CFQ-R (54, 55).

Class IV: Impaired Conductance

Class IV mutations (e.g. R117H) are associated with altered conductance and a reduced rate of chloride transport.

Class V: Reduced CFTR Level

Class V mutations (e.g. 3849 + 10kb CŸ T) lead to the production of a normal CFTR protein but at a reduced level. Examples include promoter mutations, amino acid substitutions and splicing mutations. Higher levels of correctly spliced transcripts are associated with milder disease (58). Histone deacetylase inhibitors and splicing modulators have been proposed as therapies for this class of mutations (59).

Class VI: Decreased CFTR Stability

Class VI mutations including premature termination and frame-shift mutations cause C-terminal truncations and lead to a CFTR protein with a shortened half-life (60).

Gene Therapy

In vitro experiments have been successful in achieving restoration of ion transport (Nasal Potential Difference) using either modified viral vectors or synthetic gene transfer to deliver a CFTR transgene (53). Hurdles in clinical application have included; variable expression, short duration / need for repeated dosing and immune responses to the viral vectors (53). A large, phase IIb, repeated dose, randomised, placebo controlled trial of cationic liposome mediated CFTR gene therapy is currently ongoing (61).

Whilst successes have been achieved in CFTR directed therapy, there remain both; CFTR classes for which no specific treatments exist; and residual burdens of established airway disease, for which non-CFTR therapeutic strategies might derive benefit.

1.3 Pathophysiology of CF Lung Disease

Histopathological findings in the airways of people with CF

The most prominent histopathological features of CF lung disease are bronchiectasis and air trapping that can be detected from early infancy (3, 62). CF lung disease primarily affects the airways sparing the alveoli until late in the disease (3). Additional macro-pathologic findings include bronchitis, mucopurulent plugging of bronchi, and bronchopneumonia (63). At a tissue level, glandular hypertrophy, predominance of mucous acini within glands, and goblet cell hyperplasia of the bronchial mucosa have been reported (63). Hallmarks of the disease are chronic bacterial infection and acute inflammation characterised by large numbers of neutrophils persisting within the airway lumen (64). There is lymphocytic infiltration of the submucosa (64). Infection and inflammation have been reported from early infancy (65) and bronchiectasis has been associated with elevated Neutrophil Elastase activity in bronchoalveolar lavage fluid in early childhood (66).

1.3.1 Pathogenesis Sequence

The links between ion transport defects and ASL dehydration caused by CFTR mutation and the consequent pathogenesis sequence (illustrated in Figure 1-3) of infection and inflammation in the pathogenesis of CF lung disease remain the subject of intensive investigation and controversy. We will explore each link in this sequence in greater depth and explore treatment strategies designed to address each step in the cascade.

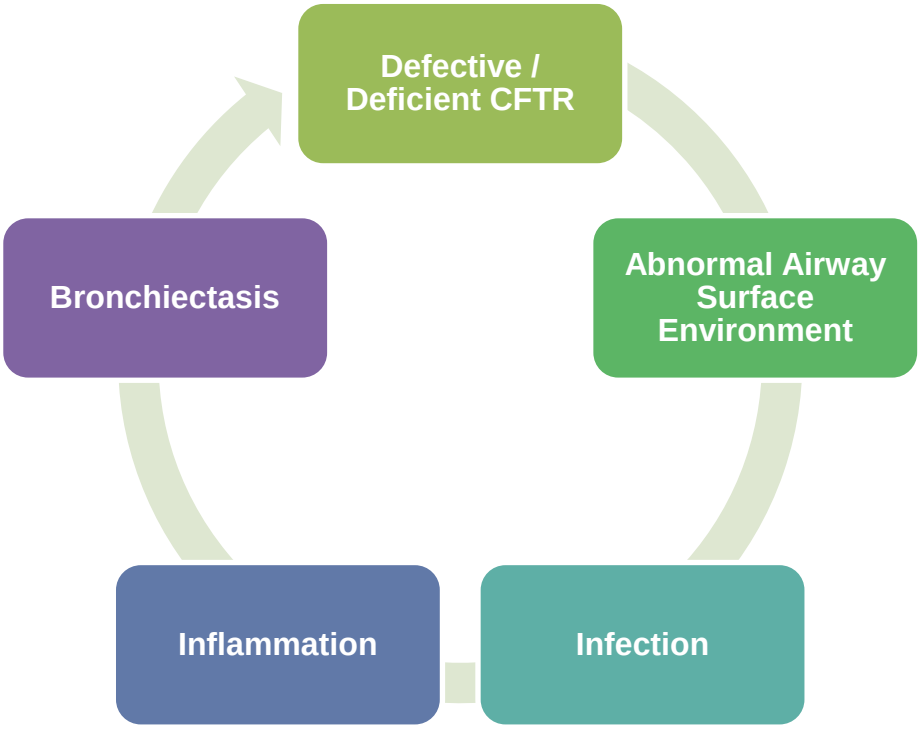

Figure 1-3 Illustration of the key steps in the pathogenesis of CF lung disease

Defective CFTR is the earliest insult in the pathogenesis of lung disease. It gives rise to an abnormal airway surface environment which is prone to infection. A vicious cycle of inflammation and infection contribute to lung damage manifested as bronchiectasis.

1.4 Airway Surface Liquid Dehydration in CF

1.4.1 Structure and Function of the ASL

Mucociliary clearance propels inhaled particles, micro-organisms and allergens from the lung towards the pharynx where they are subsequently either expectorated or swallowed. Mucus clearance requires adequate hydration of the Airway Surface Liquid (ASL); a two phase liquid system that interfaces with beating cilia comprising a mucus phase and the periciliary liquid layer (PCL) (67, 68). Studies of normal airway epithelial cell cultures under steady-state conditions suggest that the optimal depth of the PCL is approximately 7µm, corresponding to the height of the outstretched cilia and facilitating efficient ciliary beating (67-70).

The hydration of the Airway Surface Liquid (ASL) depends on balanced sodium absorption and chloride secretion (67). Sodium absorption and chloride secretion

are active transcellular ion transport processes which create the osmotic force for water movement via aquaporins and the paracellular route (71, 72).

In CF, mutation of the CFTR results in the loss of chloride efflux and sodium hyper-absorption and gives rise to airway surface dehydration and impaired muco-ciliary clearance (73-75) (illustrated in Figure 1-4). The disruption of this innate immune defence mechanism allows the development of bacterial infection and the associated neutrophil mediated inflammatory response.

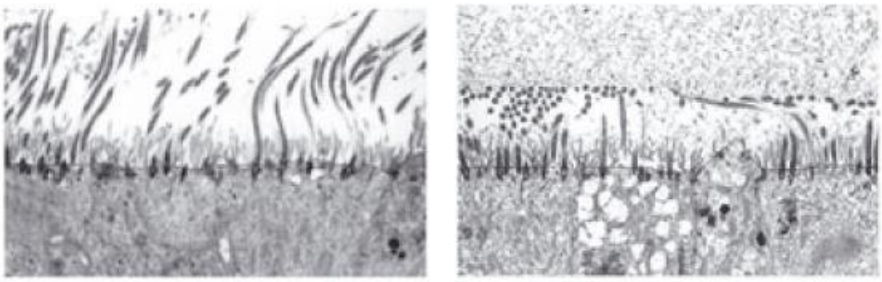

Figure 1-4 Electron Microscopy images of the ASL on well-differentiated non-CF and CF human airway cultures

Well-differentiated non-CF (left) human airway cultures demonstrate optimally extended cilia supporting a thin mucus layer. Well differentiated CF (right) human airway cultures demonstrate a depleted periciliary layer with bent over cilia and a concentrated adherent mucus layer (reproduced from Boucher (73)).

1.4.2 Ion transport in ASL regulation

The optimal hydration of the ASL is achieved via balanced trans-epithelial sodium absorption and chloride secretion (73-75). Trans-epithelial sodium absorption is mediated by the Epithelial Sodium Channel (ENaC) in the apical membrane and the Na/K ATPase localised in the basolateral membrane. Trans-epithelial chloride secretion is mediated by CFTR and Calcium activated Chloride channels (CaCC) in the apical membrane and the Na/K/2Cl co-transporter in the basolateral membrane (43, 76-78).

1.4.3 Chloride transport in ASL regulation

Trans-epithelial chloride secretion is mediated by CFTR and Calcium activated Chloride channels (CaCC) in the apical membrane (43, 76-78). Chloride enters

the cell at the basolateral membrane via Na/K/2Cl co-transporters. The electro-chemical driving force for the trans-epithelial movement of chloride is generated by the recycling of potassium through potassium-selective ion channels in the basolateral membrane (79). We reviewed the function of CFTR as an apical chloride ion channel in section 1.2.2.

Calcium-activated chloride conductance (CaCC) is recognised in airway epithelia and influences airway surface hydration via chloride secretion into the ASL (80-85). Two chloride channels have been reported to contribute to CaCC in the airways (86); TMEM16A (also known as Anoctamin 1) has been identified as the predominant component of CaCC in the luminal airway epithelial membrane (87, 88); whilst, Bestrophin 1 (Best-1) shows endoplasmic reticulum (ER) localization and facilitates receptor-mediated calcium signalling and probably serves as a chloride counter ion channel in the ER (87, 89).

1.4.4 Sodium transport in ASL regulation

Sodium absorption in the airways is mediated by the amiloride-sensitive epithelial Na+ Channel (ENaC) a hetero-trimer comprising Ŭ, ɓ, and ɔ subunits (22). The importance of ENaC mediated sodium absorption in regulating ASL hydration was emphasised by transgenic mouse studies in which over-expression of the ɓ-ENaC subunit gave rise to a CF-like lung pathology phenotype (90). ENaC expression and activity are tightly regulated (91). ENaC is inhibited by intracellular second messengers such as cAMP, calcium and PIP2 (77). Furthermore, proteolytic cleavage of ENaC by intracellular or extracellular proteases activates sodium conductance (77). Interestingly, SPLUNC1 is a secreted innate defence protein present in the ASL which acts as a soluble volume sensor and regulator of ENaC activity by protecting ENaC from proteolytic cleavage (92).

1.4.5 The Purinergic signalling system regulating ASL Volume

Trans-epithelial ion transport and ASL hydration are regulated by the neuro-humoral environment (e.g. nucleotides (39, 93), steroid hormones (94, 95), Nitric oxide (96), Acetylcholine (97)).

A purinergic signalling system is particularly important in the physiological regulation of ASL height allowing the system to be sensitive to dynamic stretch,

viral infection and inflammation (76, 98, 99) (illustrated in Figure 1-5). The dynamic volume of the ASL is sensed by the surface liquid concentration of purines such as ATP and Adenosine (76, 100). The ASL concentration of nucleotides dynamically regulates ion transport processes, balancing sodium absorption and chloride secretion to maintain a functional ASL height (76, 98, 99). For example, under static conditions, airway epithelia gradually release ATP onto the airway surface where it is converted to adenosine by cell surface exo-enzymes. Adenosine signals through the A2b purinoreceptor, regulating intracellular cAMP and affecting CFTR mediated chloride secretion and ENaC activity (101). Moreover, phasic stresses stimulate relatively large increases in ATP release onto the airway surface where a high concentration of ATP activates purinoreceptors resulting in the inhibition of sodium absorption and activation of chloride secretion via CFTR and the CaCC (67, 100).

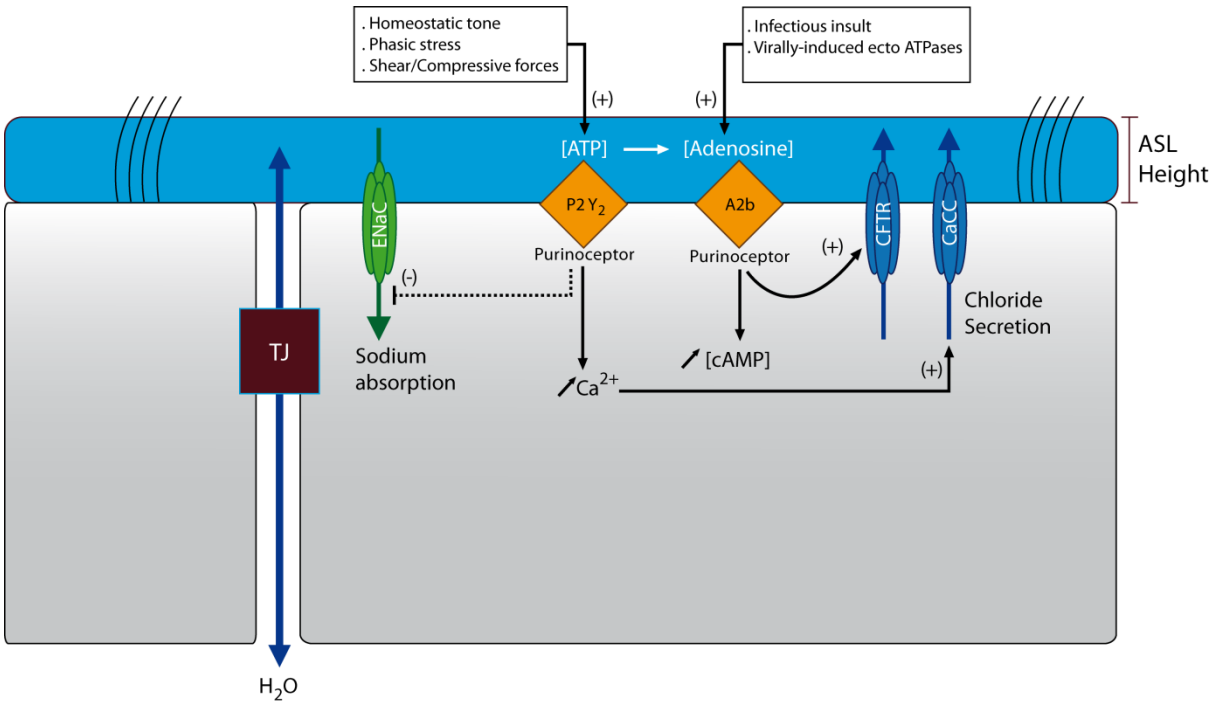

Figure 1-5 Normal regulation of Airway Surface Liquid (ASL) height

ASL volume is regulated by the surface liquid concentration of ATP and Adenosine. Under static conditions, airway epithelia release ATP onto the airway surface where it is converted to adenosine by cell surface exo-enzymes. Adenosine activates the A2b purinoreceptor resulting in increased intracellular cAMP concentration and activation of CFTR regulation of Epithelial Sodium channel (ENaC) and Chloride secretion. Phasic stress stimulates ATP release onto the airway surface where high concentration ATP activates P2Y2 purinoreceptor resulting in ENaC inhibition and activation of chloride secretion via CFTR and the Calcium Activated Chloride channels (CaCC)(reproduced from (102)).

1.4.6 ASL Regulation in Cystic Fibrosis

In CF, the dynamic regulation of ASL hydration is dramatically affected by the loss of CFTR channel function and regulatory activity. Not only does CFTR normally transport chloride and bicarbonate into the ASL, it exerts regulatory effects on both ENaC and CaCC (13, 20-43). In the absence of CFTR mediated inhibition, ENaC mediated sodium absorption is elevated by 2- to 3- fold (24) and CaCC mediated chloride secretion is amplified in CF cells (39). Furthermore, significant cross-talk links chloride secretion and sodium absorption in airway epithelia, via intracellular calcium and cAMP cascades and extracellular purines, with consequences for the regulation of ASL hydration (24, 38-41, 77, 103-107).

The overall consequence of CFTR mutation in airway epithelia is the inability to inhibit sodium absorption and drive chloride secretion. Consequently in CF, the ASL is more rapidly absorbed from airway surfaces and the airway epithelial layer is unable to maintain a functional ASL height/volume under basal conditions. This results in; ASL dehydration, inefficient ciliary beating, mucus dehydration and the adhesion of mucus plaques to the airway surface (73-75, 102). Evidence from animal experiments suggests that airway surface hydration is the most important variable in determining the efficiency of mucus clearance (73).

Airway cells can compensate for the absence of CFTR activity, especially during the motion phase, in part by increasing the release of ATP onto the airway surface, activating purinoreceptors, inhibiting sodium absorption via ENaC and activating Chloride secretion via CaCC (67). However, this means that in CF, the airway is relatively dependent on ATP as its signalling mechanism, rendering the CF airway particularly vulnerable when viral infection results in the induction of ecto-ATP-ases, upon which, the ASL volume can be seen to collapse (illustrated in Figure 1-6) (67, 102).

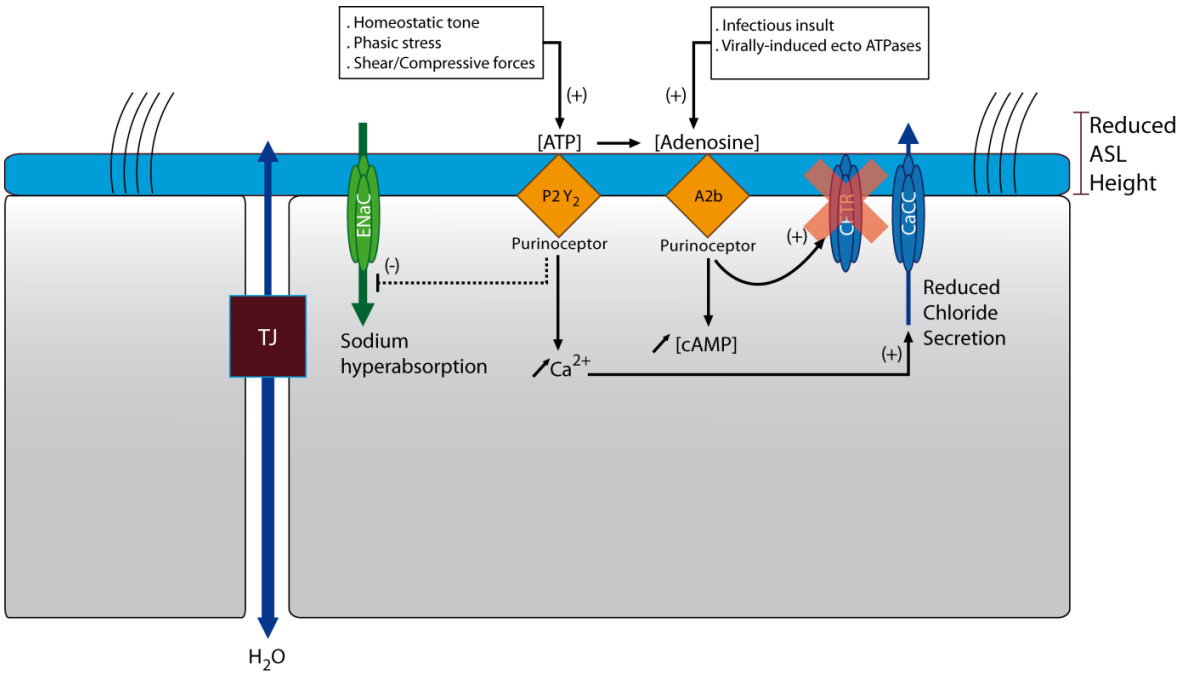

Figure 1-6 Airway Surface Liquid (ASL) height is reduced in CF

In CF, loss of CFTR results in reduced ability to inhibit sodium absorption and secrete chloride. Fluid is more rapidly absorbed from airway surfaces and the epithelium is unable to maintain a functional ASL height. The residual ASL height is relatively dependent on ATP signalling in CF, rendering the CF airway particularly vulnerable when viral infection results in the induction of ecto-ATP-ases (reproduced from (102)).

1.4.7 Pharmacological strategies to improve ASL hydration and muco-ciliary clearance in CF

Pulmozyme, also known as Dornase alfa, is a recombinant deoxyribonuclease (DNase) that cleaves extracellular DNA in the airway and improves the rheodynamics of mucus (108). Pulmozyme was tested in children and found to improve the Lung Clearance Index (LCI) significantly (108, 109).

Hypertonic saline inhalation has been demonstrated to reduce the rate of pulmonary exacerbation in older children and improve infant lung function parameters in an infant population (110, 111). It is proposed to mediate its beneficial effects on muco-ciliary clearance via rehydration of the ASL (112-114).

Mannitol is an osmotic agent administered as a dry powder by inhalation to rehydrate the ASL. In Phase III studies it was found to significantly improve FEV_1,

however, was associated with adverse effects including cough, pharyngolaryngeal pain and haemoptysis (115).

Several compounds are in development which aim to restore muco-ciliary function via ASL rehydration including; hyperosmolar agents designed to draw water into the dehydrated ASL; and ion transport modulators targeting the activation of alternative chloride transport pathways and/or the inhibition of ENaC mediated sodium hyperabsorption (116-123).

1.5 CF Airway Infection

1.5.1 Pathogenesis of CF Airway Infection

The CF airway is predisposed to bacterial infection, and airway infection is independently associated with the evolution of lung function impairment (124). Mechanistic links between CFTR mutation and the susceptibility to bacterial infection characteristic of CF have been attributed to; the chemical composition of the ASL, including pH; the characteristics of the airway epithelial surface; the mechanical function of the muco-ciliary apparatus; and impaired phagocytosis and bacterial killing by phagocytes (125-127).

The ASL is not an inert lubricating liquid. It has a rapid bactericidal effect on incident bacteria via the function of anti-microbial peptides and proteins such as human beta-defensins, lysozyme, lactoferrin, palate lung and nasal epithelial clone (PLUNC), Secretory leucocyte protease inhibitor and surfactant protein SP-A (128-131). The function of anti-microbial peptides is salt and pH-sensitive (128, 129). CFTR facilitates HCO3- transport into the ASL, and in the absence of functional CFTR, the ASL pH is reported to be significantly more acidic (128). Impaired bactericidal capacity of the ASL has been reported in CF (128, 132, 133). Furthermore, excessive binding of *Staphylococcus aureus* and *Pseudomonas aeruginosa* to the apical membrane of CF bronchial epithelia has been reported, related to an increased concentration of asialoganglioside 1 expression (134, 135).

Mucus clearance performs a mechanical innate defence function in the lung by trapping bacteria and micro-particles allowing them to be swept out of the lungs into the oropharynx. Muco-ciliary transport depends on optimal hydration of the ASL (a depth of ~7µm) to allow the cilia to be fully outstretched, beat efficiently

and allow efficient mucus clearance (69, 84). In CF, altered ion transport results in ASL dehydration, collapse of the cilia and dysfunctional mucus transport (69, 84). Thickened and hypoxic mucus plaques adhere to CF epithelia and produce a nidus for bacterial colonisation (136, 137). Furthermore, *Pseudomonas aeruginosa* product pyocyanin has been reported to disrupt ciliary function (138)

Finally, both epithelia and alveolar macrophages play a role in airway defence by bacterial internalisation and killing. A failure of CF epithelial cells to internalise and kill bacteria (139), and impaired intracellular killing of *Pseudomonas aeruginosa* by human and mouse CF macrophages have been reported (125-127).

1.5.2 Bacterial pathogens affecting the airways in CF

Early in the course of CF lung disease *Staphylococcus aureus* and *Haemophilus influenza* are most prevalent. *Pseudomonas aeruginosa* colonisation becomes more prevalent through early childhood into adulthood. *Methicillin Resistant Staphylococcus aureus (MRSA), Stenotrophomonas maltophilia, Burkholderia cepacia complex* and Multidrug resistant *Pseudomonas aeruginosa* affect a smaller percentage of patients with CF across the age range (8).

1.5.3 Conventional antibiotic management strategies

Conventional antibiotics have been used in the management of CF airway infection since the 1940ôs. Paul Di Sant'Agnese and Andersen used ñchemotherapy (é and in particularé) penicillin and drugs of the sulphonamide group, with special reference to penicillin aerosol (140)ò Current strategies to retard the onset and evolution of chronic endoluminal bacterial infection have focused on; *Staphylococcus aureus* prophylaxis; *Pseudomonas aeruginosa* eradication and chronic suppression; and treatment of acute exacerbation. The development of specific protocols to guide the appropriate antibiotic management of the CF airway has driven the evolution of ñbest practiceò and incremental improvements in lung health across the board (141).

Staphylococcus aureus (S. aureus)

S. aureus is a common early pathogen in CF (142). Colonization itself may or may not cause clinically significant pulmonary inflammation or deterioration (143, 144), but may predispose to *Pseudomonas aeruginosa* infection in a variety of ways

(145). Its pathogenicity depends on the combined action of more than 40 different extracellular toxins, enzymes, quorum sensors and cell surface proteins controlled by global regulatory elements (146-148). Small colony variants are slow growing antibiotic resistant sub-populations of *S. aureus*, which are more likely to persist in the airway and are associated with worse lung disease in children (142, 149). MRSA is characterised by resistance to penicillin (performed by ϐ-lactamase), and penicillinase-resistant compounds like methicillin and flucloxacillin (altered penicillin-binding protein PBP2a). MRSA chronic colonisation has been linked to bronchiectasis and a greater rate of FEV_1 decline (150).

Anti-staphylococcal prophylaxis with Flucloxacillin to reduce *S. aureus* carriage rate is common practice, though debated as a strategy (141, 151). The objective is to prevent *S. aureus* carriage and indirectly to prevent the acquisition of *P. aeruginosa*. Controversy has surrounded the practice since Ratjen et al. reported that patients receiving prophylaxis had significantly fewer *S. aureus* sputum isolates, but a significantly higher rate of *P. aeruginosa* acquisition (152). Interpretation of these findings was complicated by differences in the antibiotic selection employed since many of the study population received a broad-spectrum cephalosporin antibiotic, which has subsequently been more specifically associated with the isolation of *P. aeruginosa* (143). Eradication strategies for *MRSA* are the subject of on-going clinical trial evaluation (153). For example, AeroVanc is an inhaled dry powder version of the antibiotic vancomycin which is being evaluated for the treatment of MRSA airway infection (154).

Pseudomonas aeruginosa (P. aeruginosa)

Persistent lower airways *P. aeruginosa* infection causes significant morbidity and mortality in CF (155). Genotypically identical *P. aeruginosa* isolates can express different phenotypic characteristics (156). Early infections are usually with non-mucoid environmental strains which are relatively antibiotic sensitive and more amenable to eradication (157). Later in the course of disease a mucoid, antibiotic-resistant *P. aeruginosa* phenotype emerges and accompanies an accelerated decline in pulmonary function (158, 159). *P. aeruginosa* possesses several virulence factors which allow it to bind, grow in biofilm and evade the host response; type IV pili mediate attachment to host cells interacting with the glycolipid receptor asialoGM1 (135); fimbrial adhesins are involved in adhesion

and biofilm formation (160); *P. aeruginosa* binds to mucin (161). *P. aeruginosa* have quorum sensing systems (162). *P. aeruginosa* can grow in anaerobic conditions and under hypoxic stress or oxygen free radical attack *P. aeruginosa* forms a biofilm (137, 163). In biofilm *P. aeruginosa* can produce lipoproteins that induce an inflammatory response by the host cell (164). Hyper-mutation has also been reported in *P. aeruginosa* isolates from CF patients (165). Patient to patient cross-infection can occur and transmissible epidemic strains (probably acquired by droplet transmission) have been reported (156).

The optimal management of *P. aeruginosa* includes prevention, including cohort segregation (166), early detection (167), aggressive attempted eradication (168), chronic antibiotic suppression and the treatment of acute exacerbation (141, 169). Several antibiotic formulations for the treatment or chronic suppression of *P. aeruginosa* airway infection are either in use, or in the ñpipelineò including; tobramycin, used as a nebulised solution or as a dry powder; inhaled aztreonam (Cayston™) and an inhaled liposomal formulation of amikacin called Arikace™.

1.5.4 Intrinsically drug resistant CF pathogens

Burkholderia cepacia complex (Bcc) is a group of gram negative bacteria including *B. cepacia, B. multivorans and B. cenocepacia.* *Bcc* can cause cepacia syndrome; a fulminating pneumonia with a very high mortality rate presenting with high fever, severe progressive respiratory failure, leucocytosis, and elevated erythrocyte sedimentation rate (170). *Bcc* are often multi-drug resistant and sometimes pan-resistant (they produce an inducible ß-lactamase, can use penicillin as a carbon source and are even resistant to defensins) and transmissible via social or nosocomial contact (13, 171).

Nontuberculous Mycobacterial infection can complicate CF lung disease. It is often difficult to diagnose in patients with CF in view of its indolent progression and the overlap between clinical features of CF and disease caused by NTM. Furthermore, the clinical course of NTM infection in CF can be unpredictable with some patients experiencing a very indolent course and others demonstrating NTM related progressive clinical decline (13).

1.5.5 Other Bacteria relevant to CF lung disease

Since *Haemophilus influenzae* often colonises the upper airways of healthy children (172) there is controversy as to whether colonisation alone plays any role in CF. There is broader agreement that it plays a role in acute exacerbation of CF and as such should be treated (173). Debate surrounds whether *Stenotrophomonas maltophilia* causes clinically significant pulmonary deterioration (174, 175), however, it may play an indirect role by secreting ß-lactamases and inhibiting antibiotic therapy, encouraging the growth of more virulent CF pathogens (176). The clinical significance of *Achromobacter xylosoxidans* in CF lung disease is not clear; it can give rise to transient or chronic lung infection, and tends to affect people with advanced CF lung disease (173).

Developments in the field of environmental microbiology have led to the realisation that many more species of bacteria are present in both CF airways and in disease control airways than are captured by culture based methods (177). The relationships within these ñpolymicrobial communitiesò of aerobes, facultative anaerobes and obligate anaerobes and their role in CF lung disease are subjects of hot debate. Positive relationships between high pulmonary microbial diversity and pulmonary health are reported (178).

1.5.6 Infection invites a host inflammatory response

Following initial bacterial colonisation, the micro-environment becomes increasingly complicated. The inflammatory response fails to eradicate infection and chronic endoluminal bacterial infection persists alongside a prolonged, frustrated, ineffective inflammatory response.

1.6 CF Airway Inflammation

1.6.1 Pathogenesis of airway inflammation in CF

Disrupted innate defence leaves the airway in CF prone to infection and the resultant host response to infection is exaggerated, maladaptive and ineffective. Characteristics of the inflammatory response include abnormal cytokine secretion and neutrophil infiltration in the lung (179, 180). Inflammation in CF fails to eradicate the infectious insult and persists in a self-propagating cycle of neutrophil mediated acute inflammation and chronic bacterial infection, leading to tissue damage, bronchiectasis and declining lung function (3, 62-64). There is debate around the hierarchical relationship between infection and inflammation in the pathogenic sequence of airway disease in CF. Young culture negative infants with CF have been reported to have elevated IL8 concentrations in their airways (65, 181), however investigators studying young infants with CF detected by new-born screening have also provided evidence that increased inflammatory marker detection in the airway follows the development of infection in CF (182, 183).

In order to identify the cell types mechanistically responsible for exaggerated immune responses to infection in the airway in CF, Bruscia et al. studied the inflammatory response to LPS challenge in wild type and CFTR knockout mice, and mouse bone marrow chimeras transplanted with either CFTR deficient or wild type bone marrow (179). They demonstrated that enhanced pro-inflammatory cytokine secretion in this mouse model was attributable to CFTR deficiency in recruited immune cells, whereas enhanced neutrophil migration was attributable to both epithelial and recruited immune cells (179). We will consider the underlying pathogenic mechanisms operant in epithelia, neutrophils, and macrophages in CF and then go on to consider the interplay between cells types via inflammatory cytokine networks. Furthermore, we will consider anti-inflammatory strategies to ameliorate inflammation in CF.

1.6.2 Normal pro-inflammatory signalling and exaggerated signalling in CF airway epithelia

The immune system senses pathogens via conserved molecular patterns that interact with Pattern Recognition Receptors such as the Toll-like receptor family (184). These receptors are coupled to signal transduction cascades that control inducible immune response genes, most importantly, the NF-κB family of

transcription factors. NF-κB transcription factors are powerful regulators of inducible gene expression that co-ordinates innate and adaptive responses to antigenic stimuli, resulting in cytokine production to achieve pathogen clearance (illustrated in Figure 1-7) (185).

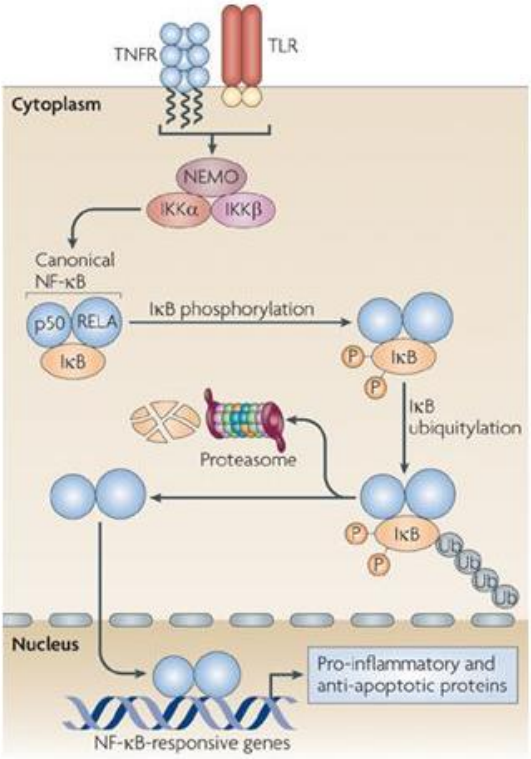

Figure 1-7 Schematic of the canonical NF-κB signalling pathway
NF-κB dimers are normally sequestered in the cytoplasm associated with IκB proteins. Pro-inflammatory signals stimulate receptors belonging to the tumour necrosis factor receptor (TNFR) or Toll-like receptor (TLR) families, which activate the IκB kinase (IKK) complex. Activation of the IKK complex (comprising IKKα, IKKβ and NEMO subunits) results IκB phosphorylation, ubiquitylation and proteasome-dependent degradation. This allows NF-κB dimers to translocate to the nucleus, bind to NF-κB sites on the promoters of target genes and activate the transcription of genes with pro-inflammatory functions (Reproduced from Pasparakis (185)).

Pseudomonas aeruginosa flagellum for example, triggers an inflammatory response by binding to TLR5 in co-operation with either TLR2, or the asialo-GM1 receptor (186). These receptors transduce a MyD88-dependent signalling cascade that activates protein kinases and a calcium-sensitive signalling pathway

(186), resulting in IL8 induction via phosphorylation of MAPKâs p38, ERK and JNK and activation of NFHB (186, 187). IL8 is a potent neutrophil chemo-attractant.

In CF epithelia, excessive NFHB activation has been observed (65, 188, 189) attended by increased IL8 concentration (190) and excessive neutrophil recruitment (64). A number of explanations for this excessive NFHB activation have been advanced. Amplified NFHB activation in response to persistent bacterial infection and pro-inflammatory cytokine stimulation (191, 192) represents a cell extrinsic explanation for this finding. However, cell intrinsic explanations have also been advanced associated with; ER stress due to the accumulation of misfolded CFTR protein; and, ER calcium store expansion and amplification of the calcium-dependent inflammatory response (192, 193).

1.6.3 NFHB activation via the Unfolded Protein Response

In a study comparing the effect of G551D versus Phe508del mutant CFTR expression on NFHB activation, a sevenfold excess in NFHB activation was attributed to ER retention of the misfolded Phe508del CFTR (194). ER retention of misfolded Phe508del CFTR leads to ER stress and activation of the unfolded protein response (UPR) (189). UPR activation leads to a reduction of protein synthesis via phosphorylation and inactivation of the translation initiation factor e1F-2Ŭ, as an adaptive mechanism to cope with ER stress (195, 196). However, in addition to switching off protein synthesis, ER stress also signals for activation of NFHB via the ER-overload response, thus, contributing to an inflammatory response (196, 197). Conversely, ER stress in the context of increased protein synthesis during an acute inflammatory response, may also affect CFTR expression itself via the UPR (195).

1.6.4 Normal calcium regulation in airway epithelia and amplification of the calcium dependent inflammatory response in CF

The intracellular calcium concentration under normal circumstances is maintained relatively low (100 nM) compared to the extracellular concentration (about 2 mM). Extracellular stimuli communicate with airway epithelial cells via G-Protein Coupled Receptors (GPCR) that generate an influx of calcium into the cell. GPCR stimulation activates Phospholipase C (198), to cleave Phosphatidylinositol 4,5-bisphosphate into Diacylglycerol (DAG) and Inositol triphosphate (IP3). IP3

activates its specific receptor (IP3R) and stimulates calcium release from the ER. Depletion of calcium from the ER activates extracellular calcium influx. Removal of calcium from the cytoplasm depends on the activation of the SERCA2 pump (199) to replenish the ER and also upon the plasma membrane calcium ATPase to extrude calcium towards the extracellular space (200).

Intracellular calcium affects almost all major cellular processes, including those involved in ASL dynamics, ciliary beat frequency (201-203), secretion of mucus and ion transport (204, 205). Enzymes and proteins participating in pro-inflammatory signalling cascades including NFκB pathway (kinases, phosphatases, transcription factors, calcineurin and calmodulin) are also calcium-sensitive.

Several aspects of intracellular calcium homeostasis are disturbed in CF airway epithelial cells. GPCR induced calcium release from intracellular stores and entry through the plasma membrane are amplified in CF airway epithelial cells (192). IP3Rs-dependent calcium release from the ER is increased as a consequence of misfolded mutant CFTR trapped in the ER (193, 206). ER calcium stores are expanded in CF epithelia, associated with ER concentration and condensation around the nucleus and IP3R clustering at the ER membrane, resulting in facilitated activation (192, 193). Depletion of calcium from the ER normally activates Stromal Interaction Molecule 1 (STIM1) which, in turn activates Calcium release-activated calcium channel protein 1 (Orai1) and transient receptor potential 6 channel (TRPC6) to activate extracellular calcium influx (207, 208). CFTR normally supresses extracellular calcium influx via a regulatory effect on TRPC6 and this suppression is lost in CF cells (209). Enhanced Orai1/STIM1 complex formation during store depletion in CF cells is also reported to contribute to increased calcium signalling (210). Furthermore, the correction of mutant CFTR folding and trafficking to the membrane using miglustat is associated with the normalisation of calcium homeostasis (193). Lastly, chronic infection itself may lead to the amplification of intracellular calcium stores, since non-CF airway epithelial cells were also reported to respond to chronic bacterial stimulation with increased ER size and calcium storage (192). Taken together, several paths lead to abnormal calcium homeostasis in CF airway epithelia, which plays a role in excessive NFκB activation via the amplification of calcium dependent inflammatory

response signalling pathways (192, 193). Over-exuberant NFκB activation and IL8 secretion by airway epithelial cells drive excessive neutrophil recruitment in the CF airway (65, 179).

1.6.5 Neutrophils in CF Airway Inflammation

The inflammatory response in the airway in CF is neutrophil dominated and associated with an excessively high neutrophil burden (180). Moreover, neutrophil effector functions including bacterial phagocytosis and killing are impaired in CF (211-213) and Neutrophil Elastase release by neutrophils causes lung damage (66).

A number of factors contribute to the excessive neutrophil population in the airway. Airway concentrations of neutrophil chemo-attractants such as IL8 (65) and Leukotriene B4 (LTB4) (214) are elevated. LTB4 and IL8 in particular negatively correlate with pulmonary function in CF (190, 214). Selectins, involved in tethering neutrophils to the endothelium in the earliest step of neutrophil transmigration into the lungs are increased in the circulations of people with CF (215, 216). Soluble ICAM-1, involved in the strong adhesion of neutrophils to endothelium is reported to be elevated in serum in people with CF (215). In co-cultures of CF neutrophils and bronchial epithelial cells, neutrophil adherence to the epithelium is increased resulting in increased IL8 secretion (217). The efferocytic removal of neutrophils from the airway is also reported to be impaired, due to Neutrophil Elastase mediated calreticulin cleavage, and resulting in the inhibition of phosphatidylserine mediated ingestion of apoptotic neutrophils (218-220).

Unfortunately, the robust neutrophil response in CF does not achieve the aim of containing and eradicating the bacterial insult. On the contrary, neutrophils get trapped in biofilms where they can self-injure (221). They have reduced phagocytic capacity (211). Defective intra-phagolysosomal hypochlorous acid production in CF neutrophils is reported to result in impaired chlorination of bacterial proteins (212). Neutrophil Elastase cleaves the chemokine receptor CXCR1 on neutrophils, disabling IL8's ability to enhance their bactericidal capacity (213).

Whilst proteases such as matrix metalloproteinases and Elastase, released by neutrophils, play an important role in phagocytosis and bacterial killing, they can also degrade the extracellular matrix and cause lung damage. The clinico-pathologic importance of Neutrophil Elastase (NE) in CF lung disease was recently emphasised by the finding that NE activity in BAL fluid in early life is associated with early bronchiectasis in children with CF (66). Free NE concentration in the airway depends on a number of factors including; the neutrophil burden; the rate of NE release (222); NE neutralisation by anti-proteases in the lung (223); the extent of neutrophil cell death by necrosis versus apoptosis (220); and the removal of dying neutrophils by efferocytosis (220). TNFŬ and IL8 in the airway drive neutrophils to release significantly increased amounts of NE, and neutrophils from people with CF release more Elastase than controls (221, 222). Free NE inactivates a range of protective immune effectors whilst stimulating mucus secretion and the liberation of the potent chemo-attractants IL8 and C5a (221).

1.6.6 Macrophages in CF

Enhanced neutrophil migration in CF mouse models was attributed both to epithelial cells and recruited macrophages (179). Furthermore, enhanced pro-inflammatory cytokine secretion was particularly attributed to CFTR deficiency in macrophages (179). CFTR is expressed at low copy number by human macrophages (126, 224). Moreover, macrophage functional defects are increasingly recognised in CF (225) including; defective cytokine secretion; impaired phagocytosis and bacterial killing; and impaired neutrophil efferocytosis.

CF monocytes were reported to secrete excess IL8 in response to bacterial LPS, due to lower thresholds for the activation of mitogen-activated protein kinases (226). This finding was corroborated by reports of abnormal TLR4 trafficking and degradation leading to increased LPS-induced activation of NFHB in CF monocytes (227).

Impaired phagocytic capacity of human CF, or CFTR inhibited, non-CF macrophages, has been reported (127, 228). Impaired intracellular bacterial killing of *Pseudomonas aeruginosa* has been reported in several studies of human and mouse CF macrophages (125-127). Though debated, this finding has been related to; CFTRôs involvement in lysosomal acidification (126, 229, 230); and,

altered ceramide concentration affecting the release of reactive oxygen species in CF macrophages (230). Defective endosome to late-endosome/lysosome maturation during LPS challenge is also reported (227).

Impaired removal of apoptotic cells (efferocytosis) by macrophages has been reported in CF (218, 219). Neutrophil Elastase was demonstrated to cleave calreticulin and inhibit phosphatidylserine mediated ingestion of apoptotic cells, thus supressing efferocytosis (220).

1.6.7 Imbalanced airway cytokine profiles in CF

Cellular crosstalk via peptide and lipid immune mediators is necessary for the co-ordination of the inflammatory response. Independent of which cell type initiates this cycle, and whether infection predates the inflammatory defect, or vice versa, the impact of CF on pro- inflammatory and anti-inflammatory cytokine balance is widely reported to be weighed in favour of pro-inflammatory cytokine production (illustrated in Figure 1-8) (231).

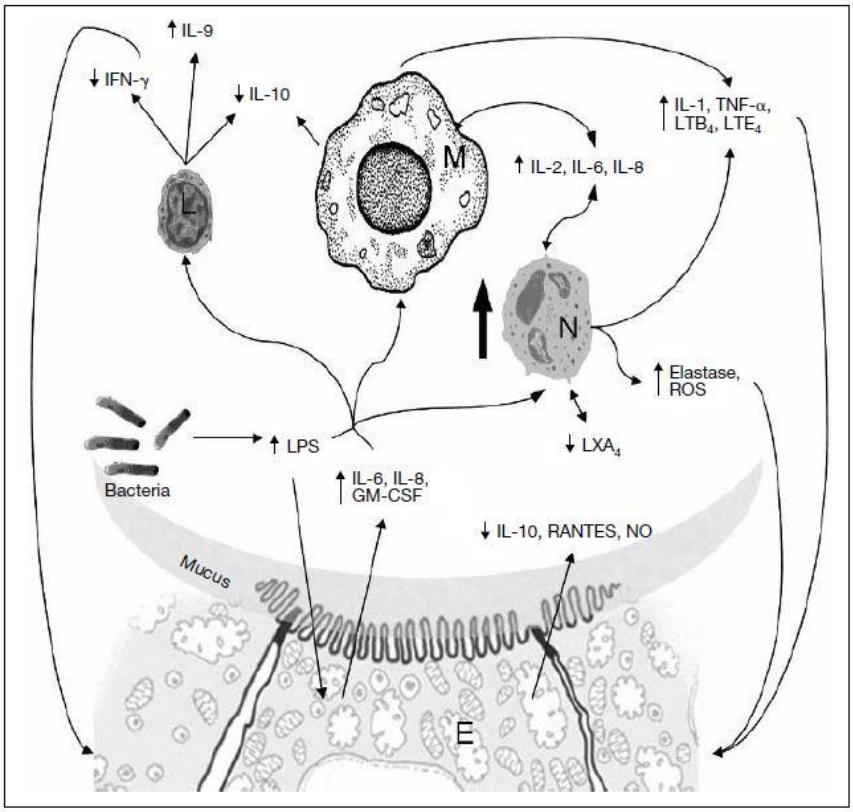

Figure 1-8 An illustration of cellular cross-talk in co-ordinating the airway inflammatory response in CF

Airway epithelial cells (E), neutrophils (N), macrophages (M), lymphocytes (L) and bacteria are depicted. TNFŬ, IL1, IL2, IL6, IL8, IL9, LTB4, LTE4, Elastase, ROS, LPS, GM-CSF concentrations are increased; (depicted by up arrows) in CF compared to normal. Conversely, Interferon-ɔ, IL10, Lipoxin A_4 (LXA_4) , RANTES, Nitric Oxide (NO) are significantly decreased (depicted by down arrows) (reproduced from Ziady et al. (13)).

Elevated airway concentrations of pro-inflammatory bacterial LPS, reactive oxygen species, IL-1ɒ and TNFŬ, in the face of reduced anti-inflammatory IL10 and TGF-ɒ concentration, promotes the activation of NFHB and drives the transcription of further pro-inflammatory cytokines including; IL6, IL8 and GM-CSF (232-236). Nitric oxide (NO) plays a role in bacterial killing and an anti-inflammatory role via NFHB inhibition (237). Impaired STAT-1 signalling in CF results in reduced production of NO by nitric oxide synthase 2 (NOS2) (237, 238).

1.6.8 Anti-inflammatory strategies

Several anti-inflammatory strategies have been evaluated for CF, however no anti-inflammatory has been universally adopted into the core treatment regime (239).

Moreover, caution has been exercised in the consideration of anti-inflammatory strategies for CF (especially pertaining to the complicating high burden of bacterial infection) in light of the lessons learned from an unsuccessful clinical trial of a leukotriene antagonist BIIL-284 (53, 240). A 24 week, placebo-controlled phase II trial of BIIL-284 in children and adults with CF was terminated prematurely in 2004, due to a significantly increased risk of adverse pulmonary events (particularly pulmonary exacerbations) in the adult patients receiving active treatment (241). Furthermore, it was recently reported that BIIL284 reduced inflammation, but at the expense of bacterial containment in mouse studies (241).

Glucocorticoids are central to anti-inflammatory therapy in several inflammatory disorders (242). Dexamethasone antagonises the pro-inflammatory transcriptional response by binding to and activating the glucocorticoid receptor. The activated receptor then binds directly to NFκB, which is bound to its own site on pro-inflammatory gene promoters. Activated glucocorticoid receptor physically hinders the phosphorylation of RNA polymerase II ï preventing the initiation of pro-inflammatory gene transcription (243). On the other hand, glucocorticoids have been reported to produce anti-secretory effects on airway epithelial cells, which would be deleterious in the context of the ASL dehydration central to airways disease pathogenesis in CF (95, 244-246). A systematic review of the risks and benefits of Inhaled corticosteroids, concluded that there is insufficient evidence to establish whether ICS are beneficial in CF, but withdrawal in those already taking them has been shown to be safe (247). The use of glucocorticoids has further been limited by adverse side effect profiles when taken systemically (cataracts and growth retardation) (248).

High-dose ibuprofen treatment has been associated with significantly lower annual rates of decline in lung function (especially in children), however, its adoption into therapy has not been universally accepted due to concerns over side effects (249, 250).

Compounds currently under evaluation include Sildenafil, KB001-A and Alpha 1 Anti-trypsin (154). Sildenafil is in Phase II studies as an anti-inflammatory, following reports that PDE type 5 inhibitors may rescue Phe508del-CFTR trafficking and attenuate inflammation in mice (154, 251). KB001-A is a

humanized monoclonal Fab fragment that targets the *Pseudomonas aeruginosa* Type III secretion system and is being tested in Phase II studies (154). Alpha 1 Anti-trypsin completed Phase II studies in 2013 (NCT01684410). It is an aerosolized protease inhibitor that targets free Neutrophil Elastase released by neutrophils in the airway (154).

Given the role played by the vicious cycle of infection and inflammation in the development of bronchiectasis in CF (illustrated in Figure 1-9); the problems of resistance to conventional antibiotics and their side effects; and the burden of morbidity and premature mortality associated with lung disease in CF novel strategies to address both bacterial infection and inflammation in CF are sought. Identifying novel strategies to meet these clinical needs rests on an improved understanding of innate defence, dysregulated inflammatory responses, host - pathogen and pathogen-pathogen interactions in the CF airway. Agents that prevent, or disrupt biofilm formation, improve the activity of existing antibiotics, or augment the host immune response are actively sought (252).

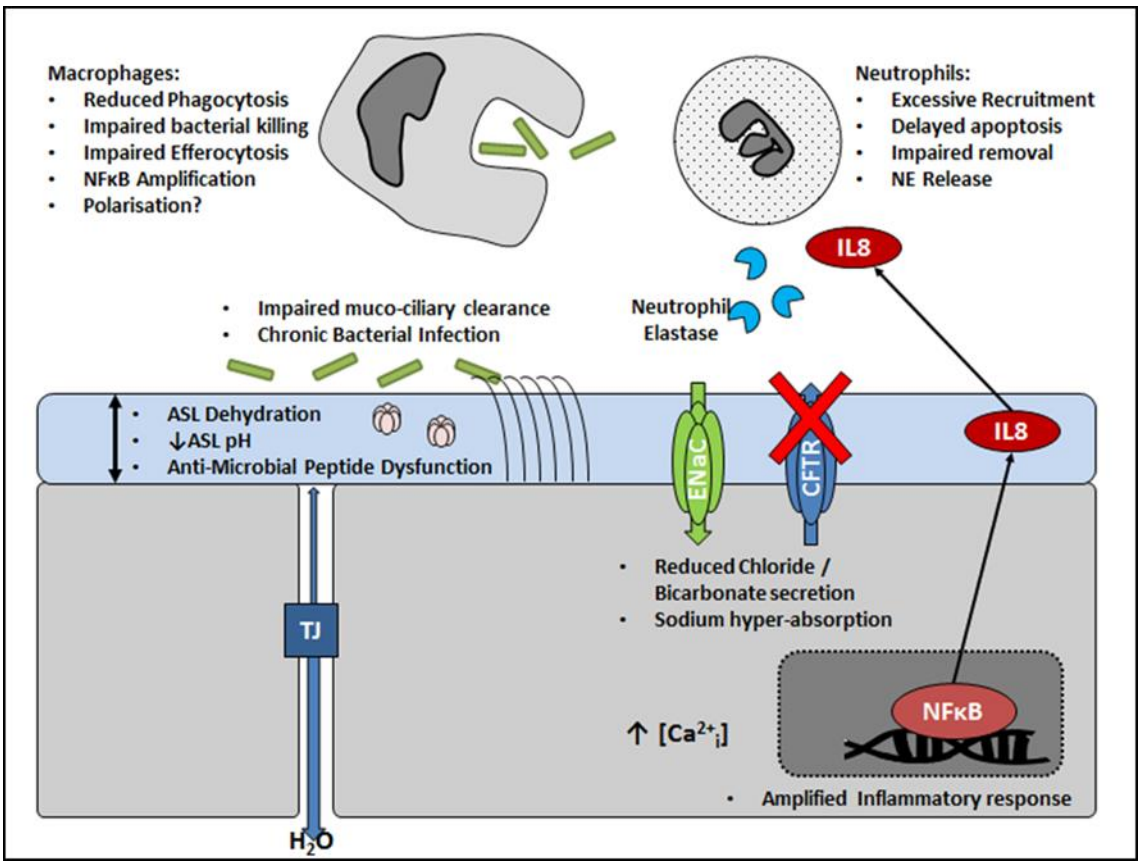

Figure 1-9 An illustration of the pathogenesis of CF airway disease

CF is caused by mutation of the gene encoding the CFTR chloride channel. This results in reduced chloride and bicarbonate secretion, sodium hyper-absorption and therefore dehydration and acidification of the ASL. Innate anti-microbial peptide dysfunction and impaired muco-ciliary clearance allow persistence of inhaled pathogens and eventually chronic endo-luminal bacterial infection. Due to amplified pro-inflammatory responses and high levels of interleukin 8 in the airway there is robust recruitment of neutrophils. The neutrophil response fails to clear the bacterial insult and the burden remains high in the airway compounded by impaired clearance of neutrophils by macrophage mediated efferocytosis. Free Neutrophil Elastase in the airway causes tissue damage and bronchiectasis ensues. Macrophages in CF have reduced phagocytic capacity, impaired bacterial killing and are impaired in efferocytosis.

1.7 Failure of the Inflammatory Response to Resolve in CF

Under normal circumstances, lung inflammation should eliminate the original threat, and then actively resolve and return the tissue to homeostasis (253). A key feature of CF, is the severe, sustained neutrophil predominant response, which persists even if the original inflammatory stimulus is removed (221). We have

considered components of the perpetual cycle of infection & inflammation including; innate predisposition to infection and the evolution of chronic endoluminal bacterial infection; and, excess neutrophil recruitment via airway epithelial and resident immune effector synthesis of pro-inflammatory cytokines. However, the underlying basis for the failure of resolution of this infection-inflammation couple remains incompletely understood. To this end we move on now to consider ñactiveò resolution of inflammation in CF.

1.8 The Active Resolution of Inflammation

1.8.1 Trans-cellular biosynthesis and the Discovery of the Specialised Pro-resolution Mediators (SPMôs)

As early as 1976 it was hypothesized that the production of eicosanoids could be the result of the transfer of a biosynthetic intermediate from a donor cell, possessing a primary oxidative enzyme such as cyclooxygenase or lipoxygenase, to an acceptor cell, that, whilst lacking the ability to produce that intermediate, was nevertheless capable of converting it into biologically active metabolites in the presence of secondary enzymes (254). In February 1984 Charles Serhan, Mats Hamberg and Bengt Samuelsson, were working on the concept of ñtrans-cellular biosynthesisò at the Karolinska Institute. They examined the metabolism of radiolabelled arachidonic acid in cellular co-incubations (255) and discovered that when 15-HPETE (an arachidonic acid derivative) was added to human leucocytes, a previously undescribed tetraene was formed (256). They elucidated the structure and later in the same year proposed the trivial name Lipoxin A (since they arose from the interactions of multiple lipoxygenase pathways) for the new compound (257). It took more than a decade for the biological action of Lipoxins to be described; as stop signals for neutrophil recruitment (258), and their role in stimulating macrophage phagocytosis of apoptotic neutrophils (259).

Dr Serhanôs lab went on over the subsequent 30 years to discover several families of previously undescribed bioactive oxygenated derivatives of Arachidonic acid (AA), Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA), formed in self-resolving exudates by transcellular biosynthesis (253, 260). In 2000, a link to the omega 3 fatty acids was established with the discovery of Resolvin E1, a bioactive oxygenated derivative of EPA (261). In 2002, three families of DHA derived bioactive mediators were discovered; the D-Series Resolvins, Protectins

and Aspirin triggered isoforms (262) . The latest family to be described in 2009 were the Maresins (263). Together, these endogenously produced, bioactive lipid mediators, biosynthesised in self-resolving inflammatory exudates, in the active resolution phase of inflammation comprise the specialised pro-resolution mediator (SPM) family (Figure 1-10).

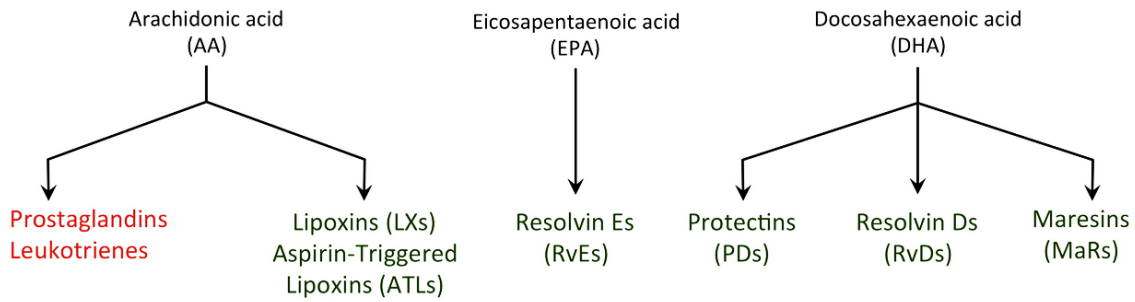

Figure 1-10 Specialised Pro-Resolution Mediators (SPMõs) from AA, DHA and EPA

SPMõs are endogenously produced self-resolving inflammatory exudates in the active resolution phase of inflammation (264). Lipoxins and their aspirin triggered isoforms are derived from arachidonic acid. E-Series Resolvins are biosynthesised from Eicosapentaenoic acid (EPA). Protectins, D-Series Resolvins and Maresins are formed from Docosahexaenoic acid (DHA) (reproduced from (265)).

1.8.2 Omega-3 & 6 Fatty acids ï Parent Compounds of the SPMõs

Whilst AA, DHA and EPA are all examples of poly-unsaturated fatty acids (PUFA), DHA and EPA are omega-3 fatty acids, whereas, AA is an omega-6 fatty acid. Omega-3 fatty acids are formed from Ŭ-Linolenic acid (LNA), and omega-6 fatty acids from Linoleic acid (LA), both essential fatty acids that must be obtained from the diet. The principal dietary sources of omega-6 fatty acids are poultry, nuts and vegetable oils. Since cold water oceanic fish concentrate omega-3 fatty acids synthesised by microalgae, they are rich sources of omega-3 fatty acids.

Omega-3&6 fatty acids must be first ingested, digested and absorbed to biosynthesise PUFA by enzymatic elongation and desaturation. PUFA are

incorporated into membrane phospholipids and liberated by Phospholipase A2 to make them available for transformation into active metabolites. In pro-resolving inflammatory exudates, DHA, interestingly, is reported to be delivered to the inflammatory site from the circulation in oedema fluid (266).

1.8.3 SPMõs and the Active Resolution of Inflammation

The acute inflammatory response is a protective mechanism that has evolved to eliminate invading organisms and be self-limited. An active resolution phase follows, that is tightly regulated and serves to restore tissue homeostasis. This active resolution phase is implemented by non-immunosuppressive pro-resolving lipid mediators (illustrated in Figure 1-11) (267).

Specialised Pro-Resolution Mediators (SPMõs) are lipid mediators which are endogenously biosynthesised in self-resolving inflammatory exudates in the active resolution phase of inflammation (264). They are potent anti-inflammatory mediators that function to terminate the local acute inflammatory response and promote the return of inflamed tissue to homeostasis (257). They mediate their effects on recruited immune effector cells and resident cells (268). The key bio-actions which define the SPMõs are;

- To stop neutrophil influx into exudates; stop transmigration and chemotaxis, block prostaglandins and leukotrienes and limit pro-inflammatory cytokine release (253)
- Non-phylogistic monocyte recruitment, uptake and removal of apoptotic neutrophils (efferocytosis) (253)

If inflammation is delayed or prolonged, insufficient or excessive in amplitude, then tissue damage results (268). CF is a disease characterised by prolonged, ineffective acute inflammation with on-going neutrophil recruitment, attendant tissue damage and failure of resolution. In this thesis we exploit advances in our understanding of ñactive resolutionò and apply this knowledge to obtain a better understanding the failure of active resolution of inflammation in CF.

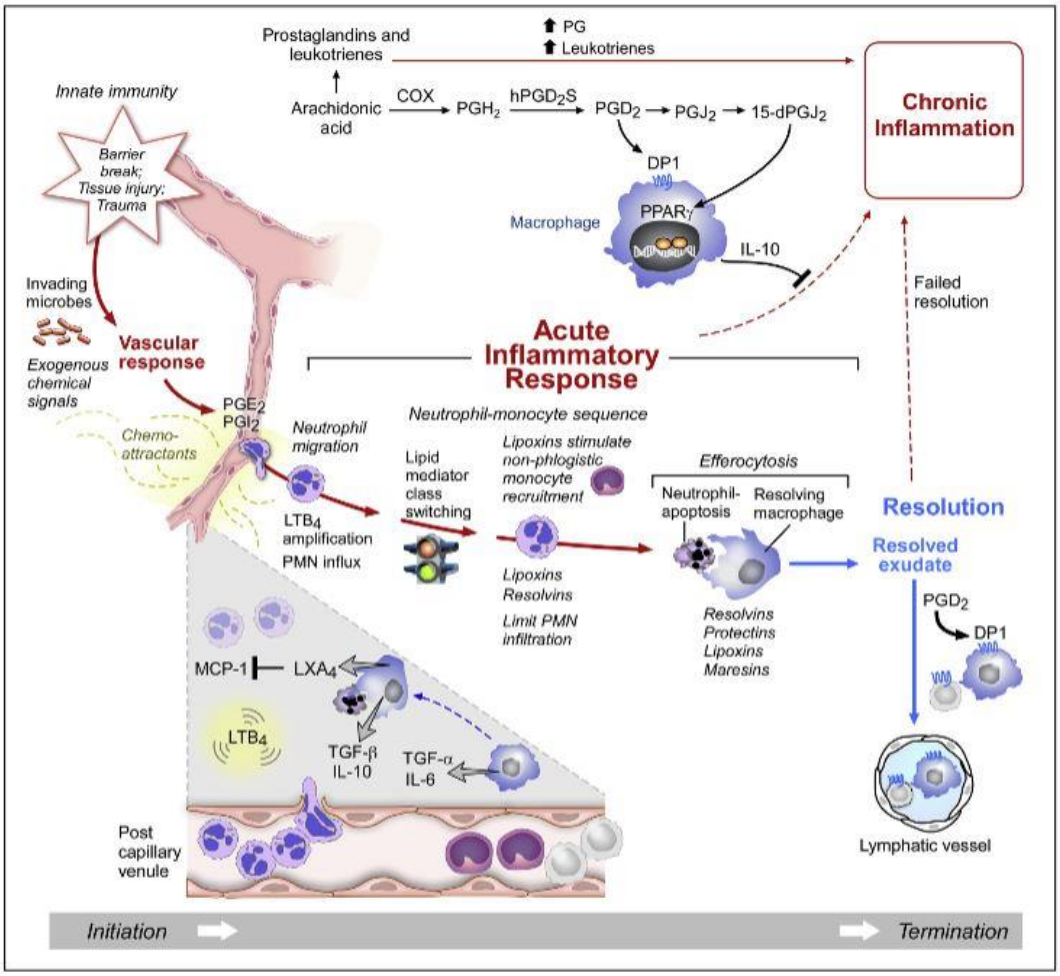

Figure 1-11 The Acute Inflammatory Response and the Role of Lipid Mediators in Resolution or Its Failure

Tissue injury or microbial invasion initiates an acute inflammatory response. Arachidonic acid derived Prostaglandins trigger changes in blood flow and Leukotrienes stimulate neutrophil recruitment. Neutrophils provide the first line of defence by migrating to sites of injury and neutralizing invading microorganisms or noxious materials by phagocytosis. Lipid mediator class switching occurs in inflammatory exudates to initiate resolution by activating Lipoxin production. LXA_4 stops LTB_4 stimulated neutrophil influx and regulates non-phylogistic monocyte recruitment. SPMs limit further neutrophil influx to the site and stimulate efferocytosis and the clearance of cellular debris by resolving macrophages. COX production of PGD2 stimulates anti-inflammatory IL-10 production. PGD2 derivative, 15-dPGJ2 activates PPARγ and activates resolution. These processes are non-redundant and therefore impaired function of any of the receptors or chemical mediators can in theory lead to failed resolution and persistent inflammation (Reproduced from Buckley et al (269)).

1.8.4 Eicosanoid Class Switching

As the fundamental properties of the SPMõs began to emerge a central conceptual question arose pertaining to the mechanisms at play in regulating the temporal maturation of inflammatory exudates from acute inflammation to active resolution. How does an exudate know when to õstopò the acute inflammatory response and õstartò active resolution?

In 2001, Levy et al described õeicosanoid mediator class switchingò in evolving inflammatory exudates (264). They demonstrated a temporal evolution of eicosanoid classes in self-limited inflammatory exudates, and correlated the eicosanoid profile with the recruitment of neutrophils into exudates, and ultimately to the decline in neutrophil numbers and transition towards monocyte / macrophage action within exudates. The sequence of eicosanoid classes appearing as an exudate matures was described as; initiation by prostaglandins; amplification mediated by leukotrienes; followed by active resolution mediated by lipoxins (264).

Furthermore, Levy et al. reported a molecular mechanism by which this õclass switchingò was achieved. Prostaglandin E2 was reported to induce 15 Lipoxygenase expression in neutrophils. Evidence was presented of PGE2õs role in effecting the switch, from 5 Lipoxygenase pathway derivative biosynthesis, to 15 Lipoxygenase pathway derivatives such as Lipoxins (264).

In this thesis, we present evidence that õeicosanoid mediator class switchingò is defective in CF.

1.9 SPMõs Nomenclature & Biosynthesis

1.9.1 15 Lipoxygenase Enzyme in Pro-Resolution Mediator Biosynthesis

The activity of the 15 Lipoxygenase (15-LO) enzyme plays a key role in the biosynthesis of; the Lipoxins (257); D- Series Resolvins (262); the Protectins (270); and the PPARõ agonists, 15-HETE, 9 & 13-HODE (271-274). SPM biosynthesis occurs by trans-cellular co-operation of two cell types, each cell contributing a separate enzymatic catalysis step. In the airway 15-LO expression can be found in; neutrophils (275), eosinophils (276), alveolar macrophages (277) and airway epithelial cells (278). Two isoforms exist, 15-LO type 1 and 15-LO type

2 (279). 15-LO is subject to transcriptional regulation by PGE2 (264), IL4, IL13, LPS and hypoxia (280, 281). Furthermore, 15-LO is also subject to translational and allosteric regulation (282, 283).

1.9.2 Lipoxins Nomenclature & Biosynthesis

LXA_4 is biosynthesised from Arachidonic Acid by the trans-cellular sequential action of 5 and either 12 or 15 Lipoxygenase enzymes (257). LXA_4 biosynthesis will be explored in detail in Chapter 4. LXA_4 is inactivated by eicosanoid oxidoreductases (284). LXA_4 interacts with a GPCR the Formyl Peptide Receptor 2 (FPR2) (285), and is also an intracellular ligand for the Aryl hydrocarbon receptor (AhR) (286, 287).

1.9.3 E-Series Resolvins Nomenclature and Biosynthesis

Resolvin E1 was the first of the Resolvin family to be discovered in 2000 (261). Resolvin E1 and E2 are synthesised from EPA by sequential enzymatic action. The action of either acetylated COX2 or cytochrome P450 enzymes generates the intermediate 18 HEPE. Subsequent action of either 5-Lipoxygenase (5-LO) or Leukotriene A4 Hydrolase gives rise to the generation of E series Resolvins 1 & 2 (Figure 1-12) (288). At least four pathways exist for the metabolic inactivation of RvE1 and these pathways are species, organ and cell type specific (289). Resolvin E1 signals via the CMKLR1 receptor in innate immune cells (290) and the LTB4 receptor 1 (BLT1) in neutrophils (291).

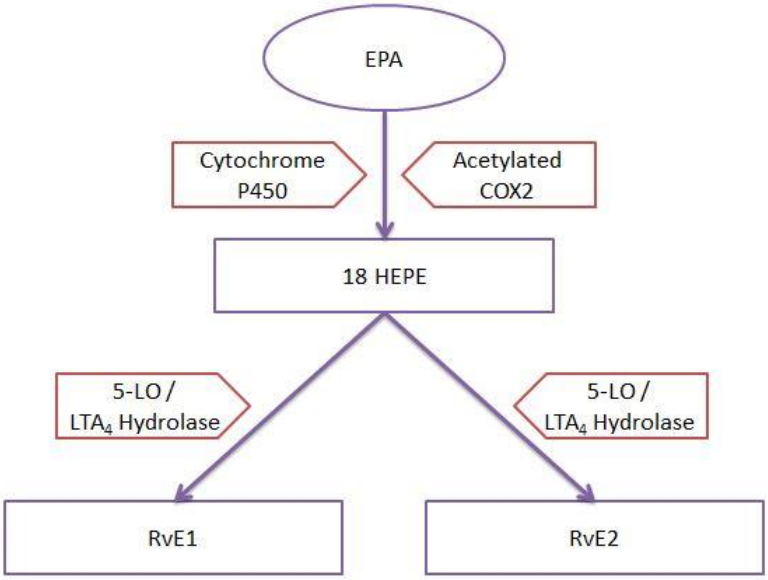

Figure 1-12 Biosynthesis of Resolvin E1 & Resolvin E2

Resolvin E1 and E2 are synthesised from EPA by sequential enzymatic action. 18 HEPE is produced by the action of either acetylated COX2 or cytochrome P450 enzymes. Subsequent action of either 5 Lipoxygenase (5-LO) or Leukotriene A4 (LTA4H) Hydrolase results in the generation of E series Resolvins 1 & 2.

Resolvin E3 (RvE3)

A third pair of E-series Resolvins (two stereoisomers) were described by Isobe and colleagues in Tokyo and collectively named Resolvin E3 (RvE3). Unlike Resolvins E1 and E2, biosynthesized via the 5-LO pathway, these metabolites are biosynthesized via the 12/15-lipoxygenase pathways. They were reported to limit neutrophil infiltration in zymosan-induced peritonitis (292).

1.9.4 D-Series Resolvins Nomenclature and Biosynthesis

There are six reported D-series Resolvins, Resolvin D1-6 (RvD1-6), first discovered in 2002 (262). Only the first two; Resolvin D1 (128 published articles indexed on Pubmed) and Resolvin D2 (18 published articles on Pubmed), are commercially available and therefore a larger body of publication has addressed their biology (1-4 published articles on Pubmed each for Resolvins 3-6).

The biosynthesis of the D-series Resolvins from DHA involves two sequential lipoxygenation steps. 15-Lipoxygenase (15-LO) catalyses the conversion of DHA

to 17R-HpDHA. A second lipoxygenation step by 15-LO at the C-7 position gives a peroxide intermediate which is either enzymatically hydrolysed to RvD1 and RvD2, or reduced to form RvD5. Alternatively, a second lipoxygenation step at the C-4 position by 5-LO gives rise to another peroxide intermediate which can either be enzymatically hydrolysed to RvD3 & RvD4 or reduced to RvD6 (Figure 1-13). Like the Lipoxins, D series Resolvins are rapidly inactivated by eicosanoid oxidoreductases (EORõs) (284, 293).

Using a ß arrestin ligand reporter system, RvD1 was revealed to signal through the GPR32 orphan receptor and ALX/FPR2 receptor (294, 295).

In this thesis we explore beneficial physiological effects of RvD1 with direct relevance for CF airways pathophysiology including ASL height regulation, IL8 production and CF alveolar macrophage function. Furthermore, we relate the production of RvD2 in sputum during pulmonary exacerbation to FEV_1 recovery.

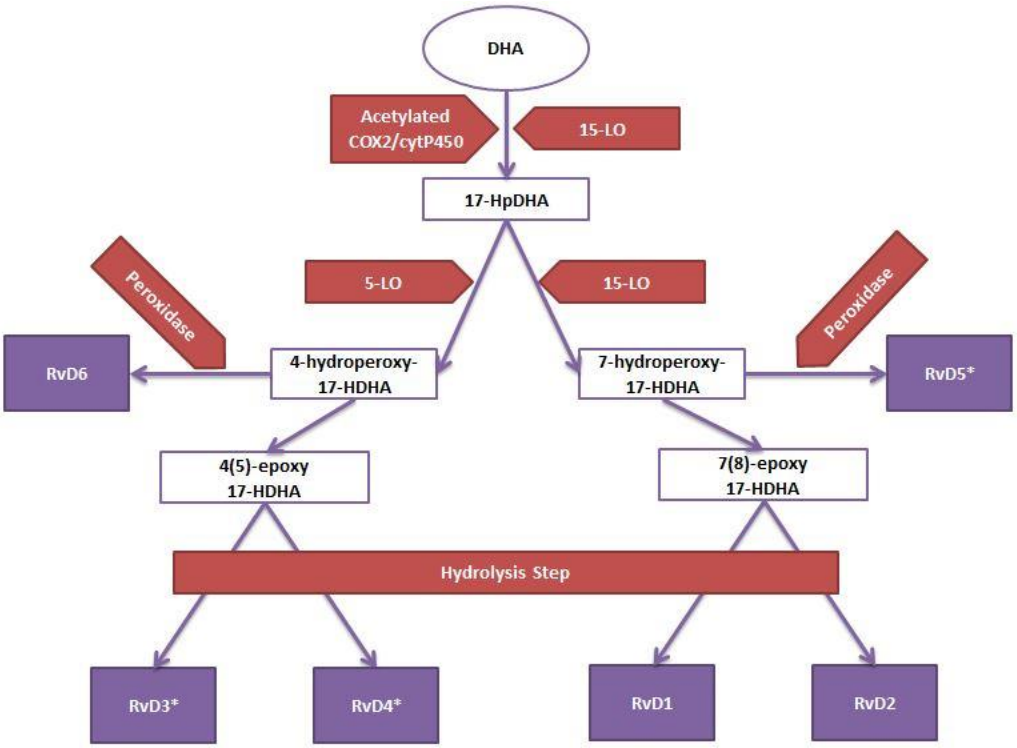

Figure 1-13 Biosynthesis of the D Series Resolvins

15-LO catalyses DHA conversion to 17-HpDHA. A second lipoxygenation step by 15-LO (right of panel) at the C-7 position gives a peroxide intermediate which is either enzymatically hydrolysed to RvD1 and RvD2, or reduced to form RvD5. A second lipoxygenation step at the C-4 position by 5-LO gives rise to another peroxide intermediate which can either be enzymatically hydrolysed to RvD3 & RvD4 or reduced to RvD6. (*Indicates compounds which are not commercially available).

1.9.5 Protectins Nomenclature and Biosynthesis

Protectins are derived from DHA and trivially named for their protective bioactivities for immune structures (they are named Neuroprotectins when generated in neural tissues). Protectin D1 is the main bioactive molecule of this group although several stereoisomers can be detected in inflammatory exudates with less potency, including Protectin Dx (PDx) (296). The first step in Protectin biosynthesis is the same as that for D-series Resolvin biosynthesis, except for the chirality at the carbon 17 position; 15-LO catalyses the conversion of DHA to 17S-HpDHA (Figure 1-14). An epoxide intermediate is formed, followed by a second enzymatic hydrolase step, giving rise to Protectin D1 (PD1). PDx is an isomer with S chirality at the carbon 10 position. PDx is generated in exudates from 17-HpDHA via a second lipoxygenation step by 5-LO (296). TLR7 stimulation was reported to enhance the biosynthesis of D-series SPMs including PD1 (297).

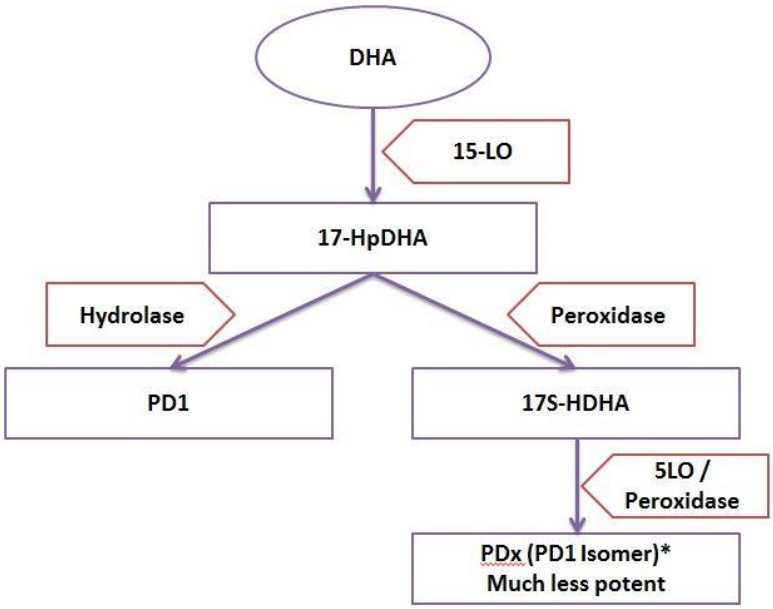

Figure 1-14 Biosynthesis of the Protectins

15-LO catalyses the conversion of DHA to 17-HpDHA. An epoxide intermediate is formed followed by a second enzymatic hydrolase step which gives rise to Protectin D1 (PD1) biosynthesis. An isomer with S chirality at the carbon 10 position (PDx) is also generated in exudates and is biosynthesised from 17-HpDHA via a second lipoxygenation step by 5-LO (296).

1.9.6 Maresins Nomenclature and Biosynthesis

There are currently two described members of the Maresin family (trivially named; Macrophage mediator in resolving inflammation); Maresin 1 and Maresin 2. The principal source of Maresins is macrophages (263). Maresins are produced from DHA by initial lipoxygenation with molecular oxygen insertion at the carbon-14 position to form a hydroperoxide intermediate. The hydroperoxide intermediate is rapidly converted to a bioactive intermediate, 13, 14-epoxy-maresin (13, 14-eMaR) (298) which is then enzymatically converted to Maresins 1 and 2 (Figure 1-15) (263, 299). The second Maresin, MaR2, was described this year.

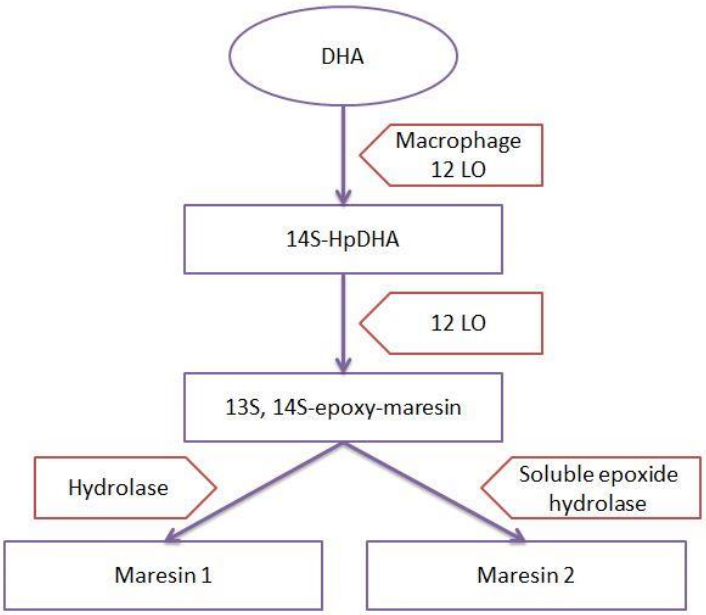

Figure 1-15 Biosynthesis of the Maresins

Maresins are produced by initial lipoxygenation with molecular oxygen insertion at the carbon-14 position to form the hydroperoxide intermediate, which is rapidly converted to 13S, 14S-epoxide-maresin and enzymatically converted to Maresins 1 and 2 (263, 299).

Maresin 1 has been described to behave as an autacoid (300, 301). Macrophage biosynthesis of Maresin 1 involves a bioactive intermediate, 13, 14-epoxy-maresin (13, 14-eMaR), which stimulates a macrophage phenotype switch from classical to alternative activation resulting in enhanced Maresin 1 biosynthesis and the inhibition of LTB_4 biosynthesis (298).

1.10 SPM Functions ï Resolution without Immune Suppression

The central effector functions which define the SPMôs are; to stop neutrophil influx into exudates (253), and to enhance non-phylogistic monocyte recruitment, and macrophage efferocytosis (253) (illustrated in Figure 1-16).

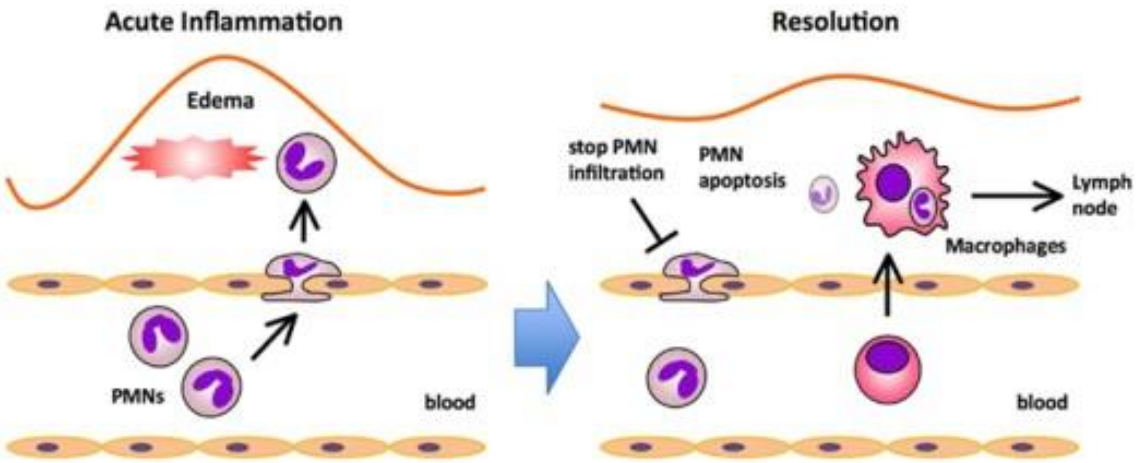

Figure 1-16 Key steps in the resolution of inflammation

The initiation and propagation phases of acute inflammation are attended by rapid infiltration of neutrophils. In the resolution phase, neutrophils undergo apoptosis and are ingested by macrophages that emigrate rapidly from the inflamed site to achieve ñTissue Resolutionò(the disappearance of tissue leukocytes and cessation of further neutrophil recruitment (268)) (reproduced from (302)).

1.10.1 SPMôs in the Regulation of Neutrophil Migration

Despite being first described in 1984, it took a further 12 years for it first to be reported that Lipoxins function as a stop signal for neutrophil recruitment (258). Subsequently, the majority of the SPMôs described have been reported to decrease neutrophil recruitment, and / or, transmigration of neutrophils into tissues, in a range of models (mouse models of peritoneal sepsis and the fMLP stimulated trans-endothelial migration model); LXA_4 (303, 304); Resolvin D1 (293); Resolvin D2 (288), Protectin D1 (296), Maresin 1 (296), Maresin 2 (299), Resolvin E1 (305), Resolvin E2 (305).

Neutrophil migration into the lung provides the first line of defence by neutralizing invading microorganisms or noxious materials by phagocytosis. Neutrophil recruitment involves migration across the endothelium, extracellular matrix and the alveolar epithelium following a chemotactic gradient (illustrated in Figure 1-17) (221). Trans-endothelial migration involves three stages; rolling adhesion, mediated by Selectins; strong adhesion, via the interaction of CD11b and ICAM-1; and migration. The neutrophil must then further migrate through the extracellular matrix occurs along fibroblasts and across the alveolar epithelial cell barrier (221).

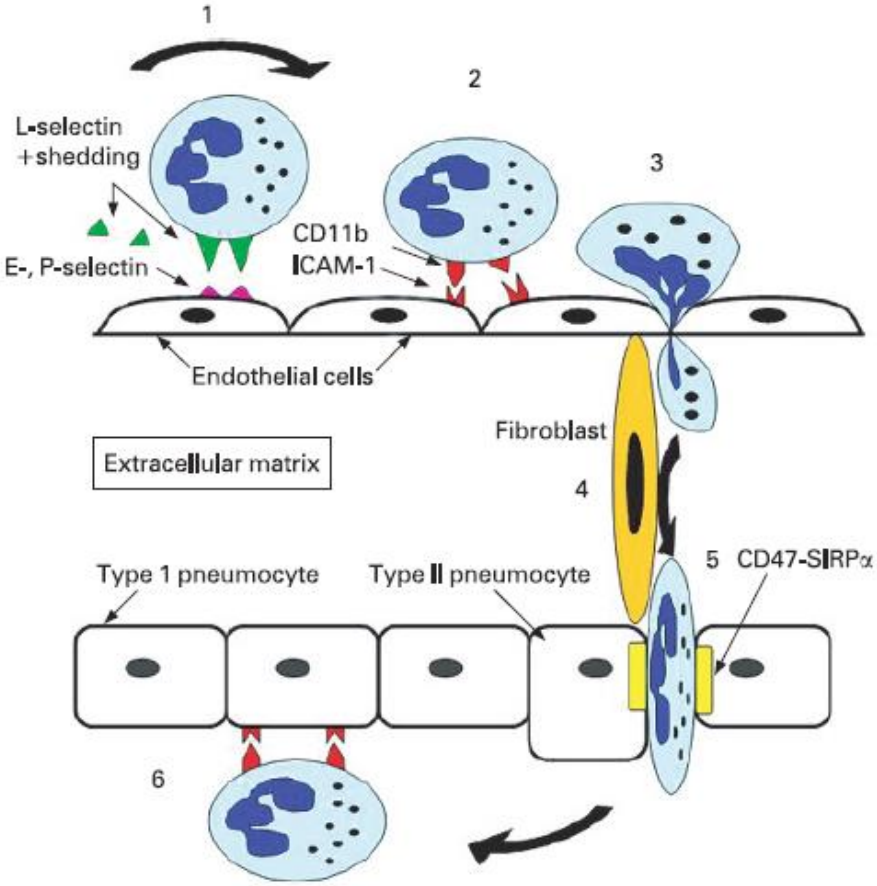

Figure 1-17 Neutrophil Migration into the lung

Neutrophil migration into the lung involves migration across the endothelium, extracellular matrix and the alveolar epithelium following a chemotactic gradient. Transendothelial migration involves three stages; (1) rolling adhesion, mediated by selectins, including E- and P-selectins on the endothelium and L-selectin on the neutrophil; (2) strong adhesion, via the interaction of CD11b and ICAM-1, and; (3) migration. (4) Migration through the extracellular matrix occurs along fibroblasts and is followed by (5) migration across the alveolar epithelial cell barrier (reproduced from Downey et al (221)).

LXA_4ôs role in regulating neutrophil recruitment has received the most attention since it was the earliest mediator to be described. LXA_4 stops neutrophil recruitment by blocking neutrophil chemotaxis, adhesion, and transmigration (304). LXA_4 inhibits chemo-attractant LTB_4 induced neutrophil-endothelial cell adhesion and potently inhibits P-selectin expression (303, 304). LXA_4 reduces CD11b/CD18 expression on the neutrophil surface (306). Furthermore, the mechanism of RvD2 reduced neutrophil infiltration was reported to be via endothelial-dependent nitric oxide production and direct modulation of leukocyte adhesion receptor expression (288).

In this thesis we explore the relative concentrations of LXA_4 and LTB_4 in the airways of children with CF, directly relevant to the regulation of neutrophil migration.

1.10.2 SPMôs enhance macrophage clearance of apoptotic neutrophils

A critical function of macrophages within the inflammatory milieu is the removal of dying cells by a specialized phagocytic process called efferocytosis (307). Efferocytosis is a key regulatory check point for the innate immune system, and its failure may contribute to disease pathogenesis and impede the active resolution of inflammation (220). More than 10^{11} circulating neutrophils are eliminated and replaced every day and the main scavengers of apoptotic neutrophils are macrophages and dendritic cells (219, 220).

First recognised in 2000, a key property of the SPMôs is to enhance macrophage phagocytosis and clearance of apoptotic neutrophils, and to stimulate monocyte recruitment; LXA_4 (258, 259), RvD1 (295), RvD2 (308), Protectin D1 (301), MaR1 (263, 309), MaR2 (299), Resolvin E1&2 (305).

Efferocytosis physically removes apoptotic cells before cell permeability is increased, allowing the release of toxic intracellular contents. In particular, neutrophils store proteases, inflammatory mediators and oxidants. ñEat meò signals are presented by the apoptotic cell, including the exposure of phosphatidylserine (normally sequestered to the inner membrane leaflet) and calreticulin (310, 311). A sequence then follows of ñtetheringò the apoptotic cell, followed by ñticklingò to engage the ñeat meò signals driving the extension of

membrane ruffles and internalisation into a large fluid filled phagosome (220). This process is inhibited by amiloride and has been reported to involve CFTR (220).

Efferocytosis has noteworthy downstream consequences for SPM biosynthesis. It is reported to drive the polarisation of macrophages towards a pro-resolution phenotype (alternative activation), stimulating the production of 15-LO via a PPARɔ/TGFƀ pathway (312) (300). Furthermore, macrophage efferocytosis promotes anti-inflammatory IL-10 and TGF-ƀ cytokine production, thus further promoting the active resolution of inflammation (307). Consistent with this concept, ñMicro-particlesò generated from human neutrophils were shown to enhance neutrophil efferocytosis by macrophages and induce de novo SPM synthesis (313). Interestingly, efferocytosis increases anti-protease secretory leucoprotease inhibitor production by macrophages (314).

SPMôs enhance macrophage mediated phagocytosis and in this thesis we explore the effect of RvD1 on CF alveolar macrophage mediated phagocytosis (known to be defective). Furthermore, efferocytosis drives SPM biosynthesis and we examine SPM biosynthesis in the contexts of CF airway disease at baseline and during pulmonary exacerbation.

1.10.3 Anti-microbial properties of SPMôs

As early as 2004 it was recognised that SPMôs improve outcomes from an infectious challenge in animal experiments (315). Karp et al. reported that mice treated with analogues of LXA_4 and subsequently challenged with *P. aeruginosa* contained that bacterial challenge more effectively (315). In a mouse model of peritonitis Spite et al. reported that RvD2 administration reduced both local and systemic bacterial burden, excessive cytokine production (IL6, IL1ƀ, IL23, PGE2, LTB_4 and TNFŬ) and neutrophil recruitment, while increasing peritoneal mononuclear cells and macrophage phagocytosis (308). These effects translated into improved survival of the mice from sepsis (308). Chiang et al. subsequently reported that in mouse models of peritoneal E. coli infection, RvD1 and RvD5 reduced bacterial titres in blood and exudates, reduced hypothermia and increased survival (316). Co-treatment of these mice with the antibiotic ciprofloxacin and RvD1 accelerated resolution and shortened resolution intervals (316). Adjuvant treatment of ciprofloxacin with a panel of SPMôs (RvD1, RvD5,

and Protectin D1) heightened host antimicrobial responses in E-Coli infected mice (316). Furthermore, in skin infections, a panel of SPMs (RvD1, RvD5, and Protectin D1) enhanced vancomycin clearance of *Staphylococcus aureus* (316). In a mouse model of aspiration pneumonia, Resolvin E1 enhanced bacterial clearance and was attended by an improvement in survival (317).

In this thesis we explore the effect of RvD1 on intracellular killing of a central CF pathogen, *Pseudomonas aeruginosa* by CF alveolar macrophages.

1.11 SPM mechanisms of action; Intracellular signalling

1.11.1 Intracellular signalling pathways; LXA_4

In human airway epithelia LXA_4 has been reported to stimulate a rise in intracellular calcium via adenylate cyclase activity and PKA activation (318). A survey of global epithelial gene expression in human enterocytes uncovered a host of genes under NF-κB control whose transcription was reduced by LXA_4 (319). In neutrophils, LXA_4 stimulation leads to an intracellular accumulation of Presqualene diphosphate (PSDP), which serves to inhibit phospholipase D activation and potently inhibit superoxide anion generation (320). In dendritic cells, Socs2 up regulation (a member of the suppressors of cytokine signalling family of proteins) plays a central role in mediating the anti-inflammatory effects of LXA_4, in a manner dependent upon both AhR and ALX/FPR2 expression (287) (illustrated in Figure 1-18). (102)

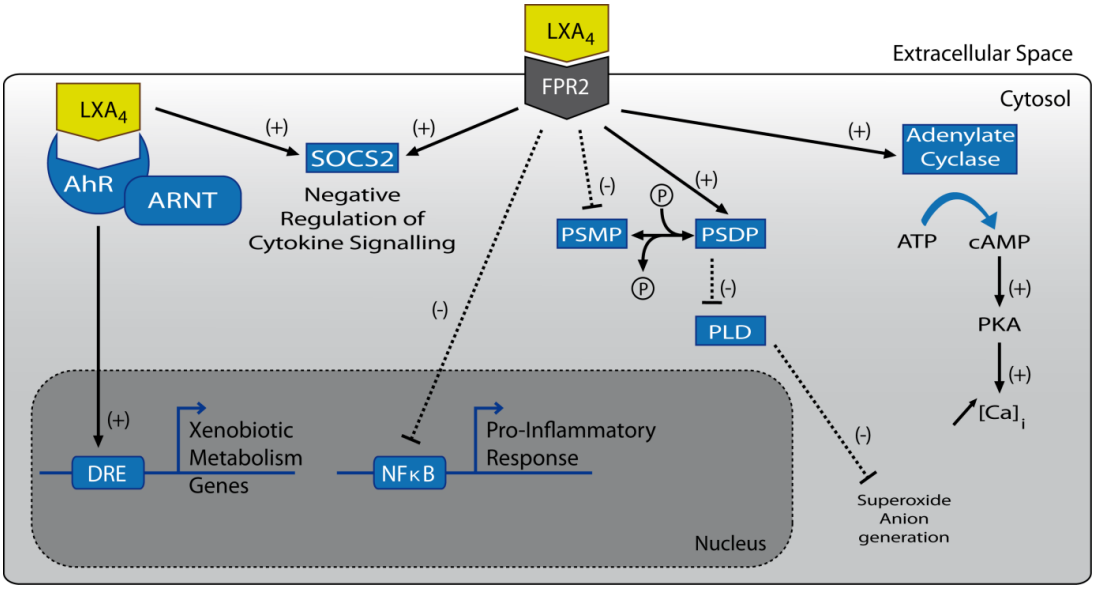

Figure 1-18 Lipoxin A_4 signalling

LXA_4 interacts with FPR2 and AhR receptors. AhR dimerizes with ARNT and regulates genes involved in xenobiotic metabolism (286, 287). LXA_4 stimulates [Ca]i increase via adenylate cyclase activity and PKA activation (318). LXA_4 reduces transcription of genes under NFκB control (319). LXA_4 stimulation leads to PSDP accumulation in favour of PSMP, inhibiting PLD activation and superoxide anion generation (320). LXA_4 stimulation results in AhR and ALX/FPR2 dependent Socs2 up-regulation and thus negative regulation of cytokine signalling (287) (Reproduced from (102)).

1.11.2 Intracellular signalling pathways; Resolvin D1

Hsiao et al recently examined the intracellular signalling pathways mediating the effects of RvD1. They reported that RvD1 strongly suppressed IL6 and IL8 production induced by a viral mimic compound (321). Pro-inflammatory signalling involving MAPKs and NFκB was supressed via RvD1 mediated inhibition of the phosphorylation of TGF-β-activated kinase 1 (TAK1), upstream of both the MAPK and NFκB pathways, by inhibiting the formation of a TAK1 / TAB1 (TAK1 binding protein) / TRAF6 (TNF receptor-associated factor 6) signalling complex (321). Furthermore, RvD1 regulates microRNA circuits, targeting cytokines and proteins involved in the immune system including; miR-146b, targeting NFκB signalling, and miR-219, which regulates 5-LO expression (255).

1.12 Pro-Resolution Receptors

1.12.1 ALX/FPR2 Receptor

FPR2 (also known as ALX) is a GPCR which conveys the pro-resolving properties of LXA_4, 15-epi-LXA_4, RvD1, aspirin-triggered Resolvin D1, and Annexin A1 (268). Unusually, the pro-inflammatory responses elicited by the antimicrobial peptide LL-37 and serum amyloid protein A (SAA) are also mediated by ALX/FPR2. ALX/FPR2 is expressed in several types of cells including; neutrophils (306), alveolar macrophages (259, 322), human bronchial epithelial cells and human bronchial biopsy sections (323). In bronchial epithelial cells the ALX/FPR2 receptor is located at the apex of the cell (323).

Structurally, ALX/FPR2 has both lipid binding and peptide biding domains (324). ALX/FPR2 differentially signals either a pro-resolution cascade, or a pro-inflammatory cascade depending upon ligand-biased receptor activation and either, hetero-dimerization of ALX/FPR2, with related FPRs (pro-inflammatory cascade), or, homo-dimerization of ALX/FPR2, to engage pro-resolving signal cascades (325).

Functionally, ALX/FPR2 deficient mice respond to an inflammatory challenge with increased acute inflammation and delayed resolution (326). It is noteworthy that LXA_4 enhances the activity of the ALX/FPR2 promoter (327) and miR-181b binds to the 3ôUTR of the ALX/FPR2 gene and regulates its expression (328).

In this thesis we examine the role of the ALX/FPR2 receptor in RvD1 mediated effects on ASL hydration.

1.12.2 Aryl hydrocarbon receptor (AhR)

LXA_4 is an intracellular ligand for the Aryl hydrocarbon receptor (AhR) (286, 287). AhR is a transcription factor which heterodimerizes with the AhR nuclear transporter protein (ARNT) upon ligand binding and interacts with dioxin response elements to regulate genes involved in xenobiotic metabolism (286, 287).

1.12.3 GPR32 Receptor

RvD1 binds to GPR32 (also known as the RvD1 receptor DRV1) (295). RvD1ôs receptor activation is reported to be concentration dependent, i.e. low

concentration RvD1 employed GPR32 to block neutrophil-endothelial cell interaction, whereas high concentration effects were mediated via ALX/FPR2 (295). GPR32 expression is reported in peripheral blood leukocytes, vascular tissue and small airway epithelial cells (295, 321). In this thesis we were not able to evaluate the role of GPR32 in RvD1 mediated physiological effects in the CF airway since pharmacological tools have not yet been developed to interrogate the contribution of this receptor.

1.12.4 Peroxisome proliferator-activated receptor gamma (PPARɔ)

PPARɔ is a ligand-activated transcription factor that is up-regulated during the resolution of inflammation and mediates the anti-inflammatory effects of a number of essential fatty acid derived lipid mediators (329, 330). Potent PPARɔ ligands include Prostaglandin type pro-resolving lipids (331, 332), 12 and 15 Lipoxygenase pathway products (274, 331, 332), SPM parent compounds EPA and DHA (332), and non-enzymatic oxidation products of linoleic acid (332).

In this thesis we describe a relationship between PPARɔ agonist 15-d-PGJ_2 and recovery of FEV_1 during pulmonary exacerbation of CF. We also develop methods to quantify PPARɔ expression in CF alveolar macrophages.

1.12.5 SPMõs awaiting Receptor assignments

RvD2 stimulated vasoprotective prostacyclin in a pertussis toxin sensitive manner, implicating a role for GPCRõs (288). Protectin D1 is reported to specifically bind to human neutrophils, but its cognate receptor has not yet been established (268). Resolvin E2 interacts with an unidentified leukocyte GPCR (333).

1.13 SPMõs in Lung Infection and Inflammation Models

The biological roles of SPMõs are thought to be non-redundant, tissue and cell type specific (253). Nevertheless, the specific functions of individual mediators within the family in different tissues, contexts and disease states remain areas of active research. Therefore we consider the reported actions of SPMõs specifically in a pulmonary infectious or inflammatory context.

Pulmonary benefits have been reported for LXA_4 (315, 318, 334), RvD1 (335-337), Protectin D1 (338, 339) and Resolvin E1 (305, 317). A pulmonary role for LXA_4 was reported in mice challenged with *P. aeruginosa*. Mice treated with analogues of LXA_4 contained the bacterial challenge more effectively (315). In a mouse model of acute lung injury, LPS induced lung injury and cigarette exposure RvD1 administration was reported to have beneficial effects, including; blocking leukocyte infiltration and reduced cytokine levels (335), promoting alternative macrophage activation, and stimulating neutrophil efferocytosis (336), and attenuation of NFHB activation (337). In mouse and human asthma studies, Protectin D1 was reported to protect from lung damage, airway inflammation and hyper-responsiveness (338), and promoted apoptosis of neutrophils in BALF (339). Protectin D1 was generated by human asthmatic patients and detected in exhaled breath condensate (338). In mouse models, Resolvin E1 administration enhanced the resolution of established neutrophil-mediated pulmonary injury (305). In a mouse model of aspiration pneumonia, Resolvin E1 decreased lung neutrophil accumulation, enhanced bacterial clearance, decreased lung tissue levels of several pro-inflammatory chemokines and cytokines and was attended by an improvement in survival (317).

Pulmonary benefits of SPMõs reported in animal models stimulated us to examine the human disease specific roles of these compounds. These reports informed the design of the ñPro-Resolution mediators in CF exacerbationò(PRINCE) study reported herein.

1.14 SPMõs and Active Resolution in CF

The acute inflammatory response is a protective mechanism that has evolved to eliminate invading organisms and be self-limited (267). SPMõs serve to terminate the local acute inflammatory response and promote the return of inflamed tissue to

homeostasis (257). These processes are non-redundant and therefore impaired function of any of the receptors or chemical mediators involved in enacting the resolution process can in theory lead to failed resolution and persistent inflammation (269). If inflammation is delayed or prolonged, insufficient or excessive in amplitude, then tissue damage results (268). CF is a disease characterised by prolonged, ineffective acute inflammation with ongoing neutrophil recruitment, attendant tissue damage and failure of resolution.

In contrast to the key functions of SPMõs; to stop neutrophil influx into exudates, limit pro-inflammatory cytokine release and enhance macrophage phagocytosis of apoptotic neutrophils (253), in CF, pro-inflammatory cytokines are abundant, neutrophils are present in excess and macrophage phagocytosis is impaired (340, 341).

A number of aspects of SPM biology in CF have been studied to date. A significant body of work has looked at the metabolism of parent compounds AA and DHA. LXA_4 biosynthesis has been explored in CF and will be specifically addressed further in Chapter 4. The physiological effects of LXA_4 have been examined in CF models and SPMõs have been detected in studies of sputum from adults with CF. Lastly, the role of the pro-resolution PPARɔ receptor has been examined in CF models.

1.14.1 Essential Fatty Acid Imbalance in CF

The parent compounds of the SPMõs are essential fatty acids and in order to contextualize studies reported herein of SPM biosynthesis in CF we must first review the literature on the SPM parent compounds in CF. As early as the seventies, fatty acid abnormalities were described in CF (342). Decreased tissue LA concentration is accompanied by increased AA concentration. This has been attributed to increased metabolism of LA to AA via increased expression of ɑ5- and ɑ6-desaturase enzymes in CF cells. Furthermore, pathological regulation of Arachidonic acid release by Phospholipase A2 (PLA2) has been reported in CF (343). Basal cytosolic PLA2 activity was reported to be increased whereas restoration of CFTR delivery to the membrane restored normal activity (344). DHA concentration has also been reported to be reduced in CF tissues. Several lines of evidence suggest that DHA deficiency in CF is of clinico-pathological

significance. AA / DHA imbalance in CF is thought to play a role in the excessive production of AA derived mediators such as prostaglandins and leukotrienes, since DHA is reported to suppress α5- and α6-desaturase enzyme expression. Furthermore, experimental addition of DHA was found to decrease desaturase enzyme expression and normalize LA to AA metabolism (345-347) in culture systems, and high dose oral supplementation of DHA was reported to reverse PUFA abnormalities and ameliorate CF related pathology including pulmonary inflammation in CFTR null mice (348).

There are similarities between animal descriptions of essential fatty acid deficiency and CF related pathology; liver steatosis, increased caloric needs, increased bacterial colonisation and decreased immune responses (349). Strandvik and colleagues found an association between serum concentrations of LA and DHA and CFTR mutation severity (350).

Prompted by these findings, several small studies have evaluated the effect of DHA supplementation in CF and larger scale studies are in progress. There are reports of improvement in fatty acid levels, decreases in inflammatory markers, improvement in pulmonary function tests and decreased exacerbations (345).

1.14.2 LXA_4 biosynthesis in CF

Platelets from CF patients were reported to generate 40% less LXA_4 than controls, an observation that was replicated by CFTR inhibition (351). In this thesis we will examine the LXA_4 synthetic capacity of airway epithelial cells and isolated alveolar macrophages in CF.

1.14.3 SPMs measured in airways samples from people with CF

LXA_4 concentration in airways samples from patients with CF has been variously reported as absent, significantly suppressed, or not significantly different from disease controls (315, 352, 353). A significant increase in LXA_4 concentration was measured in patients following antibiotic therapy (354). The presence of Resolvin E1 in respiratory tract secretions from adult patients with CF was associated with better lung function (353). In this thesis we will examine LXA_4 synthesis in the airways of children with CF.

1.14.4 Beneficial physiological effects of LXA_4 in CF

In CF models, LXA_4 has been shown to; restore airway surface liquid height by enhanced Calcium activated Chloride secretion (318, 355) and ENaC inhibition (356); stimulate tight junction formation (357); decrease neutrophilic inflammation, pulmonary bacterial burden, and disease severity (315); and enhance repair (358) (illustrated in Figure 1-19). Uniquely, LXA_4 is reported both to restore airway surface liquid hydration, and counter-regulate the pro-inflammatory program of the CF airway environment. Mechanistically, in CF airway epithelial cells, LXA_4 interacts with the ALX/FPR2 receptor to stimulate an intracellular calcium increase (318) that activates a compensatory calcium-activated chloride secretory mechanism, and overcomes the lack of CFTR mediated chloride transport to restores ASL height in vitro (355). The increased intracellular calcium concentration generated in CF airway epithelial cells by LXA_4 occurs via ALX/FPR2 stimulation, and results in apical ATP release via Pannexin channels, resulting in P2Y11 purinoreceptor stimulation (359).

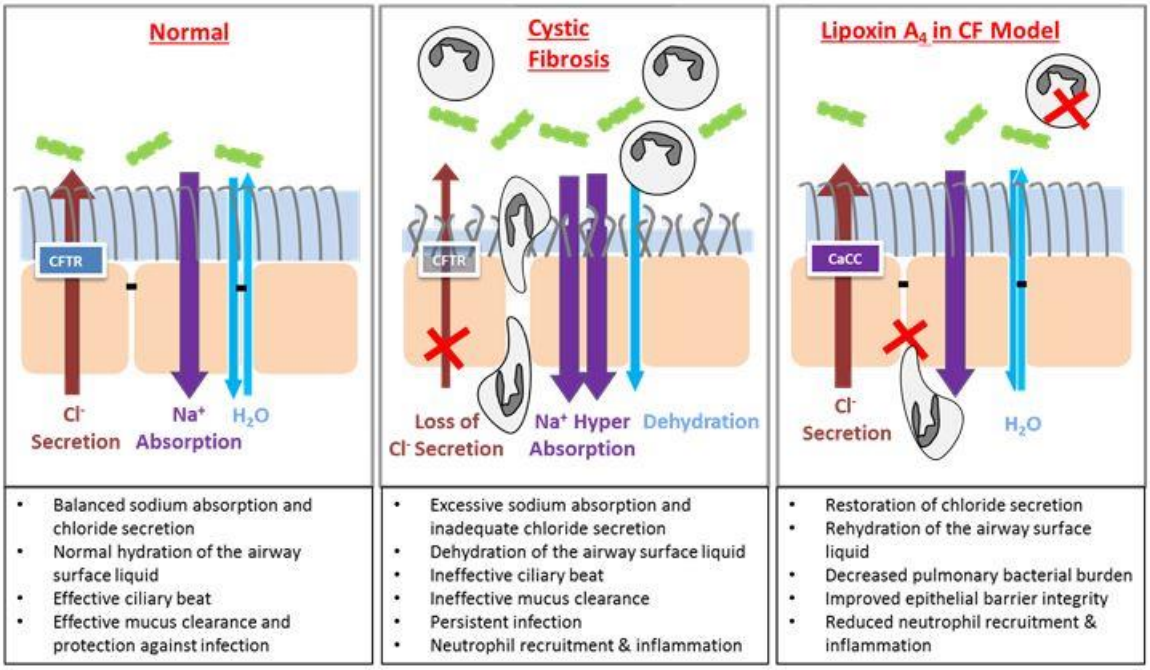

Figure 1-19 An illustration of normal airway physiology, the pathophysiology of CF, and the physiological properties of LXA_4 in CF models

LXA_4 augments airway epithelial innate defence by stimulating tight junction formation (357); enhancing Calcium activated Chloride secretion (318), ENaC inhibition (356) and restoring airway surface liquid height in CF airway epithelial cells (355). LXA_4 has pro-resolution effector functions and reduces pulmonary bacterial burden (315), limits neutrophil recruitment (360) and reduces IL8 secretion by airway epithelial cells (323).

1.14.5 PPARɔ plays a role in the resolution of inflammation and is disrupted in CF

PPARɔ is up-regulated in the active resolution phase and mediates the anti-inflammatory effects of essential fatty acid derived lipid mediators (329, 330). Disrupted PPARɔ function has been reported to play a role in CF lung disease (331). In CFTR knockout mice the expression of PPARɔ was reported to be down-regulated at the RNA and protein level, and associated with reduced nuclear localisation (361). PPARɔ agonist pioglitazone was reported to normalize pulmonary infiltration of neutrophils and pro-inflammatory cytokine levels in the lungs of CFTR-deficient mice challenged with Pseudomonas (362). Recent studies demonstrated a role for PPARɔ in the expression of carbonic anhydrases and bicarbonate transport in the lung (363).

In this thesis we describe a relationship between the production of PPARɔ agonist 15-d-PGJ_2 in sputum and the extent of recovery of FEV_1 following pulmonary exacerbation of CF. We also develop methods to quantify PPARɔ expression in CF alveolar macrophages.

1.15 SPMôs in Clinical Drug Development Programmes

The effect of LXA_4 inhalation has been evaluated in a pilot study of eight asthmatic and healthy adult subjects. The challenge was tolerated, had no adverse effect on pulse or blood pressure and demonstrated favourable effects on specific airway conductance (364). More recently, a clinical trial of topical 15(R/S)-methyl-LXA_4 for infantile eczema found that the LXA_4 analogue relieved eczema severity (as effectively as a topical steroid) and improved quality of life without apparent adverse events (364, 365).

The Pharmaceutical industry have reported that Resolvin E1 and synthetic analogues have been formulated for oral, IV and subcutaneous routes of administration and that Phase I clinical studies have demonstrated potency across a range of inflammatory disease models including asthma, colitis, rheumatoid arthritis, atherosclerosis, dry eye and retinopathy (103). Phase I ï II clinical trials demonstrated efficacy of a Resolvin E1 analogue for the treatment of dry eye and phase III studies are reported to be underway (366, 367).

Furthermore, Maresin 1 was reported to be in a clinical drug development programme (253) and Protectin D1 is in clinical development for the treatment of neurodegenerative disease (253).

1.16 Aims and Outcomes of this study

Given the roles played by ASL dehydration, infection and inflammation in the development of bronchiectasis in CF novel strategies to rehydrate the ASL and improve muco-ciliary clearance, compliment conventional antibiotics and combat excessive inflammation are sought. Our group had previously demonstrated beneficial physiological effects of LXA_4 in CF bronchial epithelial cell model systems, including the restoration of ASL height via stimulation of the specific ALX/FPR2 receptor (85, 356, 359). RvD1 was reported to be an agonist at the ALX/FPR2 receptor (295) and to attenuate NFκB activation in mouse models of lung injury (337). Furthermore, SPM enhance phagocyte function (253) and bacterial clearance (316). Therefore, we aimed to investigate the potential of RvD1 as a candidate therapeutic in CF.

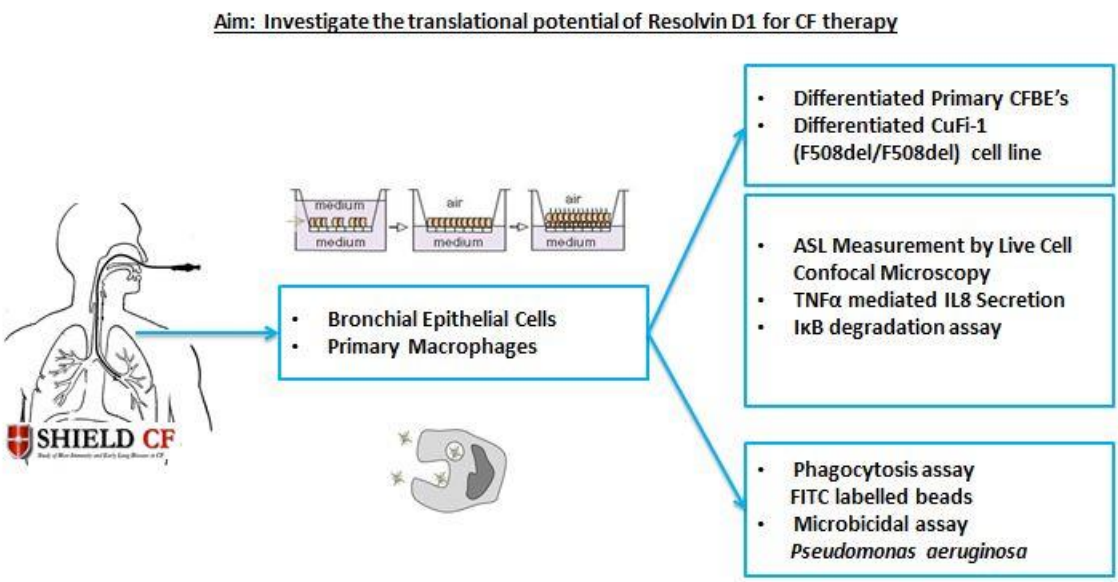

Figure 1-20 Illustration of the aim to investigate the translational potential of RvD1 for CF therapy

Using live cell confocal microscopy we demonstrate that RvD1 increases ASL height in primary CF bronchial epithelia and the CuFi-1 cell line. By Western blotting and ELISA, we show that RvD1 preserves the integrity of IκB, and attenuates IL8 secretion stimulated by TNFŬ in a CF bronchial epithelial cell line. In CF, impaired alveolar macrophage phagocytosis and bacterial killing are reported (125-127, 228). Using primary CF alveolar macrophages we

demonstrate that RvD1 enhances macrophage phagocytic capacity and improves intracellular killing of *Pseudomonas aeruginosa*.

The underlying basis for the failure to actively resolve inflammation in the CF airway remains incompletely understood. We noticed significant overlap between the pathophysiological characteristics of inflammation in the CF lung, and the reported functions of SPMõs in the active resolution of inflammation. In particular, SPMõs limit neutrophil recruitment and enhance tissue neutrophil clearance (253). In CF, neutrophil recruitment is excessive, the airway burden is high and inflammation is prolonged and ineffective leading to failed bacterial containment and tissue damage (221). LXA_4 is the first of a series of SPMõs to be synthesised in the resolution phase of inflammation, and is generated by a ñclass switchò in eicosanoid lipid mediator synthesis via up-regulation of 15-LO (264). Some controversy surrounded prior reports of reduced LXA_4 concentration in CF airway samples (315, 352). It was our aim to understand the role of eicosanoid mediator class switching in the failure to actively resolve inflammation in CF lung disease.

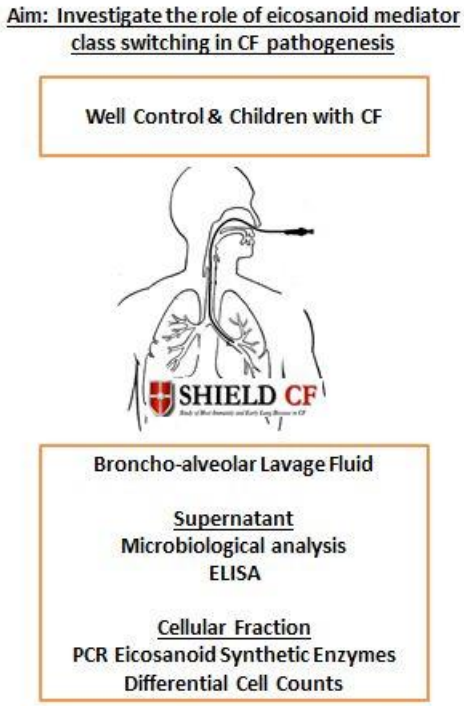

Figure 1-21 Illustration of the aim to investigate the role of eicosanoid mediator class switching in CF pathogenesis

Using BAL samples from children with CF and controls we measured eicosanoids expressed before (LTB_4) and after the ñclass switchò towards active resolution (LXA_4). Furthermore, we quantified eicosanoid synthetic enzymes in the cellular phase of BAL by qPCR. We demonstrate impoverished 15-LO type 2 gene expression, associated with a depressed LXA_4 / LTB_4 ratio in the lower airways of children with CF.

In order to further investigate how defective 15-LO expression in alveolar macrophages could result in the failure to synthesise LXA_4 we develop a method to isolate a purified alveolar macrophage population from the BAL of children with CF and controls. Furthermore using this method we present pilot data on 15-LO expression and macrophage polarisation in this enriched population. Using qPCR, Western blotting and ELISA, we go on to investigate the regulation of 15-LO expression and activity in a CF bronchial epithelial cell line and find that it is broadly similar to that found in a non-CF bronchial epithelial cell line (NuLi-1).

With an accumulating body of experimental evidence to suggest that SPMôs could present therapeutic opportunities in CF lung disease, we aimed to investigate

whether pro-resolution lipid mediator expression could be associated with clinically meaningful indicators of lung health in children with CF. In interventional studies on animal models of infection, SPMõs; accelerated resolution, and lowered antibiotic treatment requirements (316). Acute exacerbations of infection in the CF airway vary in terms of their outcomes, including, variable duration of exacerbation and variable extent of recovery of lung function parameters including FEV_1 (368, 369). Using the lipidomic approach and spirometry, we evaluated the relationships between Pro-Resolution lipid mediator expression in sputum during pulmonary exacerbation, and the extent of FEV_1 recovery following antibiotic treatment for CF Pulmonary Exacerbation.

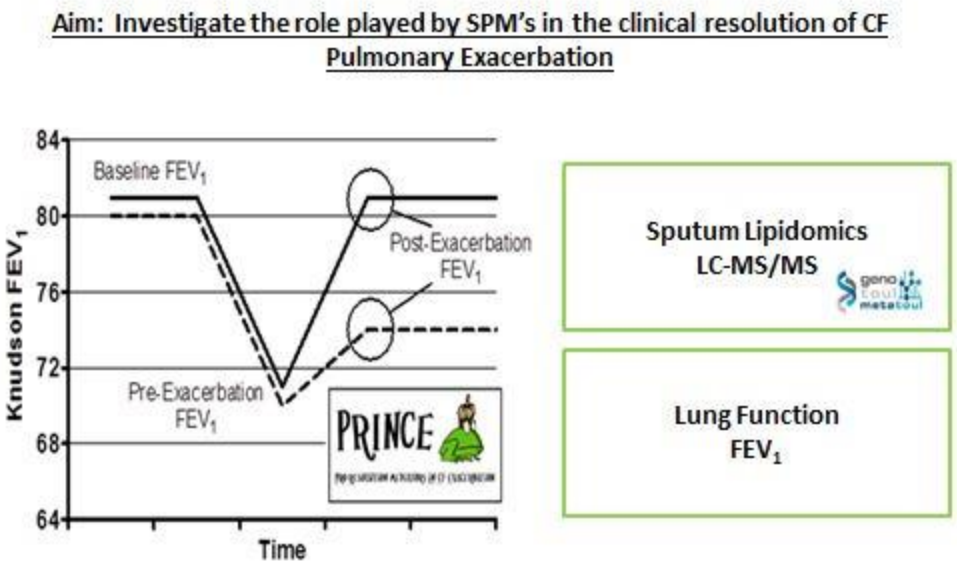

Figure 1-22 Illustration of the aim to investigate the role played by SPMõs in the clinical resolution of CFPE

*Graph adapted from Collaco et al. (368)

We report significant positive associations between RvD2 and the PPARɔ agonist 15-d-Prostaglandin-J2 measured in sputum during CFPE and the full recovery of FEV_1 post exacerbation. Whilst these findings are novel, and require validation in a larger population of both children and adults with CF, they suggest potentially exciting new avenues for the development of antibiotic adjuvant therapies in CF.

CHAPTER 2

MATERIALS AND METHODS

2 Materials and Methods

2.1 The SHIELD CF Study

The SHIELD CF Study (Study of Host Immunity and Early Lung Disease in Children with CF) is a longitudinal study established by Drs McNally & Linnane around the CF preschool surveillance bronchoscopy programs at Our Ladys Children Hospital, Crumlin (OLCHC), The National Childrens Hospital, Tallaght and University Hospital Limerick. Children with CF were recruited when they attended for their first annual surveillance bronchoscopy and consented to the collection of blood, urine, bronchoalveolar lavage fluid, bronchial brushings and clinical data parameters as part of this longitudinal study. Consent was renewed at each annual surveillance visit. Children with CF remained in the study until they were able to reliably expectorate sputum for microbiological surveillance of their lung disease and graduated from the pre-school surveillance bronchoscopy program. Children without CF undergoing bronchoscopy for clinical reasons were recruited as paediatric controls.

2.1.1 Inclusion & Exclusion criteria & Treatment Characteristics of SHIELD CF participants in the work reported in this thesis

Children were excluded from the studies reported in this thesis if they were taking leukotriene receptor antagonists or treatment dose antibiotics in the two weeks prior to bronchoscopy. Annual surveillance assessments were undertaken when the children were in a stable clinical condition. As a matter of local prescribing policy, NSAIDs, inhaled steroids and oral corticosteroids are not routinely prescribed for young children with CF. No included participant was on long term oral corticosteroids. As a matter of pragmatism (given the high rate of inhaled corticosteroid prescription among referrals to a tertiary paediatric respiratory clinic), it was not possible to completely exclude all control children on low dose inhaled corticosteroids, however, investigation for moderate / severe asthma was not listed as an indication for bronchoscopy among the included control group (370).

2.1.2 Bronchoscopy, BAL Collection and Storage

Flexible fibre optic bronchoscopy and BAL collection were performed as previously described (371). Patients were fasted for a minimum of four hours prior to the procedure. All procedures were conducted in the operating room under general

anaesthesia. Prior to introduction of the bronchoscope, lignocaine 4% was applied to the airway above the vocal cords, and lignocaine 1% to the vocal cords and lower airway to provide local anaesthesia. The maximum dose of lignocaine did not exceed 7mg/kg. Supplemental oxygen, pulse oximetry, continuous ECG recording and intermittent non-invasive blood pressure monitoring was carried out throughout. Before the introduction of the bronchoscope a control lavage was performed using sterile saline to control for bacterial contamination of the instrument. The bronchoscope was introduced via a laryngeal mask airway to prevent contamination of the bronchoscope and introduction of pathogenic bacteria into the lower airways from above. Suction was not performed until the tip of the bronchoscope was past the carina. In order to control for variations in dilution, BAL samples were only included if the 4ml/kg total instillation volume was strictly adhered to and a return of 40-60% volume was attained.

BAL was performed by instilling two aliquots of 1ml/kg sterile 0.9% saline into both the right middle lobe and lingula. All four samples were pooled, sealed in a sterile container and transported to the laboratory on ice. BAL was centrifuged at 3000rpm and the supernatant and cell pellet aliquoted and frozen at -80ºC for subsequent experiments.

After the procedure, patients were observed in the recovery area for at least 30 minutes with pulse oximetry, ECG monitoring and blood pressure measurement until fully alert and breathing without difficulty.

2.2 PRINCE Study

In order to investigate a role for Pro-resolution Mediators as adjuvant therapeutics for the treatment of CF pulmonary exacerbation in childhood, we designed a prospective paediatric observational cohort study; The Pro-Resolution Mediators in CF Exacerbation (PRINCE) study (Figure 2-1).

2.2.1 PRINCE Study Population

We invited eligible inpatients attending OLCHC with Cystic Fibrosis Pulmonary Exacerbation (CFPE) (commencing in February 2013) to participate. Cases were identified by the investigator (FR) by liaising with the multi-disciplinary respiratory team and the admitting resident medical staff. Since this was the first study (to our

knowledge) to examine sputum pro-resolution mediator profiles of children with airways disease we did not have any information about the extent or timing of the production of such mediators in children without CF. We therefore expanded the study protocol to recruit an additional group of inpatients without CF, attending OLCHC from whom sputum could be obtained (e.g. community acquired pneumonia, Non CF bronchiectasis with infective exacerbation, children with tracheostomy in situ) as a ñDisease Controlò population. The study was publicised within the hospital and among staff.

To be included in this study, participants with CF must:

- ¶ Have a confirmed diagnosis of Cystic Fibrosis
- ¶ Be able to co-operate with spirometry and expectorate sputum
- ¶ Complete at least part of their treatment for CFPE as an inpatient
- ¶ Have a pre-exacerbation baseline* (*defined as best reliable FEV_1 recorded in the 3 months prior to exacerbation) FEV_1> 50% predicted
- ¶ Fulfil Fuchs Criteria for Cystic Fibrosis Pulmonary exacerbation (372)

To be included in this study, control participants must:

- ¶ Be productive of sputum
- ¶ Complete at least part of their treatment as an inpatient
- ¶ Previous diagnosis of other comorbid condition not specifically excluded

2.2.2 Study Design & Sample Size Estimation

Primary End-Point: A description of the relationship between airway concentrations of Pro-Resolution lipid mediators and the extent of FEV_1 recovery.

We based our initial sample size estimates on a study which looked at the concentration of one SPM (LXA_4) at the start and end of exacerbation. In that study of LXA_4 change after completion of antibiotic treatment for CFPE (354) a statistically significant (P = 0.003) change in LXA_4 concentration from 19.7 - 39.6 ng/ml was observed with 18 patients. For this prospective study, we estimated that we would need a minimum of 23 patients to to detect a mean difference of 10ng/ml in the concentration of one SpM.

Based on previous studies of FEV_1 recovery during exacerbation (368), in order to relate SPM production to our primary outcome - the kinetics of FEV_1 recovery to baseline - we estimated that we would need to recruit a minimum of 60 patients.

**Statistical support was provided by the National Children's Research Centre via Dr. Gloria Crispino O'Connell.*

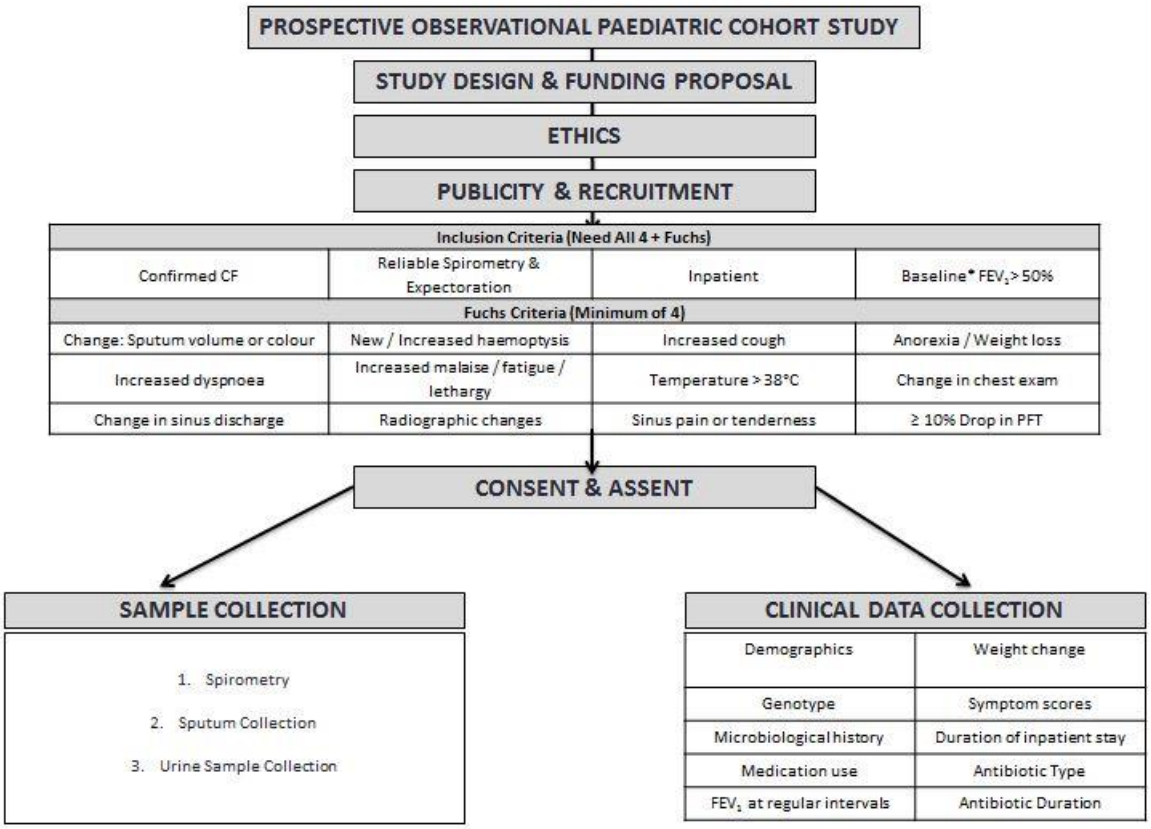

Figure 2-1 Overview of the conduct of the ñPRINCEò study

2.2.3 PRINCE Clinical Data Collection

Clinical data was recorded onto an anonymised standard data collection form including; comorbid conditions, age, sex, prior microbiology history and regular medications. For those children hospitalized for CFPE the following additional data was collected; weight at baseline and during the course of CFPE, treatment antibiotics for CFPE, length of antibiotic course and CF mutations. A Respiratory and Systemic Symptoms Questionnaire© (Boehringer Ingelheim) was administered on participants being treated for CFPE at intervals during the course of their infection (days (1-2), (4-5), & (6-12)).

2.2.4 Spirometry

Spirometry was performed by the investigator (FR) on PRINCE study participants with CF at intervals during the course of their infection (days (1-2), (4-5), (6-8) & (11-12)). Historical spirometry measurements performed by the pulmonary function lab at OLCHC were recorded from the patientôs clinical notes. An

EasyOneÊ Diagnostic portable spirometer was used to perform the measurements. The equipment was checked daily for validation and quality control. Patients were asked to wear a nose clip and perform at least three forced expiration manoeuvres in a sitting position until at least three acceptable spirograms were obtained with between manoeuvre variability of less than 0.15L for FVC and FEV_1 (373). The participantôs sex, and most recent height and weight recorded during this admission were entered into the spirometer and Forced expiratory volume in one second (FEV_1) measured in litres was expressed as a percentage of that predicted, with calculations performed by the spirometerôs software based on reference values used by the spirometerôs manufacturer.

2.2.5 PRINCE Study Sputum Collection & Processing

Sputum consists of a mixture of mucus, inflammatory and epithelial cells, and cellular degradation products from the lower respiratory tract (374). Sputum collection and processing was performed by the investigator (FR). Patients were asked to blow their nose, rinse their mouth and swallow water before expectoration to reduce salivary contamination (374). They were then asked to spontaneously expectorate sputum into a sterile container. This was then capped tightly, placed on ice and transported immediately to the lab for processing. Sputum samples were collected on a once off basis from control participants and at intervals during the course of their infection (days (1-2), (4-5), (6-8) & (11-12)) from participants with CF.

Only sputum plugs were used (salivary dilution represents a significant sampling variable (375)). An unprocessed portion of sputum was immediately placed into cold HPLC grade methanol, frozen at minus 80 degrees and kept under those conditions until transported to Toulouse on dry ice for analysis by LC-MS/MS.

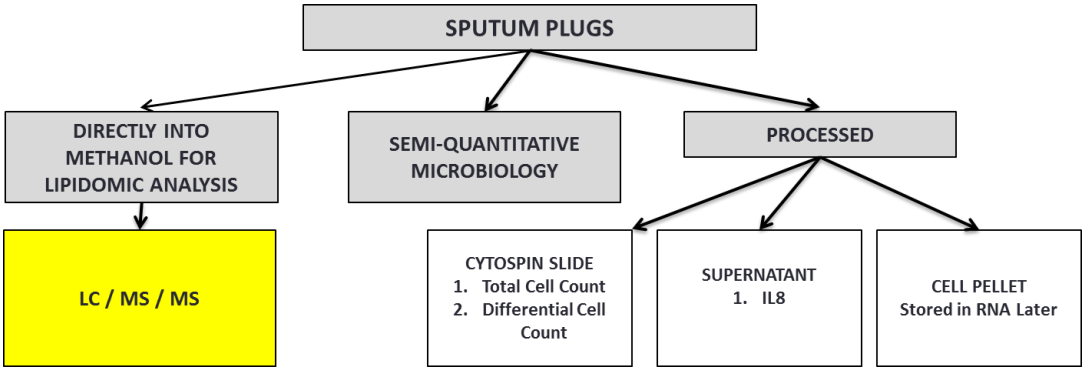

Figure 2-2 Overview of the sputum sample processing algorithm for the ñPRINCEò study

The remaining sputum was then processed according to a modified version of the validated protocol of Pavord at al. (376) to obtain a supernatant fraction for the quantification of IL8 and a cell pellet in order to obtain a total cell count (Figure 2-3). Dithiothreitol (Sputasol) was used to improve cell dispersion. It is a sulphydryl agent which produces mucolysis by opening disulphide bonds crosslinking glycoprotein fibres. Compared with mechanical dispersion it is reported to result in easier, quicker, and more reproducible cell counts. DTT is reported to be compatible with IL8 measurement and not to cause appreciable cell activation (376, 377).

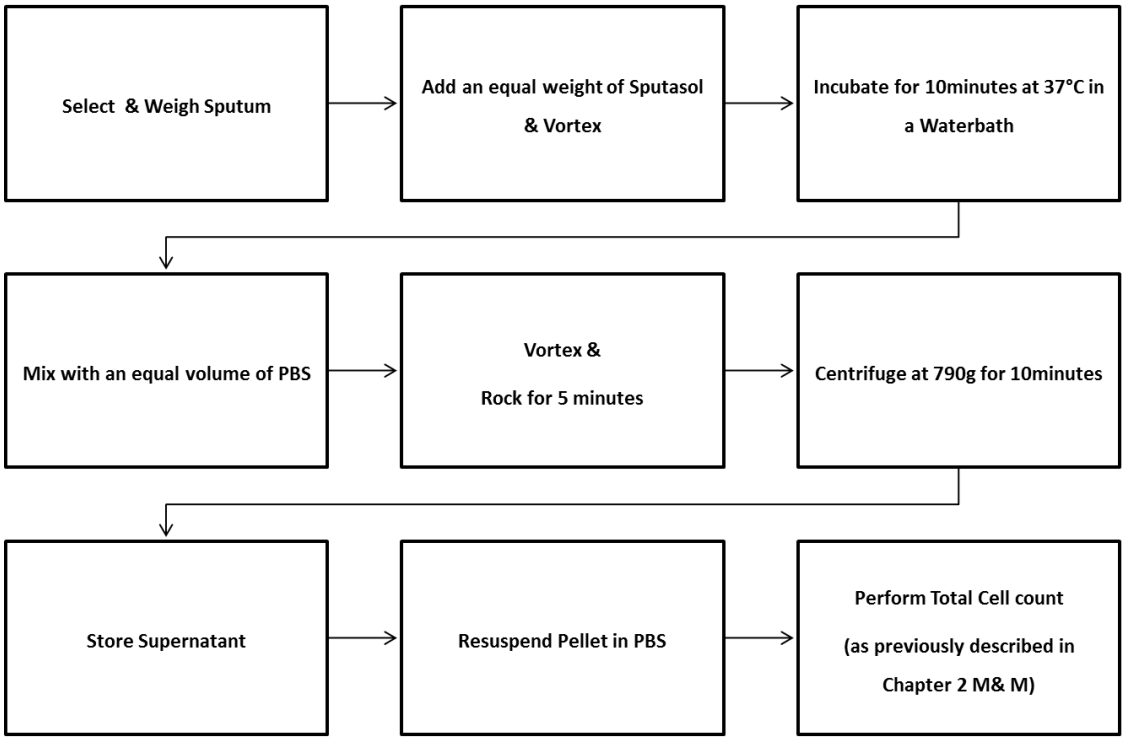

Figure 2-3 Flow chart illustrating the method used to process sputum for the PRINCE study

A modified version of the protocol used by Pavord et al. (376)

2.3 Methodologies common to both SHIELD CF & PRINCE Studies

2.3.1 Ethics

Ethical approval for the SHIELD CF study was granted by each participating hospitalôs research ethics committee. Ethical approval for the conduct of the PRINCE study was obtained from the recognised ethics committee of Our Ladyôs Childrenôs Hospital, Crumlin. An amendment to the original PRINCE study application was approved by the same body to allow us to recruit a control cohort.

2.3.2 Consent, Assent and Confidentiality

Written and oral information were provided with the use of information leaflets. Written assent was sought and obtained from the participants in the PRINCE study. Written consent was obtained from the parent / legal guardian of participants of both studies prior to enrolment. Specific provisions were made to maintain patient confidentiality and for the observation of the Data Protection

guidelines. Data and biological samples were pseudo-anonymised at collection by allocation of a study participant number.

2.3.3 Microbiological testing of airway samples

For the SHIELD CF Study an aliquot of pooled BAL was sent directly from theatre to the microbiology lab at OLCHC. For the PRINCE Study a separate sputum sample was sent directly to the microbiology lab at OLCHC. Conventional bacterial studies were performed according to Clinical Pathology Accreditation guidelines. Selective media for the detection and enumeration of *Staphylococcus aureus, Methicillin resistant S. aureus, Burkholderia cepacia, Haemophilus influenzae* and *P. aeruginosa* were employed. Viral studies were performed on BAL samples including, Indirect Fluorescent Antibody test for Adenovirus, Parainfluenza 1, 2 & 3, Influenza A & B, Respiratory Syncytial Virus. Testing for Mycobacterium tuberculosis also was performed on BAL.

2.3.4 Total & Differential Cell Counts

Total and differential cell counts were obtained by the trypan blue exclusion method and light microscopy. Total cell counts were measured by 1:1 dilution in trypan blue and instillation into disposable CountessÊ cell counting chamber slides. Automated cell counts were evaluated by the Countess® Automated Cell Counter (InvitrogenTM).

For differential cell counts, cytospin slides were prepared with $5x10^5$ cells. The slides were stained with hematoxylin and eosin and examined to evaluate homogeneity of the sample. Representative areas of the sample were selected and counted using an eyepiece graticule with mesh and Tally counter. At least 300 consecutive cells were counted at high power magnification and the results were expressed as percentages of the total nucleated cell count. For the SHIELD CF study, differential cell counts were evaluated by a single consultant histopathologist Dr Michael McDermott, at OLCHC.

Neutrophil and macrophage counts were calculated using the absolute cell count of the raw BAL and the % neutrophils or macrophages reported on the differential.

2.3.5 Sample Transportation

For the PRINCE study lipid mediator analysis by LC ï MS/MS, samples were transported on dry ice to the Lipidomic Platform in Toulouse, and immediately replaced in minus 80 degrees storage upon receipt in Toulouse.

2.4 Liquid Chromatography / Tandem Mass Spectrometry (LC-MS/MS)

Chromatography (gas, liquid or thin layer) is used to separate compounds of interest before mass spectrometry analysis. Mass spectrometry (MS) is a technique that is used to determine the mass, elemental composition and chemical structure of molecules. The target molecules are ionised to form charged particles and introduced into the mass spectrometer. Separation is performed according to their mass to charge ratio using electromagnetic fields under vacuum. The separated ions are then recorded by a detector, and the signal is converted into a mass spectrum (378).

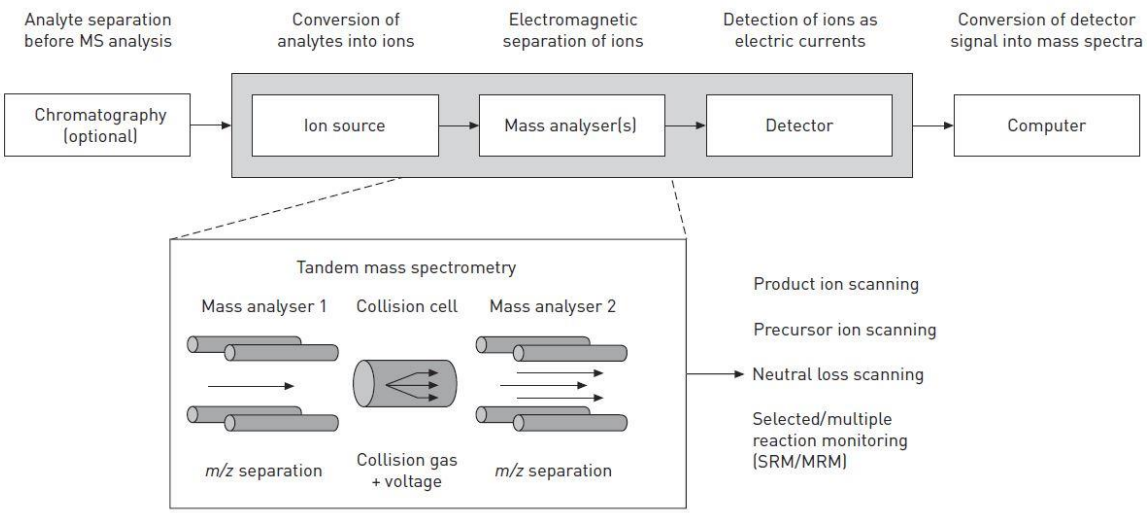

Figure 2-4 Schematic Illustration of the Mass spectrometry method (Reproduced from Wheelock et al. (378)).

For the PRINCE Study, lipid mediators were quantified by the metabolo-lipidomic approach by the Lipidomic Platform at Toulouse by the previously published Liquid Chromatography / Tandem Mass Spectrometry method (379).

2.4.1 Extraction

For extraction, sputum was crushed with a FastPrep ®-24 Instrument (MP Biomedical) in 500 µL of HBSS (Invitrogen) and 5 µL of internal standards (Deuterium labelled compounds). After 2 crush cycles (6.5 m/s, 30 s), 20 µL was withdrawn for protein quantification and 300 µL of cold methanol was added. After centrifugation at 900g for 15 min at 4°C, supernatants were transferred into 2 mL 96-well deep plates and diluted in water to 2mls. Samples were then submitted to solid phase extraction (SPE) using HRX 96-well plates (50 mg/well, Macherey Nagel), pre-treated with methanol (2mL) and equilibrated with 10% methanol (2 mL). After sample application, the extraction plate was washed with 10% methanol (2 mL). After drying under aspiration, lipids mediators were eluted with 2 mL of methanol. Prior to LC-MS/MS analysis, samples were evaporated under nitrogen gas and reconstituted in 10 µL of methanol (379).

2.4.2 LC-MS/MS

LC-MS/MS analysis of eicosanoids was performed by our collaborator Dr Justine Bertrand-Michel at the Lipidomic Platform in Toulouse as previously described (379). Lipid mediators were separated on a ZorBAX SB-C18 column (2.1 mm, 50 mm, 1.8 µm) (Agilent Technologies) using Agilent 1290 Infinity HPLC system (Technologies) coupled to an ESI-triple quadruple G6460 mass spectrometer (Agilent Technologies). Data was acquired in Multiple Reaction Monitoring mode with optimized conditions (ion optics and collision energy). Peak detection, integration and quantitative analysis were done using Mass Hunter Quantitative analysis software (Agilent Technologies) based on calibration lines built with commercially available eicosanoid standards (Cayman Chemicals).

No data was available regarding chemical stability of PUFA metabolites in airways samples, however a guide to their chemical stability in human serum after 5 days, when preserved in methanol and stored at -20°C and from data reported in the Human Serum Metabolome project (380, 381), is presented in Table 2-1, for the purposes of understanding their broad chemical stability characteristics in human tissues. The Limit of Detection for each compound by this technique was determined by the Lipidomic Platform in Toulouse and reported in Table 2-1, as previously published (379).

Table 2-1 An indication of the chemical stability and limits of detection of key Pro-Resolution PUFA metabolites examined in the PRINCE study

As no data was available regarding chemical stability of PUFA metabolites in airways samples, stability (expressed as % change in concentration at 5 days) is here reproduced from a study on the chemical stability of lipid mediators in human serum after 5 days, when preserved in methanol and stored at -20°C (381) and from the Human Serum Metabolome project (380, 381). *Limit of Detection was determined by the Lipidomic Platform in Toulouse (as previously published (379)).

Compound	**Stability (381)** **(% change at 5 days)** **Degradation**	**Limit of Detection*** **(379)**
LXA_4	-5.9%	2.5pg
LXB_4	-5.5%	4.9pg
RvD1	-1.1%	2.5pg
RvD2	+1.1%	2.5pg
18-HEPE	Not given (RvE1 -13.1% RvE3 -4.4%)	2.5pg
7-MaR1	-17.8%	4.9pg
PDx	Not given (PD1 -10.4%)	1.3pg

2.5 Epithelial Cell culture

2.5.1 Immortalised Epithelial Cell Line Source

Immortalised NuLi-1 and CuFi-1 cell lines were used to model human bronchial epithelium. These cell lines were sourced from Prof Joseph Zabner at the University of Iowa. These cell lines were generated by enzymatically isolating primary cells from the bronchial epithelium of human donor lungs. The NuLi-1 (normal lung, University of Iowa) cells were derived from the airway epithelium of a human male with normal genotype (382). CuFi-1 (cystic fibrosis, University of Iowa) cells were derived from human airway epithelium of a female with Phe508del / Phe508del genotype (382). Cells were immortalised by transduction the reverse transcriptase component of telomerase, hTERT, and human papillomavirus type16 (HPV-16) E6 and E7 genes (382). These cell lines have the advantage that when grown at the air-liquid interface, they are capable of forming polarized differentiated epithelia, exhibiting trans-epithelial resistance and maintaining the ion channel physiology expected for the genotypes. They have also been demonstrated to retain NFκB responses to lipopolysaccharide (382).

2.5.2 Primary CF Bronchial epithelial cells

The methods to establish primary epithelial cell cultures were developed by Dr Gerry Higgins and Dr Valia Verriere in the Urbach lab and were performed as previously published (85). Bronchial brushings were obtained from the SHIELD CF study cohort at bronchoscopy. For the studies performed in this thesis all participants had Phe508del / Phe508del CFTR mutation genotype. The airway brush with adherent cells was placed directly into Eagles Minimum Essential Media (EMEM) (Gibco®) supplemented with 5 ml Penicillin/Streptomycin (Gibco®), 80 µg tobramycin (Gibco®), 2.5 µg Fungizone (Gibco®) and 5 µg gentamicin (Gibco®) and transported on ice to the cell culture suite.

The bronchial brush with cells attached was transferred to 5ml of BEGM in a 15 ml tube. The media was agitated with a pipette to detach the cells from the brush. The cells were pelleted at 1000 rpm for 5 min at 4^0C and then resuspended in 3-4 ml BEGM Primary media. Cells were incubated in a collagen coated T25 flask at 37^0C in 5% CO_2 (Sanyo CO_2 Incubator) in BEGM Primary media. For the first 3 days after seeding, unattached cells were removed, pelleted and reseeded into a fresh T2 flask to improve yield from the retrieved cells (85).

2.5.3 Epithelial Cell Culture Media Used

Cell Line Bronchial Epithelial Cell Growth Medium (BEGM)

Cell line BEGM was prepared by taking 500mls of Bronchial Epithelial Cell Basal Medium (BEBM, Lonza Clonetics®) and adding BEGM® Single Quots Kit® (Lonza Clonetics®) containing; Bovine Pituitary Extract 2ml, Insulin 0.5ml, Hydrocortisone 0.5ml, Retinoic Acid 0.5ml, Transferrin 0.5ml, Tri-iodothyronine 0.5ml, Epinephrine 0.5ml, and Human Epidermal Growth Factor 0.5ml. The gentamicin-amphotericin B aliquot from this kit was discarded and replaced with 500µl (10mg/ml) Gentamicin (Gibco®, Invitrogen). Finally, 2.5mls of Fungizone (Gibco®, Invitrogen) is added.

Primary Bronchial Epithelial Cell Growth Medium (BEGM)

Primary BEGM was prepared by taking 500mls of Bronchial Epithelial Cell Basal Medium (Lonza Clonetics®) and adding BEGM® Single Quots Kit® (Lonza Clonetics®) containing; Bovine Pituitary Extract 2 ml, Insulin 0.5 ml, Hydrocortisone 0.5 ml, 0.5 ml, Retinoic Acid 0.5 ml, Transferrin 0.5 ml, Tri-iodothyronine 0.5 ml, Epinephrine 0.5 ml and Human Epidermal Growth Factor 0.5 ml. The gentamicin-amphotericin B aliquot from this kit was discarded and replaced with 500µl (10mg/ml) Gentamicin (Gibco®, Invitrogen). Antibiotics and anti-fungals were added in the following quantities; 5 ml of Penicillin - Streptomycin (Gibco®, 5000 units of penicillin and 5000 µg of streptomycin per ml); 500 µl Gentamicin 10 mg/ml (Gibco®) ; and, 2.5 ml fungizone (Gibco®).

Cell Line Day 2 Media (Day 2)

Cell line Day 2 Media was prepared by mixing 500mls of Dulbecco's Modified Eagle Medium (DMEM, Gibco®, Invitrogen) and 500mls of F12 Nutrient Mixture (HAM) (GibcoÈ, Invitrogen). To this base UltroserÊ G serum substitute (PALL Life Sciences) suspended in 20mls of sterile water was added. The mixture was supplemented with antibiotics and antifungals; 10mls Penicillin - Streptomycin 100X Solution (5000 units of penicillin and 5000 µg of streptomycin per ml) (GibcoÈ, Invitrogen), 1ml Gentamicin (10mgùml) (GibcoÈ, Invitrogen), and 5mls Fungizone® Antimycotic Liquid (Gibco®, Invitrogen) 250 µg /ml amphotericin B. The combined mixture is filter sterilized.

Primary 50/50 Bronchial Epithelial Media (Primary 50/50)

Primary 50/50 medium was prepared by adding 250mls of Dulbecco's Modified Eagle Medium (DMEM) (Gibco®) to 250 mls of Bronchial Epithelial Cell Basal Medium (BEBM) (Lonza Clonetics®). The base medium was supplemented by adding BEGM® Single Quots Kit® (Lonza Clonetics®) containing; Bovine Pituitary Extract 2 ml, Insulin 0.5 ml, Hydrocortisone 0.5 ml, Retinoic Acid 0.5 ml, Transferrin 0.5 ml, Tri-iodothyronine 0.5 ml, Epinephrine 0.5 ml and Human Epidermal Growth Factor 0.5 ml. The gentamicin-amphotericin B aliquot from this kit was discarded. To this mixture is then added; 5 ml of Penicillin - Streptomycin (Gibco®), 500 µl Gentamicin 10 mg/ml, (Invitrogen) 2.5 mls fungizone (Gibco®), 0.5 ml BSA (1.5mg/ml) (Sigma Aldrich®, USA) and 25 µl of retinoic acid (Sigma Aldrich®, USA). The media was filter sterilised and stored at 4 ^{0}C until ready for use.

2.5.4 Collagen coating of culture surfaces

Bronchial epithelia were cultured on collagen coated surfaces. 50mg of Collagen from human placenta (Bornstein & Traub Type IV, Sigma-Aldrich®) was dissolved in 83.3mls of sterile distilled water and 166.6µl of glacial acetic acid (Fisher). This stock solution was filter sterilised and stored at 4ęC. Collagen stock solution was used at a working concentration of 60µg/ml by diluting 1:10 with distilled water. Prior to seeding cells, the liquid collagen was aspirated from the plastic surface and the surface was rinsed thrice with sterile DPBS.

2.5.5 Bringing cells up from liquid nitrogen

NuLi-1 and CuFi-1 cell stocks were stored in liquid nitrogen. To bring cells up from storage the vial of cells was removed from the liquid nitrogen and placed in a water-bath to thaw at 37ęC. The cells were placed in a 15ml tube containing 10mls of pre-warmed BEGM media and centrifuged at 1100rpm for 5 minutes at 4ęC. The supernatant was discarded and the cell pellet re-suspended in 10ml of BEGM, inverted to mix, and the contents poured into a rinsed, collagen coated, labelled T75 flask (Corning®). Cells were grown to 70-80% confluence (as assessed by light microscopy (Olympus CKX41)) in flasks in a Sanyo CO_2 Incubator at 37ęC in 5.0% CO_2. The culture medium was changed every 2-3 days.

2.5.6 Splitting and seeding cells

Bronchial epithelial cells grown in flasks were used to set up two kinds of cell preparations depending on the experimental protocol;

- Non-polarised bronchial epithelial cell monolayers, grown submerged under media on collagen coated plastic in 6 well plates
- Polarised, differentiated bronchial epithelial cell preparations, grown on collagen coated hanging cell culture inserts (Millipore) at air-liquid interface

To split cells, the old media was aspirated cells were washed twice with calcium free sterile DPBS. 5-7 ml of Trypsin / EDTA (Gibco®) was introduced and the flask was returned to the incubator (Sanyo CO_2 Incubator: 37ęC in 5.0% CO_2) for 5 minutes. The flask was tapped to aid detachment. Once the cells were detached, 1 ml Fetal Bovine Serum (FBS) (Gibco®) was added to the flask to neutralize the trypsin. Detached cells were aspirated and transferred into a fresh 15 ml tube and pelleted at (Eppendorf Centrifuge 5810R) 1100rpm for 5 minutes, 4ęC. The cell pellet was re-suspended in pre-warmed BEGM media and apportioned for seeding cells into collagen coated (and rinsed) 6 well plates or hanging cell culture inserts (Millipore), with the remainder returned to a new flask.

2.5.7 Feeding Undifferentiated Monolayers

Media was pre-warmed to 37°C in the water-bath. Every 2-3 days, the old media was aspirated and discarded and monolayer cultures were fed with 2mls per well of BEGM media. Cells were incubated at 37°C in 5.0% CO_2 (Sanyo CO_2 Incubator).

2.5.8 Liquid-Liquid Interface Cultures in Hanging Cell Culture Inserts

Media was pre-warmed to 37°C in the water-bath. Cells were incubated at 37°C in 5.0% CO_2 (Sanyo CO_2 Incubator). Prior to achieving confluence, cultures were fed with 1.5 ml of BEGM in contact with the basolateral side of the membrane and 500µl of BEGM added to the apical compartment. Following confluence, the media was changed to Day 2 media for cell lines, and 50/50 media for primary cells to aid differentiation. Media was changed every 2 days. A period of liquid-liquid interface growth is essential to achieve differentiation.

2.5.9 Measuring Trans-Epithelial Electrical Resistance (TEER)

Once cells in hanging cell culture inserts had achieved confluence, regular TEER readings were obtained using an EVOMXÊ probe (World Precision Instruments, Sarasota, FL). For Liquid-Liquid cultures, the probe of the EVOMXÊ was placed in contact with the media on either side of the membrane in order to obtain a resistance measurement. Following the Air-Liquid transition, 0.5mls of media was transferred from the basolateral compartment into the apical compartment in order to perform this resistance measurement. The media was then removed from the apical compartment to restore the air interface.

2.5.10 Air-Liquid Interface Cultures

When tight junctions began to form in the cultures, evidenced by increasing TEER (>200q /cm2) the cultures were switched to air-liquid interface. In this arrangement, the apical compartment is exposed to air and media is placed in contact with the basolateral side of the porous support. The air-liquid phase is employed to achieve polarisation, cell differentiation and ciliation. Preparations were considered to be fully differentiated and polarised when they had achieved TEER values >700q /cm2.

2.5.11 Bronchial Epithelial Cell Treatments & Assays

Differentiated and polarised epithelial cell cultures were used for; airway surface liquid height measurements, measurements of IL8 secretion, measurements of 15-Lipoxygenase (15-LO) mRNA expression and activity in bronchial epithelia. Undifferentiated monolayers were used for; measurements of IKB, ALX/FPR2 and 15-LO protein expression.

Resolvin D1 was used at a concentration of 1nM or 100nM and obtained from Cayman Chemical, MI. LXA_4 was used at 1nM and sourced from Calbiochem, Merck. The peptide Boc-Phe-Leu-Phe-Leu-Phe; Boc-2 (Phoenix pharmaceutical, Belmont, USA) was used at 10µM as a specific pharmacological inhibitor of the ALX/FPR2 receptor. BAPTA-AM (Molecular probes, Leiden, Netherlands) was used in calcium free DPBS at a concentration of 10µM as an intracellular calcium chelator. CFTRinh-172 (Sigma) was used as an antagonist of the CFTR channel at a concentration of 20ÕM. TNFŬ (eBiosciences, San Diego, USA) was used at a concentration of 80ng/ml to stimulate IL8 secretion. To measure total 15-LO

activity by an epithelial monolayer 5(S), 6(R)-dihydroxy-7, 9-trans-11, 14-cis-eicosatetraenoic acid (DiHETE) (Cayman Chemical) was used as a 15-LO substrate at a concentration of 250ng/ml. Prostaglandin E2 (Cayman Chemical) was used at a concentration of 100nM to try to up-regulate 15-LO transcription.

2.6 Primary Alveolar Macrophage Culture

2.6.1 Primary Alveolar Macrophage Medium

Primary Alveolar Macrophage Medium was prepared using RPMI 1640 (Biosciences) as a base and adding; 5mls of 10% Foetal Bovine Serum (Heat inactivated at 56°C for 30 mins in water bath, Sigma), 500µl L-Glutamine 2mmol/l (Gibco), and 1ml Penicillin - Streptomycin 100X Solution (5000 units of penicillin and 5000 µg of streptomycin per ml) (Gibco®, Invitrogen).

2.6.2 Enriching Primary Alveolar Macrophages from BAL Cell Pellet

BAL fluid from both children with CF and controls was collected and processed to obtain a cell pellet as per SHIELD study protocol (Section 2.1.2). BAL pellet cells were re-suspended in primary alveolar macrophage medium at a concentration of 0.5 million viable cells per ml and placed in a T25 flask, 6 or 96 well tissue culture plates (depending on the experimental protocol) and incubated (humidified, 37.2°C, 21% oxygen, 5% CO_2) overnight to attach to tissue culture coated plastic. The following morning non adherent cells suspended in the medium were aspirated and discarded. The adherent cells washed twice with pre-warmed calcium and magnesium free DPBS. For some experiments analysis was performed directly on the cells adherent to plastic. Where indicated, Non enzymatic cell dissociation solution (Sigma) was applied to detach the cells (2mls/T25 flask) and allowed to incubate in the cold for 5-10 minutes. The flask was then tapped to aid detachment. Detached cells were collected in preparation for experiments in a 15ml tube and enumerated by trypan blue exclusion.

2.7 Alveolar Macrophage Functional Assays

2.7.1 Phagocytosis Assay

The phagocytic capacity of CF alveolar macrophages was measured by their ability to engulf IgG & FITC labelled beads using a Phagocytosis Assay kit (Cayman Chemical, Ann Arbour, MI) according to the manufacturerôs instructions.

Macrophages were enriched by adherence to tissue culture coated plastic and cultured in complete macrophage medium in 12 and 96 well plates. Macrophages were exposed to the Latex beads ï rabbit IgG-FITC complex added to the culture medium for 1 hour in the dark at 37^0C in 5.0% CO_2 (Sanyo CO_2 Incubator). The tissue culture plates were centrifuged for 5mins at 400g and the supernatant was discarded. To quench non-specific staining the cells were washed with a trypan blue quenching solution, centrifuged, the supernatant discarded and then subjected to analysis.

The degree of phagocytosis in each sample was analysed quantitatively by measuring the fluorescence intensity of engulfed FITC labelled complexes in triplicate using a fluorescence plate reader (Synergy MX Biotek~® Instruments, Winooski, VT). Qualitative fluorescence microscopy experiments were conducted in parallel with a concurrently obtained light microscopy image overlaid in order to visualise the cells.

2.7.2 Bactericidal assay against Pseudomonas aeruginosa (PA01)

CF alveolar macrophages were isolated by adherence to tissue culture plastic and cultured in complete macrophage medium in a 12 well plates overnight as previously described. *P. aeruginosa* strain PAO1 was obtained from Professor Fergal OGara, University College Cork, Ireland. Strain PAO1 is a wound isolate and is the most widely used *P. aeruginosa* laboratory strain (383).

PAO1 was grown in LB broth and then switched to antibiotic free macrophage medium overnight at 37°C shaking. The bacteria were grown up to log phase with an optical density of ~0.6. Upon reaching log phase bacteria were equalised to an Optical Density of 0.1 in fresh antibiotic free macrophage medium warmed to 37°C. Macrophages were washed twice with antibiotic free macrophage medium to remove all traces of antibiotic. PAO1 at an optical density of 0.1 (concentration of 2x10^14 CFU/ml) was then added to alveolar macrophage preparations. The inoculating time was 30 minutes, after which the apical medium was removed, the cells were washed with medium and gentamicin (400µg/ml) was applied for one hour to kill extracellular adherent bacteria. The macrophages were then washed twice and treated with Triton-X 100 (0.4% v/v) for 15min to lyse the macrophages release internalised bacteria.

Quantification of the viable intracellular bacterial load within macrophages was performed by serial dilution of the lysate 1 in 10 in antibiotic free macrophage medium. The lysate dilution series was plated out on LB agar and incubated at 37ÁC overnight. Colony Forming Units were counted (accurate range Ó30 & Ò300) and the results were expressed as colony forming units per ml by correction for dilution factors.

2.8 ELISAôs

2.8.1 Lipoxin A_4 ELISA

LXA_4 was measured by ELISA using the LXA_4 EIA Kit (Oxford Biomedical Research) according to the manufacturerôs instructions. The test kit operates on the basis of competition between an enzyme conjugate and the LXA_4 in the sample for a limited number of antibody binding sites.

50µl of neat sample was added to the rat anti-LXA_4 antibody pre-coated 96 well plate. 50µl of diluted lyophilized LXA_4 ï HRP conjugate was added to each well. The plate was incubated at RT for 1 hour to allow competition for binding sites to take place. The microplate was then washed with the supplied wash buffer 5 times using an Automated Plate Washer (TRI-Continent Scientific Medical Supply Co. Ltd) to remove all unbound material. The bound enzyme conjugate was detected with TMB. After 30 minutes at room temperature the absorbance was read at 650nm using a plate reader (Synergy MX Biotek~® Instruments, Winooski, VT). The extent of colour development was inversely proportional to the amount of LXA_4 in the sample or standard. Quantitative results were obtained by comparing the absorbance reading of the wells of the samples against a standard curve. Specificities of antisera are given in Table 2-2.

2.8.2 Leukotriene B4 ELISA

Leukotriene B_4 (LTB_4) was measured by ELISA using the LTB_4 EIA Kit (Cayman Chemical, MI) according to the manufacturerôs instructions. Briefly, the assay principle is based on the competition between LTB_4 (in the sample / standard) and an LTB_4-acetlycholinesterase conjugate (tracer) for a limited amount of LTB_4 anti-serum. The ELISA plate is pre-coated with a mouse monoclonal anti-rabbit IgG. Samples and standards are applied to the wells and incubated with a fixed amount of tracer and antiserum. LTB_4-antiserum complexes bound to the IgG on the plate reduce the binding of tracer-antiserum complexes to IgG on the plate. The tracer

interacts with Ellmanôs reagent and generates a signal measured by spectrophotometric absorbance at 412nm. The intensity of this signal is inversely proportional to the amount of free LTB_4 in the sample or standard. Quantitative results are obtained by comparing the absorbance reading of the wells of the samples against a standard curve. Specificities of antisera are given in Table 2-2.

Table 2-2 Cross-Reactivity of Anti-Sera quoted by manufacturers of ELISA Kits

	LTB_4 EIA Kit Cayman Chemical	**LXA_4 EIA Kit Oxford Biomedical Research**
LXA_4	Not given	100%
15-epi-LXA_4	Not given	24%
Lipoxin B_4	Not given	1.0%
LTB_4	100%	<0.01%
5(S)-HETE	6.6%	<0.1%
5(R)-HETE	3.7%	<0.1%
20-hydroxy LTB_4	2.7%	Not given
15(R)-HETE	0.98%	0.1%
15(S)-HETE	0.4%	0.1%
6-trans-12-epi LTB_4	0.31%	Not given
6-trans LTB_4	0.11%	Not given
5,6-DiHETE	0.07%	5.0%
Leukotriene C4/D4/E4	<0.01%	<0.01%
Glutathione, 20-carboxy LTB4 , 19(R)-hydroxy Prostaglandin B2	<0.01%	Not given

2.8.3 Interleukin 8

IL8 was measured by an in-house ELISA using mouse anti-IL8 capture antibody, and biotinylated goat anti-IL8 detection antibody (R&D Systems, Abingdon, Oxon, UK) by the previously published method (384).

Vollerôs Buffer

0.16g Sodium Carbonate (15mM)

0.294g Sodium Bicarbonate (35nM)

Make up to 100mls with double distilled water and adjust pH to 9.6.

The capture antibody (Human CXCL8/IL-8 monoclonal mouse IgG antibody, MAB208, R&D systems) was diluted 1:125 in Vollerôs Buffer and 100Õl was

applied to each well of a 96 well plate. The antibody was allowed to adhere to the plate at 4°C overnight. The plate was washed thrice with DPBS-0.05% Tween and blocked for 1 hour at RT using 300µl per well of blocking buffer (1% BSA in DPBS-0.05% Tween). A standard curve was prepared using a known concentration of IL8 standard diluted in DPBS. 100µl of sample and standards were loaded and incubated at room temperature for 2hours. The plate was then washed thrice and incubated with 100µl per well of detection antibody (BAF208, goat polyclonal anti-human IL8 biotinylated antibody, R&D systems) for 2 hours at room temperature. The plate was again washed thrice and incubated with 1:2000 streptavidin Horseradish Peroxidase. The plate was incubated for 30mins at room temperature in the dark, washed thrice, and then developed with 2,2'-azino-bis (ABTS). The absorbance was read at 405nm in an automated plate reader. Quantitative results were obtained by comparing the absorbance reading of the wells of the samples against a standard curve.

2.8.4 Neutrophil Elastase Activity Assay

Neutrophil Elastase (NE) activity was measured as described previously (371), by mixing 50εl of the chromogenic substrate MeOSuc-AAPV-pNA, with each sample and measuring absorbance at 405 nm over time at 37 °C in a 96-well microplate reader. The concentration of NE in samples was determined by comparison against a standard curve of purified NE (371).

2.9 PCR

In order to quantify mRNA expression of specific genes by PCR, three steps were required; extraction of RNA from cell lysates; RNA reverse transcription into cDNA; and quantification by Real Time PCR.

2.9.1 RNA Isolation

Total RNA was extracted in an RNase free environment from cells using RNAqueousÈ kit (Ambion Inc., Austin, TX) according to the manufacturerôs instructions. The principle of this column method (385) relies on the ability of glass fibres to bind nucleic acids in concentrated salt solutions. Samples are disrupted in a high concentration guanidinium salt solution that lyses cells and inactivates endogenous RNases. The lysate is diluted with an ethanol solution to allow RNA to bind to the glass fibre filter in the filter cartridge. Most other cellular contents

flow through the filter cartridge. The bound RNA is then eluted in a very low ionic strength solution (385).

Briefly, cells in suspension were pelleted, whereas, adherent cells were detached and pelleted. Lysis binding solution was applied to the cells and the cells were vigorously disrupted by shear force, drawing the lysate through an orange (25G) needle several times to homogenise the sample. An equal volume of 64% Ethanol was added to the lysate and the combined mixture was applied to a filter cartridge assembled in a collection tube. The cartridge was then washed thrice with the provided wash solutions and then the bound RNA was eluted into a clean, labelled collection tube with Elution solution.

The quantity and quality of isolated RNA was analysed by spectrophotometry using a Nanodrop (Thermo-scientific). The principle of this test relies on absorption of ultraviolet light (260nm) by RNA / DNA. Quality was assessed by the 260/280 ratio, given that protein impurities absorb UV light at 280nm. Samples with ratios closer to 2.0 were considered to be higher in purity.

2.9.2 Specific provisions for RNA Isolation from Primary Alveolar Macrophages

Macrophages release RNases (especially when viability declines or activation occurs) therefore Lysis Binding Solution must be applied as soon as possible to the isolated cells. Detached cells were pelleted at high speed (3000rpm, 10 mins) and then resuspended in 700µl of Lysis Biding Solution (RNAqueous Phenol Free total RNA Isolation kit Biosciences).

In addition to improve recovery of RNA, 500µl of LBS was applied to the remaining cells still attached inside the T25 flask, scraped with a cell scraper and aspirated. The combined lysate was then subjected to homogenisation by shear force using an orange needle and stored at -80°C until isolated.

2.9.3 Reverse Transcription

RNA was reverse transcribed into complementary DNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturerôs instructions.

Briefly, total isolated RNA of interest is combined with buffer, random primers, Deoxyribonucleotide triphosphate Mix (dNTPs; dATP, dCTP, dGTP and dTTP), and Reverse Transcriptase enzymes. The resultant mixture is placed in a Thermal Cycler (Applied Biosystems) and undergoes Denaturation, Primer Extension, cDNA synthesis and Reaction Termination steps. cDNA was then stored at -80°C until analysis by quantitative PCR.

2.9.4 Real Time PCR

Real time PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan® Probes (Table 2-3).

Briefly, the principle of the method is as follows; a pre-determined total quantity of cDNA of interest is combined with PCR Mastermix containing a polymerase enzyme (Applied Biosystems) and the target Taqman(R) probe. The Taqman(R) probe is an oligonucleotide sequence with a fluorescent probe attached to the 5' end and a quencher to the 3' end. During amplification, the probe binds to the target amplicon sequence; the polymerase replicates the target template and cleaves the fluorescent probe, separating it from the quencher. The increase in fluorescence intensity is proportional to the number of cleavage cycles, which is also directly related to the initial amount of target in the sample.

Table 2-3 Taqman(R) Probes used

All probes used were supplied by Applied Biosystems, and 18S was used as a housekeeper probe. *The selected probes were chosen in view of previous reports of their differential expression characteristic of M1 and M2 polarised macrophages (329).

Human Taqman(R) Probe	**NCBI Reference Sequences:**
ALOX5 (5 Lipoxygenase)	NM_000698.3 NM_001256153.1 NM_001256154.1
ALOX12 (12 Lipoxygenase)	NM_000697.2
ALOX15 (15 Lipoxygenase-1)	NM_001140.3
ALOX15B (15 Lipoxygenase-2)	NM_001039130.1 NM_001039131.1 NM_001141.2
LTA4H (Leukotriene A4 Hydrolase)	NM_000895.2 NM_001256643.1 NM_001256644.1
M1 Macrophage Polarisation Markers (329)*	
IDO1 (Indoleamine 2,3-dioxygenase 1)	NM_002164.5
IL-12 p35 (Interleukin-12 p35)	NM_000882.3
M2 Macrophage Polarisation Markers (329)*	
CCL22 (CC Chemokine type 22)	NM_002990.4
PPARɔ (Peroxisome Proliferator-activated receptor ɔ)	NM_015869.4 NM_005037.5 NM_138712.3 NM_138711.3

2.9.5 Calculation of Relative Fold Expression

The cycle threshold for each target gene was normalized using the cycle threshold (Ct) of the endogenous control (18S). Relative fold expression for each target was calculated as ($2^{(-\Delta\Delta Ct)}$) in comparison to a reference sample.

2.10 Western Blotting

In order to quantify the expression of a target protein Western Blotting was performed. This involves several steps; protein extraction, quantification and solubilisation, separation on the basis of molecular weight by electrophoresis, transfer onto a membrane, blocking, probing with primary and secondary antibodies, and detection.

2.10.1 Whole cell protein extraction

The cells of interest were washed thrice with DPBS and placed on ice. The protein was then harvested by scraping with a cell scraper in the presence of RIPA buffer (Sigma-AldrichÈ), supplemented with HaltÊ protease and phosphatase inhibitor cocktail (Thermo, UK). The lysed cell suspension was collected by pipette and placed into a labelled Eppendorf and centrifuged at 14,000rpm for 20 minutes at 4ęC. The protein suspended in the liquid phase was aspirated and stored at -80°C.

2.10.2 Membrane expressed protein of interest extraction (Non-Denatured)

The ALX/FPR2 receptor is a cell membrane associated protein. In order to quantify its expression in bronchial epithelial cells we modified our protein extraction protocol to improve the yield of membrane associated proteins. The cells of interest were washed thrice with DPBS and placed on ice. Protein was harvested by scraping in the presence of RIPA buffer (Sigma, UK), supplemented with HaltÊ protease and phosphatase inhibitor cocktail (Thermo, UK) as above with the addition of an extra detergent N- lauroylasacrcosine sodium salt (Sigma-Aldrich®, USA). The cell lysate and remaining cell fragments were aspirated, vortexed and placed on ice for 30 minutes. Furthermore, the lysate was then subjected to sonication at 50% amplitude for 20 sec pulses (Sonics-Vibra Cell™). Lammelli buffer (Sigma-Aldrich®, USA) was added in a 1:1 ratio to the protein lysate prior to storage at 4^0C.

2.10.3 Bradford assay

Determination of the protein concentration of the cell lysate was performed by Bradford assay (386) using a Pierce BCA Protein Assay Kit (Thermo Scientific, USA) according to the manufacturerõs instructions.

Briefly, the prepared Bradford Reagent was gently mixed in the bottle and brought to room temperature. Bovine Serum Albumin was used to prepare protein standards by dilution in DPBS. 10µl of each protein standard sample (known concentration in the range 0.1 ï 1.4mg/ml) or sample of unknown concentration was placed in duplicate per well of a 96 well plate. 10µl of DPBS buffer was added to the blank wells. 200µl of Bradford Reagent mixture was added to each well, gently agitated on a plate shaker for 60 seconds and the placed in an incubator at 37^0C for 30 min. The plate was removed from the incubator and left to cool before the absorbance was measured at 595nm on the plate reader (Synergy Mx, Biotek® Instruments, Highland Park, Winooski, VT). The unknown protein concentrations were determined by plotting a standard curve of the net absorbance versus protein concentration of the standards and interpolating the unknown values.

2.10.4 Protein denaturation

Protein (30µg per well) was solubilised in Lamelli Buffer (Sigma-Aldrich®, USA) and where indicated, denatured at 95°C for 5mins (Stuart, Block heater, SBH130DC, UK).

2.10.5 Preparation of a Tris-glycine SDS-polyacrylamide gel

The gel is made by polymerising acrylamide monomers into a network of cross linked chains giving a pore size which can be customised to suit the size of the molecules being separated (387). Sodium Dodecyl Sulphate (SDS) is used as an anionic detergent used to linearize proteins and impart a negative charge to the protein. Following the application of an electric field, the mobility of a protein then depends on its length and mass to charge ratio. The gel has two components, a stacking gel and a resolving gel. Reagents used in the preparation of a gel are described in Table 2-4.

Table 2-4 Preparation of reagents for a Tris-glycine SDS-polyacrylamide gel

Reagents	
30% Acrylamide	(Sigma-Aldrich®)
1.5M Tris (pH 8.8)	Dissolve 181.71g Trizma base (Sigma®) in 800mls distilled water. Adjust pH to 8.8 with 1mol/l HCl and then make up to 1 litre (Stored at 4ęC).
0.5M Tris (pH 6.8)	Dissolve 60.57g Trizma base ((Sigma®) in 800mls distilled water. Adjust pH to 6.8 with 1mol/l HCl and then make up to 1 litre (Stored at 4ęC).
10% Sodium Dodecyl Sulfate (SDS)	10g SDS (Sigma Aldrich®) is dissolved in 100mls of distilled water.
10% Ammonium persulphate (10% (w/v) APS)	1g ammonium persulfate (Sigma-Aldrich®) is dissolved in 10 mL of H_2O (stored at -200C).
Tetramethylethylenediamine (TEMED)	(Sigma Aldrich®)

Gel reagents were assembled in the quantities indicated in Table 2-5. The TEMED is added last to initiate polymerisation. The glass plates are assembled in a BioRad® rig with the resolving gel being introduced into the space between the glass plates prior to setting. The gel is covered with a thin film of water to prevent the gel drying out and allowed to set for 20 ï 45 minutes using the excess gel in the tube to gauge when the gel has set. The layer of water is then removed and the stacking gel is prepared according to the composition indicated in Table 2-5. The stacking gel is introduced on top of the set resolving gel, up to the level of the front glass plate and a comb is introduced into the rig to form the wells.

Table 2-5 Quantities of reagents used for the preparation of resolving and Stacking Tris-glycine SDS-polyacrylamide gels.

	10% Resolving Gel	12% Resolving Gel	5% Stacking Gel
ddH2O	2.7 ml	2.3 ml	1.4 ml
30% acrylamide	2.3 ml	2.8 ml	330 µl
Tris	1.8 ml 1.5M Tris (pH8.8)	1.75 ml 1.5M Tris (pH8.8)	250 µl 0.5M Tris (pH6.8)
10% SDS	70 µl	70 µl	20 µl
10% APS	70 µl	70 µl	20 µl
TEMED	2.8 µl	2.8 µl	2 µl

2.10.6 Electrophoresis

The gasket (BioRad®) was assembled for electrophoresis and placed in a tank ensuring a good seal between the gasket and the gels. The wells were rinsed with running buffer (prepared as indicated in Table 2-6) and the remaining running buffer was poured into the gasket / tank. Solubilised protein was loaded into the wells of the gel alongside a SpectraÊ Multicolour Broad Range Protein Ladder (Thermo-Scientific, MA) used to identify the molecular weight of a protein revealed in a specific band. The gel was run at 80mV (BioRad PowerPac™) for 20 minutes to allow the protein to stack down into the resolving gel and then increased to 100mV. The gel was run until the loading dye front fully migrated through the gel.

2.10.7 Protein Transfer

Protein is transferred from the gel onto a PVDF membrane (Thermo Scientific, USA) in the following manner. Transfer buffer is prepared with the composition described in Table 2-6 and cooled to 4°C. The PVDF membrane is soaked in methanol. The gel containing the separated protein is removed from the electrophoresis gasket and assembled into the transfer cassette (BioRad®) in contact with the PVDF membrane, sandwiched between sponges and filter paper. The cassette is closed taking care to remove bubbles and compact it completely, and placed into a transfer rig. The tank is filled with transfer buffer (with an ice pack to cool the buffer) and protein is transferred at 100mV for 1 hour.

Table 2-6 Composition of running and transfer buffers.

	Running Buffer Quantity	Transfer Buffer Quantity
Glycine (Sigma®)	14g	14.4g
Trizma base (Sigma®)	3g	3.03g
SDS (Sodium Dodecyl Sulphate) (Sigma Aldrich®)	1g	-
Methanol	-	200mls
Distilled Water	1 litre	800mls

2.10.8 Membrane Blocking & Antibody Incubation

The specific conditions for membrane blocking are described (Table 2-7) for each Western Blotting experiment. Primary and secondary antibody pairs, incubation conditions and concentrations were optimised for each experiment.

Table 2-7 Antibodies and conditions used in Western blotting experiments

Antibodies	Primary	Secondary	Conditions
Anti- 15 Lipoxygenase 1	ab80221 (abcam) Rabbit Polyclonal IgG 1:1000 1hour at RT	ab97051 (abcam) Goat poly-clonal Anti-Rabbit IgG Antibody 1:6000 1 hour at RT	Blocked and probed in BSA
Anti- 15 Lipoxygenase 2	ab23691 (abcam) Rabbit Polyclonal IgG 1:625 1hour at RT	ab97051 (abcam) Goat poly-clonal Anti-Rabbit IgG Antibody 1:10,000 1 hour at RT	Blocked and probed in BSA
IĬB	Mouse monoclonal anti-IĬBŬ antibody (gift from Dr Sarah Doyle's lab) 1:2500 (5% Milk/0.1% P-BST) Overnight at 4°C	Anti-Mouse IgG rabbit polyclonal HRP linked secondary antibody (Abcam, Cambridge, MA) 1:5000 (5% Milk/0.1% P-BST) 1 hour RT	Protein denatured Blocked in 5% Milk/0.1% P-BST 1 hour RT
ALX/FPR2	Rabbit polyclonal anti-FPRL1 antibody (ab63023, Abcam, Cambridge, MA) 1:2000 (2% Milk/0.1% P-BST) Overnight at 4°C	Anti-rabbit IgG HRP linked secondary antibody (Cell Signalling, USA) 1:2500 (5% Milk/0.1% P-BST) 1 hour RT	Non-denatured protein Blocked in 5% Milk/0.1% P-BST 1 hour RT
Ŭ-Tubulin	Mouse monoclonal Anti-alpha Tubulin antibody (Abcam, Cambridge, MA) 1:5000 (5% Milk/0.1% P-BST) 1 hour RT	Anti-Mouse IgG rabbit polyclonal HRP linked antibody (Abcam, Cambridge, MA) 1:5000 (5% Milk/0.1% P-BST) 1 hour RT	

Following antibody exposures the membranes were washed for 15 minutes 3-4 times with PBS-0.1% Tween on a rocker. In order to reprobe for loading control proteins, membranes were stripped using Restore™ PLUS Western Blot Stripping Buffer (Thermo-Scientific, MA), re-blocked, and exposed to primary & secondary antibody pairs for the appropriate loading control protein.

2.10.9 Detection by Enhanced Chemi-luminescence

ECL substrate Super Signal West Dura chemi-luminescent (Thermo, USA) was used to develop the blots. The substrate was prepared according to the manufacturer's instructions and placed in a 50ml tube with the membrane for 5 minutes. The membrane was then placed between two acetate sheets in a cassette and closed. In the darkroom, the membrane was exposed to X-ray film to detect luminescence and the X-ray film was developed and fixed.

2.10.10 Band densitometry

Band densitometry was performed using Image J (NIH, USA) and results were expressed as Relative Band Intensities following normalisation by the loading control band.

2.11 Confocal Fluorescence Microscopy

Confocal microscopy is an imaging technique that is used to achieve high optical image resolution and gather 3-dimensional information. The technique involves point scanning of the laser excitation followed by a spatially filtered fluorescence emission signal emitted back from the focal point onto the confocal point. The image is constructed in a point-wise manner with the illuminating laser scanning rapidly from point to point one a single focal plane synchronised with the aperture. The scan is then repeated for multiple depth-wise focal planes to generate a 3-dimensional image. The spatial filtering is the key principle in enhancing the depth wise optical resolution by optical slicing (illustrated in Figure 2-5) (388).

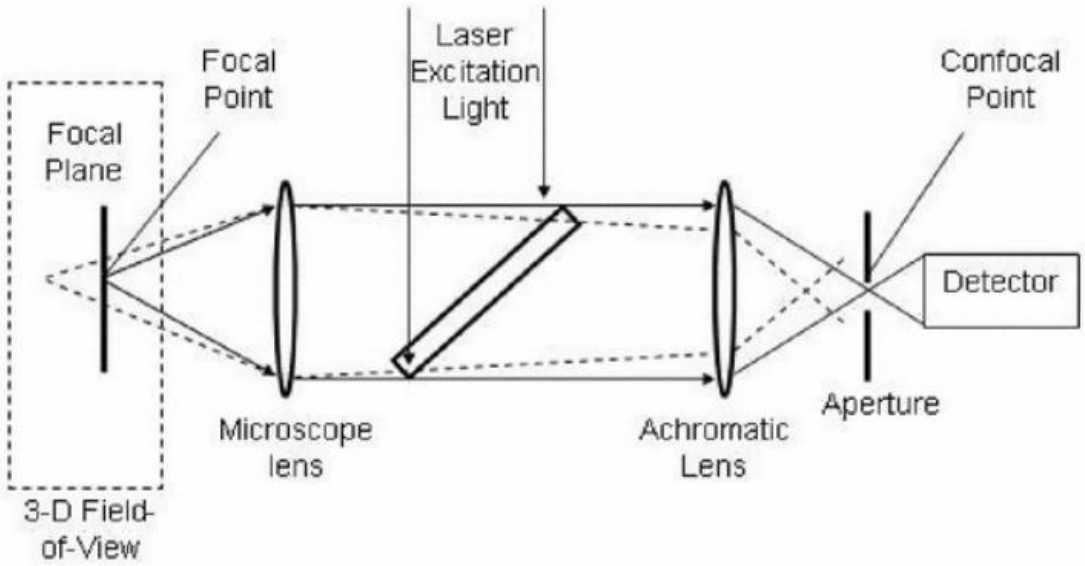

Figure 2-5 The principle of confocal microscopy using a pinhole aperture as a spatial filter

Confocal microscopy involves point scanning of the laser excitation. The pinhole aperture at the confocal point allows only the emitted fluorescent signal from the specific focal point and plane to pass through to the detector (solid lines), and filters out light emitted from outside the focal point (dashed lines) (reproduced from (388).

Furthermore, fluorescent proteins discovered in marine organisms led to the development of exciting biological imaging techniques (389). In fluorescence microscopy, electrons within fluorescent molecules are excited to a higher energy state by light of a certain wavelength within an excitation spectrum. They quickly lose this energy and drop back down to their original energy level with the emission of light with a characteristically longer wavelength in a phenomenon known as the Stokes shift (390).

2.11.1 ASL Height Measurement by Live-cell Confocal Fluorescence Microscopy

Airway surface liquid (ASL) height measurements were performed as previously published by Verriere et al., based on the method of Tarran et al. (85, 391). Cell monolayer cultures used for ASL measurement had obtained a minimum TEER of 800q/cm2. 24 hours prior to imaging, 8Õ Texas redÈ- dextran (10,000MW, Invitrogen, Auckland, NZ) at a concentration of 2mg/ml was applied to the centre of the insert. 0.7mls of Fluorinet^{TM} electronic fluid Perfluorocarbon-72 (FC-72, 3M,

St. Paul, USA) was added to the apical compartment of the insert. Perfluorocarbon-72 is immiscible with the ASL and was used to prevent ASL evaporation during the confocal scanning experiments (85).

The ASL image was captured with a Zeiss LSM 510 Meta using a 40X objective and constructed in a point-wise manner. The illuminating laser (555nm) scanned rapidly from point to point on a single focal plane (XY) measuring 160µm x 160µm, serially repeated over 30µm at depth intervals (Z-plane) of 1µm to generate a 3-dimensional image. Images were captured from 3 randomly selected fields on each culture insert after screening the insert for homogeneity.

2.11.2 ASL Image Analysis

Images were analysed using Zeiss LSM Image Browser (Carl Zeiss Micro-Imaging GmbH, Germany). Measurements were taken of the ASL height in the Z plane at nine equally spaced, pre-specified points (defined by their XY co-ordinates) from each image. Each biological repeat represents the mean of 27 height measurements per culture insert (nine measurements from each of three fields).

2.12 Statistics

Graphpad prism® was used to perform statistical analyses. The specific statistical tests used are described with each result. Results are presented as mean and standard error of the mean (SEM) unless specifically stated otherwise. When we wished to evaluate the distribution of biological data the standard deviation (SD) was calculated. Fisher's exact test was used for categorical variables. Student t-test was used to compare pairs of continuous variables. One-way ANOVA was used to compare the means of three or more unmatched groups of continuous variables. Newman-Keuls was used as a Post-test to compare all pairs of means following a one-way ANOVA. Two-way ANOVA was used to determine how a variable was affected by two factors. Following two-way ANOVA, the Bonferroni method was used as a Post-test, incorporating correction for multiple comparisons. Correlations were analysed using the Spearman test. Results were reported as statistically significant with P value <0.05*, <0.01**, <0.001***.

CHAPTER 3

THE ROLE OF RESOLVIN D1 IN CYSTIC FIBROSIS AIRWAY DISEASE

3 The role of Resolvin D1 in Cystic Fibrosis airway disease

3.1 Introduction

There are unmet needs in CF therapy for treatments; to rehydrate the airway, improve muco-ciliary clearance, strengthen the host immune response, prevent neutrophil mediated inflammatory tissue destruction, and augment the effectiveness of existing antibiotics.

3.1.1 ASL Regulation

Normal mucus clearance from the airways relies on adequate hydration of the Airway Surface Liquid (ASL) (Illustrated in Figure 3-1). This is achieved via balanced sodium absorption, mediated by the Epithelial Sodium Channel (ENaC) in the apical membrane and the Na/K ATPase localised in the basolateral membrane, and chloride secretion, mediated by CFTR and CaCC chloride channels in the apical membrane and the Na/K/2Cl co-transporter in the basolateral membrane (76). Water follows passively via aquaporins in the cell membrane and the paracellular route. ASL is composed of a periciliary fluid layer (PCL) and a mucus layer riding on top of the PCL. The normal balance between Na absorption and Cl^- secretion maintains a PCL height of 7-10mm which corresponds to the height of outstretched cilia. A PCL height less than 6 mm results in compression of the cilia by the mucus layer and disrupted muco-ciliary transport. Thus the regulation of ASL height by trans-epithelial ion and water transport within a very narrow range of a few microns is critical for effective muco-ciliary clearance (73).

Trans-epithelial ion transport and ASL hydration are regulated by the neuro-humoral environment (e.g. nucleotides (39, 93), steroid hormones (94, 95), Nitric oxide (96), Acetylcholine (97)). Dynamic stretch, viral infection and inflammation, for example, affect the ASL by increasing the ASL concentration of nucleotides, which in turn regulate ion transport processes to maintain a functional ASL height (76, 98, 99). In CF, the ASL is dehydrated due to the loss of chloride secretion through CFTR and the sodium hyper-absorption which has been postulated to be consequent to the loss of ENaC down-regulation by CFTR (67).

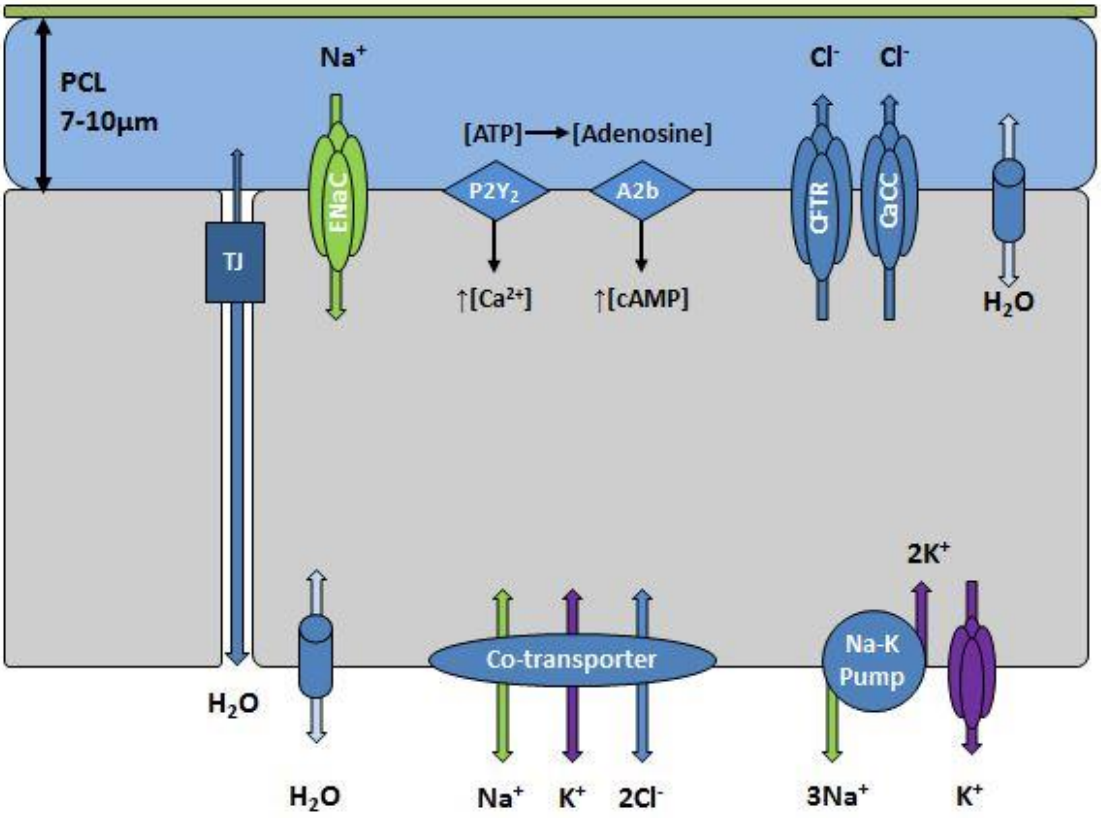

Figure 3-1 Schematic illustrating the regulation of Airway Surface Liquid (ASL) height

Adequate hydration of the PCL is achieved via balanced trans-epithelial sodium absorption and chloride secretion. Sodium absorption is mediated by apical Epithelial Sodium Channels (ENaC) and basolateral Na/K ATPase pump. Chloride secretion is mediated by apical CFTR and CaCC chloride channels and the basolateral Na/K/2Cl co-transporter. Water follows passively via aquaporins and the paracellular route. Apical Purino-receptors ($P2Y_2$ and A2b) regulate trans-epithelial ion transport via calcium and cAMP and subsequently affect ASL hydration.

3.1.2 Inflammation

Excessive NF-κB activation has been observed in CF (65, 188, 189) and variously attributed to; persistent bacterial infection and pro-inflammatory cytokine stimulation (191); Intrinsic imbalances between pro and anti-inflammatory cytokine profiles in CF (231); ER stress due to the accumulation of misfolded CFTR protein (196); and, ER calcium store expansion and amplification of the calcium dependent inflammatory response (192, 193). Degradation of I-κB in the cytosol

releases NFHB and permits its translocation to the nucleus where NFHB binds to its site in the promoter region of the IL-8 gene, and rapidly up-regulates synthesis of the pro-inflammatory cytokine, IL8 (187). IL8 is a potent neutrophil chemo-attractant and is secreted by both tissue resident cells and recruited immune effector cells. Higher IL8 has been associated with worse lung function in CF (190). Tumour necrosis factor Ŭ (TNFŬ) is a potent activator of the pro-inflammatory nuclear transcription factor NFHB and its synthesis is induced by LPS acting through Toll-like receptor 4 (392). It is secreted by mononuclear phagocytes, natural killer cells, endothelial cells, mast cells, activated lymphocytes, and neutrophils. TNFŬ concentration was inversely correlated to FEV_1 in CF (214).

3.1.3 Phagocytosis and bacterial killing in CF

Macrophage phagocytosis plays an important role in bacterial containment and the resolution of acute inflammation by engulfing bacteria and apoptotic neutrophils. In CF, impairments in alveolar macrophage-mediated phagocytosis and bacterial killing are described (125-127, 228).

3.1.4 Resolvin D1

Resolvin D1 (RvD1) is an endogenously produced ñSpecialised Pro-Resolutionò lipid mediator involved in the ñactive resolutionò phase of inflammation (262). The biosynthesis of RvD1 from DHA involves two sequential lipoxygenation steps; 15 Lipoxygenase catalyses the conversion of DHA to 17R-HpDHA, and then 15 Lipoxygenase generates a peroxide intermediate which is enzymatically hydrolysed to RvD1 (288). RvD1 stops neutrophil infiltration in both mouse models, and a model of fMLP stimulated trans-endothelial migration, and enhances macrophage phagocytosis of apoptotic neutrophils (288, 293). RvD1 was reported to attenuate NFHB activation in a mouse model of lung injury (337). In a mouse model of cigarette smoke exposure RvD1 promoted differentiation of alternatively activated (M2) macrophages and stimulated neutrophil efferocytosis (336). Using a b arrestin ligand reporter system, RvD1 was revealed to signal through the GPR32 orphan receptor and ALX/FPR2 receptor (294, 295). Resolvins are rapidly inactivated by eicosanoid oxidoreductases (284, 293).

3.1.5 SPMâs in ASL Regulation

Our group previously demonstrated that the related SPM - LXA_4 restores ASL height in CF bronchial epithelial cell models via stimulation of the ALX/FPR2 receptor (85). The LXA_4 mediated ASL rehydration is achieved by; apical ATP secretion via Pannexin-1 channels, and activation of P2RY11 purinoreceptors, leading to calcium activated chloride secretion (393), and; inhibition of airway epithelial sodium absorption through ENaC (356).

3.1.6 ALX/FPR2 Receptor

ALX/FPR2 conveys the pro-resolving properties of LXA_4 and RvD1 (268). ALX/FPR2 is expressed in several types of cells including human bronchial epithelial cells and bronchial biopsy sections (323). It is noteworthy that LXA_4 enhances the activity of the ALX/FPR2 promoter, and enhances the apical membrane localisation of ALX/FPR2 in CF cells (327, 356).

3.2 Aims & Hypotheses

We aimed to investigate the potential of RvD1 as a candidate therapeutic for CF airways disease. We set out to investigate the impact of RvD1 upon three key pathologic aspects of CF airways disease; airway surface liquid hydration; NFHB mediated inflammation, and; macrophage phagocytosis - key to neutrophil efferocytosis and bacterial clearance. We investigated the following hypotheses;

- RvD1 induces an airway surface liquid (ASL) height increase in normal and CF airway epithelia via ALX/FPR2 receptor stimulation
- RvD1 attenuates NFHB activation and agonist stimulated IL8 secretion by CF airways epithelial cells
- RvD1 restores phagocytic and bactericidal capacity to CF alveolar macrophages

3.3 Results

3.3.1 Resolvin D1 restores ASL height in CF bronchial epithelial cell line CuFi-1

In order to test the hypothesis that RvD1 induces an ASL height increase in CF airway epithelia, we cultured CuFi-1 bronchial epithelial cells as polarised, differentiated epithelial layers and stained the ASL with Texas red®-dextran. Using live cell confocal microscopy we visualised the ASL and then measured the height of ASL on CuFi-1 cell preparations using Zeiss LSM Image Browser (Carl Zeiss Micro-Imaging GmbH, Germany).

**Dr Gerard Higgins taught me confocal microscopy. Master's student Ahmad Moukachar shadowed me in the lab during the initial phase of these ASL experiments and in that capacity had hands on experience sitting side by side with me doing the cell culture, treatments and capturing the confocal microscopy images. Ahmad made a parallel set of measurements on the early ASL images. The data I present here are my own independent measurements.*

The mean height of ASL on CuFi-1 cells under non-stimulated (NS) conditions was low (5.7 ± 0.3µm) (n=8 inserts) (Figure 3-2A). The three dimensional structure of the ASL visualised upon vehicle control treated CuFi-1 preparations had a patchy, discontinuous appearance (Figure 3-2B). 30 minutes basolateral exposure to 1nM RvD1 increased the ASL height on CuFi-1 cells (7.1 ± 0.2µm vs. 5.7 ± 0.3µm, $P<0.01$) (n=6 inserts) (Figure 3-2A). Treatment with a higher concentration of RvD1 (100nM) also resulted in an increase in ASL height compared to vehicle control conditions (8.3 ± 0.3µm vs. 5.7 ± 0.3µm, $P<0.001$) (n=5 inserts) (Figure 3-2A). After exposure to RvD1 (100nM) the ASL was fuller, with greater continuity (Figure 3-2B).

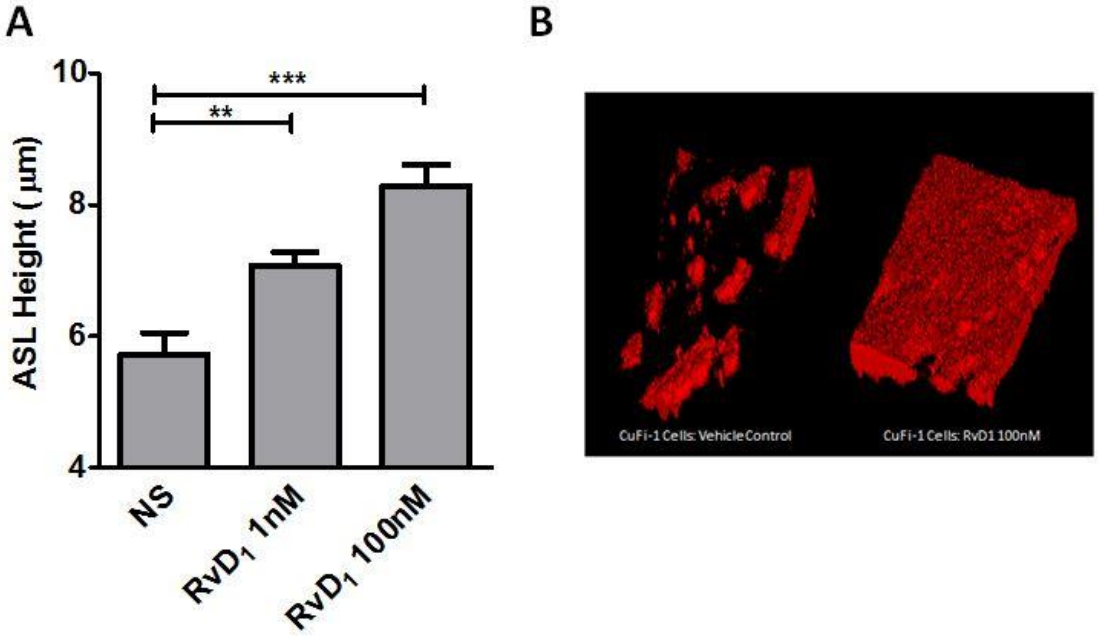

Figure 3-2 Resolvin D1 (RvD1) restores airway surface liquid (ASL) height in CuFi-1 differentiated bronchial epithelial cells

ASL height in µm was measured by live cell confocal fluorescence microscopy using Texas red®-dextran to stain the ASL. (A) Differentiated CuFi-1 bronchial epithelial cell preparations were stimulated with either vehicle control (NS), RvD1 1nM or 100nM 30 minutes prior to image acquisition. Untreated CuFi-1 cells (NS) demonstrated reduced ASL height at 5.7µm and disrupted architecture. RvD1 1nM and 100nM restored ASL height on CuFi-1 cells to 7.1µm** and 8.3µm*** respectively. (B) Representative images of the 3 Dimensional structure of the ASL on CuFi-1 differentiated bronchial epithelial cells treated for 30mins with vehicle control (Left of panel) and RvD1 100nM (Right of panel). Under control conditions the ASL is low in volume, patchy and disrupted. Following treatment with RvD1 the ASL height and volume were significantly increased, and the continuity was restored. (Results are presented as Mean ± SEM, *P<0.05/**P<0.01/***P<0.001, n= 5-8 inserts, Student t test). **Ahmad Moukachar provided technical assistance. Data presented are my own independent measurements.*

3.3.2 Resolvin D1 restores ASL height upon CuFi-1 bronchial epithelial cells via ALX/FPR2 receptor stimulation and intracellular calcium mobilisation

We hypothesised that RvD1 induces an ASL height increase via ALX/FPR2 receptor stimulation. To investigate the role of ALX/FPR2 in the elevation of ASL height observed in CuFi-1 cells after RvD1 treatment (1nM) we used Boc2 as a pharmacological inhibitor of the ALX/FPR2 receptor (323).

Boc2 treatment alone did not significantly alter ASL height in CuFi-1 cells (5.9 ± 0.6µm vs. 5.7 ± 0.3µm under NS conditions) (n=6 inserts) (Figure 3-3A). Pre-treatment of CuFi-1 cells with Boc2 completely prevented the elevation in ASL height seen with 1nM RvD1 alone (5.7 ± 0.5µm; RvD1 (1nM) + Boc2 vs. 7.1 ± 0.2µm; RvD1 (1nM), $P<0.05$) (n=7 inserts) (Figure 3-3A).

Two pathways for chloride secretion are commonly recognised in the airways; cAMP-dependent (CFTR mediated) and calcium dependent secretory pathways (CaCC) (43). Sodium absorption in the airways is mediated by the amiloride-sensitive epithelial Na+ Channel (ENaC). ENaC has been reported to be inhibited by intracellular calcium (394-396).

In order to investigate the role played by intracellular calcium in the elevation in ASL height induced by RvD1 in CuFi-1 cells, we used BAPTA-AM to chelate intracellular calcium (Figure 3-3B). A non-significant reduction in ASL height compared to vehicle control was recorded following BAPTA-AM treatment (5.2 ± 0.4µm vs. 5.7 ± 0.3µm; NS, P=NS) (n=4 inserts) (Figure 3-3B). The RvD1 mediated increase in ASL height was completely prevented by co-administration of the intracellular calcium chelator BAPTA-AM (4.9 ± 0.2µm; RvD1 + BAPTA vs. 7.1 ± 0.2µm; RvD1, $P<0.001$) (n=5 inserts) (Figure 3-3B).

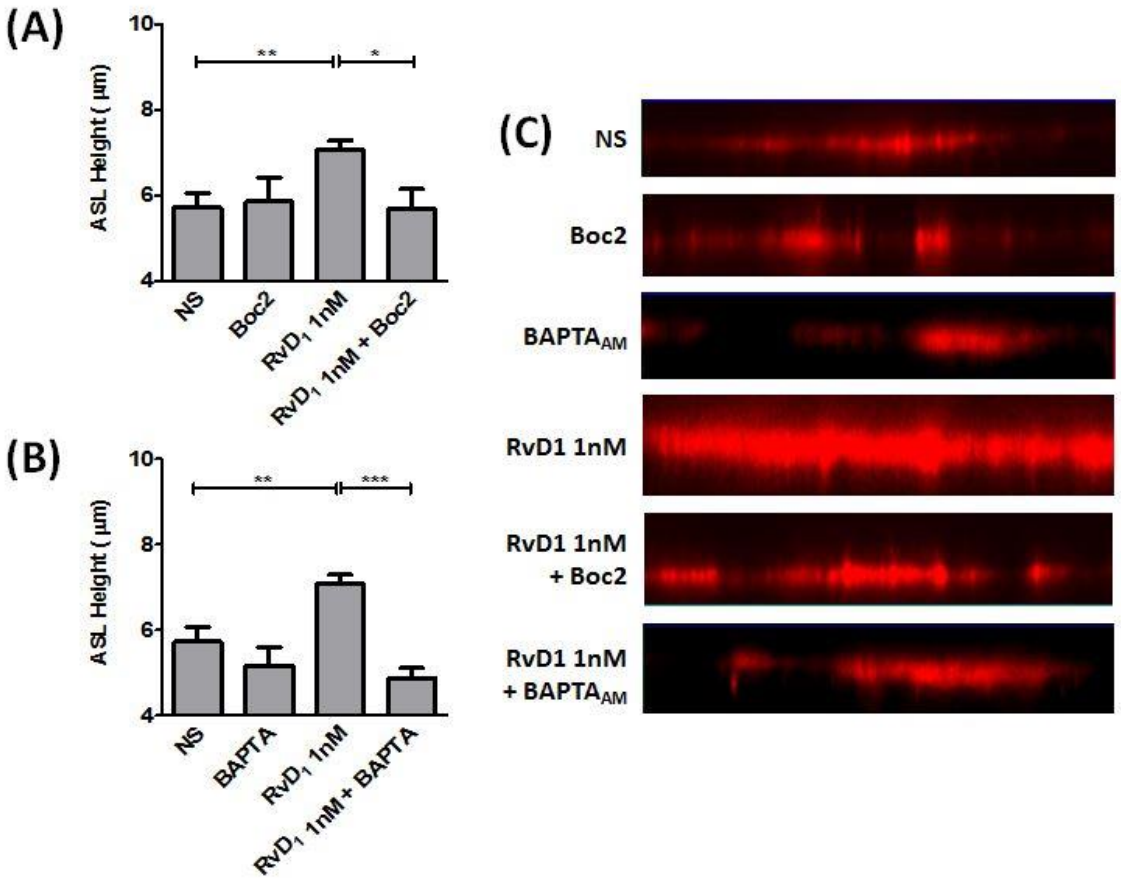

Figure 3-3 Resolvin D1 (RvD1) restores airway surface liquid (ASL) height in CuFi-1 differentiated bronchial epithelial cells via ALX/FPR2 receptor stimulation and intracellular calcium mobilisation

(A) CuFi-1 differentiated bronchial epithelial cells were treated with either vehicle control (NS) or with Boc-2 (10µM) (Specific ALX/FPR2 receptor antagonist) alone, or as a 20 minute pre-treatment, followed by RvD1 (1nM) for 30mins. The RvD1 associated increase in ASL height was completely abolished by the ALX/FPR2 receptor antagonist Boc2. (B) CuFi-1 differentiated bronchial epithelial cells were treated with either vehicle control (NS) or with BAPTA-AM (10µM) (intracellular calcium chelator) alone, or as a 20 minute pre-treatment, followed by RvD1 (1nM) for 30mins. The RvD1 associated increase in ASL height was completely abolished by the intracellular calcium chelation using BAPTA-AM***. (C) Representative images are shown. (Results are presented as Mean ± SEM, *P<0.05/**P<0.01/***P<0.001, n= 5-8 inserts, Student t test). **Ahmad Moukachar provided technical assistance for the Boc2 experiments. Data presented are my own independent measurements.*

3.3.3 Resolvin D1 restores ASL height in polarised, differentiated Primary CF Bronchial Epithelial Cells

In order to corroborate that RvD1 increases ASL height in CF epithelial cells as demonstrated in CuFi-1 cell lines, we cultured Primary CF bronchial epithelia (CFBE), obtained from children with CF by bronchial brushing, as polarised, differentiated epithelial layers at air-liquid interface.

**Primary bronchial epithelial cells were obtained via the SHIELD CF Study set up by Drs Paul McNally and Barry Linnane. Myself, Paul McNally, Des Cox, Ahmad Zaid, Sheila Javadpour, Peter Greally and Basil Elnazir performed bronchoscopy for the SHIELD CF study. Dr Gerard Higgins taught me primary cell culture and Lab technician, Coral Fustero provided technical assistance in primary CF bronchial epithelial cell culture for these experiments.*

Under vehicle control conditions (NS), the ASL height in CFBEs was low (3.7±0.2µm) (n=6 inserts) (Figure 3-4A). 30 minutes exposure to RvD1 (1nM) elevated ASL height on CFBE (6.8±0.3µm; RvD1, vs. 3.7±0.2µm; NS, $P<0.001$, n= 7 inserts). Bronchial Epithelium derived from 2 separate children homozygous for the Phe508del CFTR mutation (Figure 3-4A).

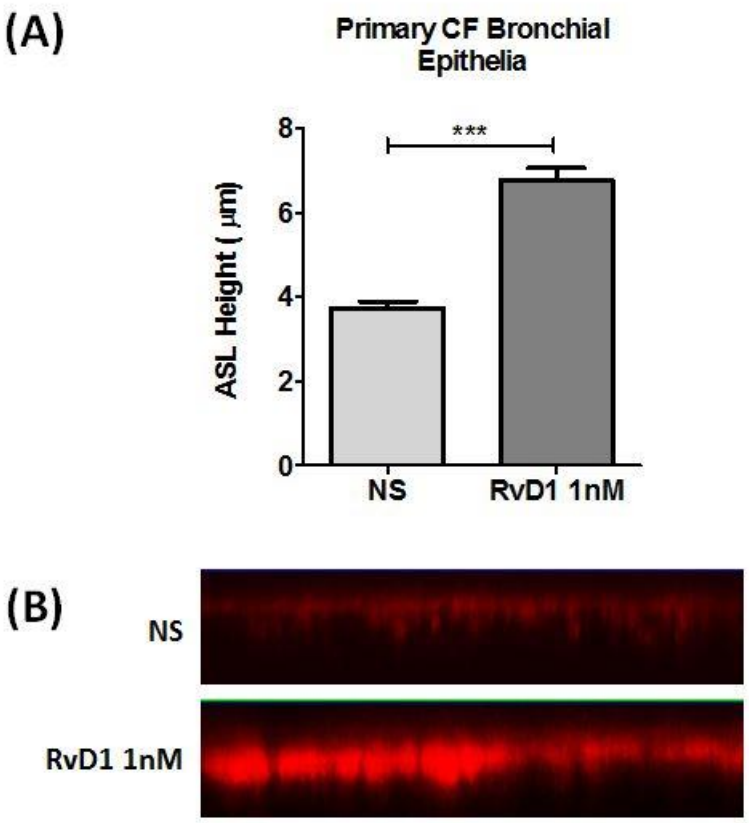

Figure 3-4 Resolvin D1 (RvD1) restores airway surface liquid (ASL) height in polarised, differentiated Primary CF Bronchial Epithelial Cells

ASL height in µm was measured by live cell confocal fluorescence microscopy using Texas red®-dextran to stain the ASL. Differentiated Primary CFBE preparations were stimulated with either vehicle control (NS), or RvD1 1nM 30 minutes prior to image acquisition. (A) Untreated CFBEõs (NS) demonstrated low ASL height at 3.7±0.2µm. RvD1 1nM increased ASL height on CFBE to 6.8±0.3µm***. (B) Representative images are shown. (Results are presented as Mean ± SEM, ***P<0.001, n= 6-7 inserts prepared from Bronchial Epithelium derived from 2 separate children with CF, Student t test). **Primary bronchial epithelial cells were obtained via the SHIELD CF Study. Dr Gerard Higgins taught me primary cell culture and Lab technician, Coral Fustero provided technical assistance in primary CF bronchial epithelial cell culture for these experiments. Data presented are my own independent measurements.*

3.3.4 Resolvin D1 does not affect net ASL in non-CF NuLi-1 cells, but restores ASL height loss associated with CFTR inhibition

We hypothesised that RvD1 would induce an ASL height increase in both normal and CF airway epithelia. In order to investigate the effect of RvD1 on ASL height in non-CF bronchial epithelial cells, we cultured NuLi-1 cells as polarised, differentiated bronchial epithelial cell layers. Again we used the same protocol to visualise the ASL and measure its height. NuLi-1 cell preparations were stimulated with either vehicle control or RvD1 (1 & 100nM). The ASL height upon vehicle control treated NuLi-1 cells measured 7.2 ± 0.3µm (n=11 inserts) (Figure 3-5B). This was significantly higher than the ASL measured under non-stimulated conditions in CuFi-1 cells (5.7 ± 0.3µm, P<0.01) (Figure 3-2A). ASL height did not change significantly in NuLi-1 cell preparations following RvD1 exposure in NuLi-1 cell preparations (7.8 ± 0.3µm; RvD1 (1nM, n=13 inserts) & 7.6 ± 0. 3µm; RvD1 (100nM, n=7 inserts), P=NS) (Figure 3-5B).

Three key ion transport pathways contribute to the hydration of the ASL in airways epithelia; cAMP-dependent chloride secretion via CFTR; calcium dependent chloride secretion via CaCC, and sodium absorption via ENaC. CFTR has been reported to regulate both CaCC (stimulation) and ENaC (inhibition) channel activity (*see section 1.2.3*). In order to further examine the effect of RvD1 on ASL height regulation in the setting of CFTR inhibition, we pharmacologically inhibited CFTR in NuLi-1 cells using CFTR inhibitor 172.

Selective CFTR inhibition resulted in a significant reduction in ASL height in NuLi-1 cell preparations (4.7 ± 0.3µm vs. 7.2 ± 0.3µm (NS), P<0.001) (n=6 inserts) (Figure 3-5B). RvD1 (100nM) treatment significantly elevated ASL height in CFTR inhibited NuLi-1 cells (6.7 ± 0.3µm; CFTR inh + RvD1 (100nM) vs. 4.7 ± 0.3µm; CFTR inh alone, P<0.001) (n=6 inserts) (Figure 3-5B).

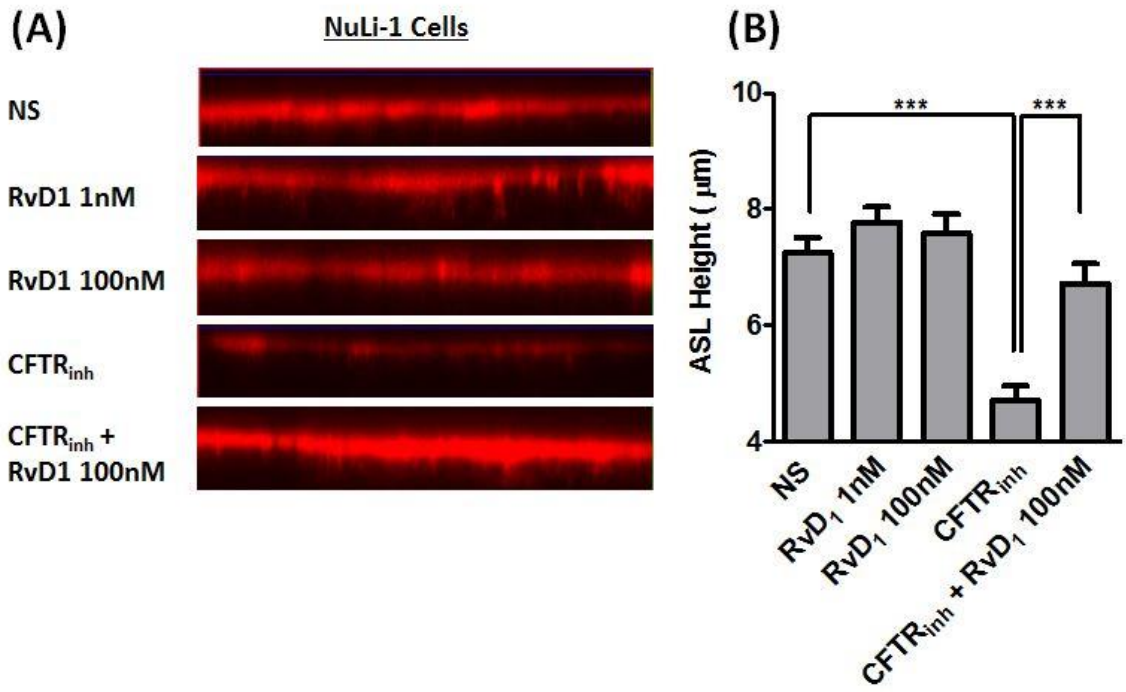

Figure 3-5 Resolvin D1 (RvD1) does not affect overall airway surface liquid (ASL) height but restores ASL height loss associated with CFTR inhibition in NuLi-1 differentiated bronchial epithelial cells

ASL height in µm was measured by live cell confocal fluorescence microscopy using Texas red®-dextran. Differentiated NuLi-1 bronchial epithelial cell preparations were stimulated with either vehicle control (NS), RvD1 (1nM), RvD1 (100nM) alone, CFTR inhibitor 172 (20µM) alone, or RvD1 (100nM) in combination with CFTR inhibitor 172 (20µM) 30 minutes prior to image acquisition. (A) Representative images are presented. (B) Mean ASL height in vehicle control treated preparations was 7.3µm. Mean ASL height did not change significantly when treated with 1nM RvD1 (7.8 µm) or 100nM RvD1 (7.6 µm). CFTR inhibition significantly reduced ASL height to 4.7µm***. Concurrent treatment of CFTR inhibitor treated preparations with RvD1 (100nM) resulted in significant increase of the ASL height to 6.7µm***. (Results are presented as Mean ± SEM, ***P<0.001, n=6-13 inserts, Student t test). **Ahmad Moukachar provided technical assistance. Data presented are my own independent measurements.*

3.3.5 Resolvin D1 attenuates TNFŬ induced IL8 secretion by CuFi-1 cells via preservation of IHB

We hypothesised that RvD1 attenuates NFHB activation and agonist stimulated IL8 secretion by CF airways epithelial cells. In order to investigate this hypothesis we treated CuFi-1 epithelial cells with TNFŬ alone (in order to stimulate IL8 secretion), or in combination with RvD1 (100nM). Under these conditions, we measured apical IL8 secretion by ELISA and quantified IHB expression by Western blotting (Figure 3-6).

Baseline IL8 secretion by CuFi-1 epithelial cells measured 169 ± 54ng/ml (Figure 3-6A). A significant induction of IL8 secretion was achieved by treatment with TNFŬ (80ng/ml) (546 Ñ 39ng/ml vs. 169 Ñ 54ng/ml, P<0.001) (Figure 3-6A). Treatment with RvD1 prevented the stimulation of IL8 secretion induced by TNFŬ in CuFi-1 cells (283 ± 44ng/ml vs. 546 ± 39ng/ml, P<0.01, n=6-7) (Figure 3-6A).

TNFŬ treatment of CuFi-1 cells resulted in a significant degree of IHB degradation (0.67 Ñ 0.06; TNFŬ vs. 1.07 ± 0.04; NS, P<0.01) (Figure 3-6 B&C). Co-treatment with RvD1 significantly prevented IHB degradation (0.93 Ñ 0.05; TNFŬ + RvD1 vs 0.67 Ñ 0.06; TNFŬ, P<0.05, n= 4-5, representative blots are presented) (Figure 3-6 B&C).

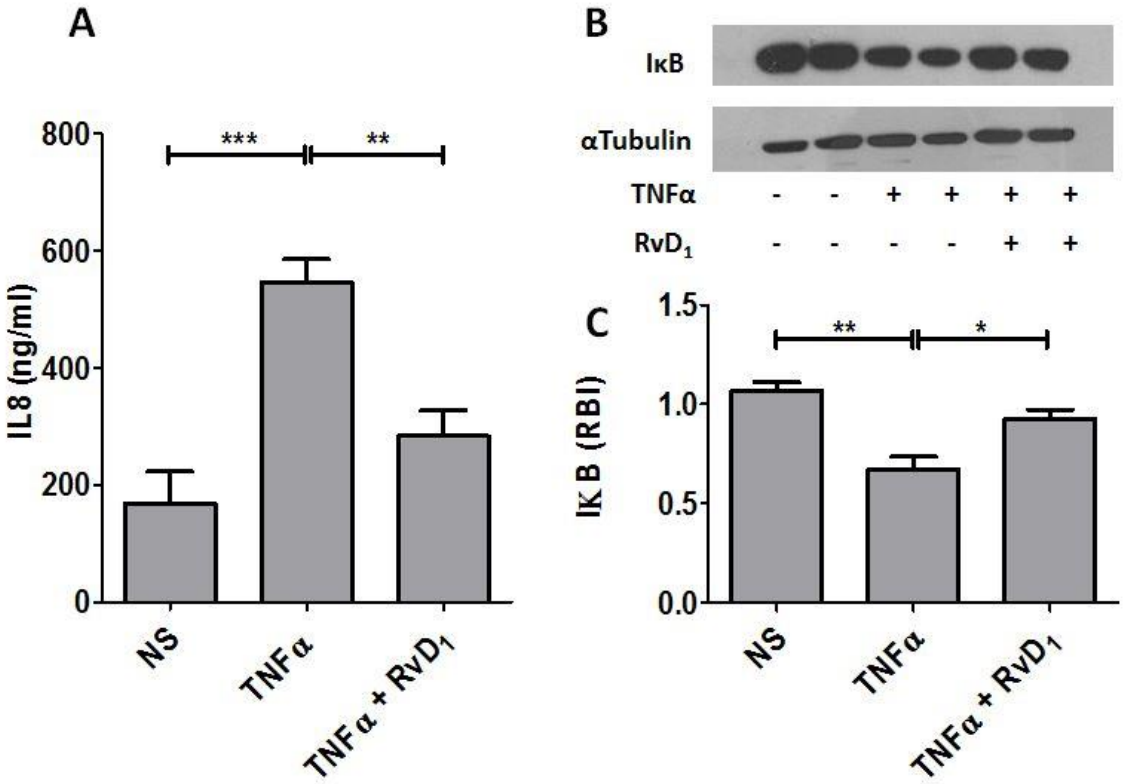

Figure 3-6 Resolvin D1 (RvD1) attenuates TNFŬ induced IL8 secretion by CuFi-1 cells via preservation of IκB

(A) CuFi-1 differentiated bronchial epithelia cells were exposed to vehicle control (NS) or TNFŬ (80ng/ml) for 24 hours to induce IL8 secretion, either alone or in combination with RvD1 (100nM). Apical IL8 concentration was measured by ELISA. Baseline IL8 secretion by CuFi-1 cells measured 169 ±54 ng/ml. Following stimulation with TNFŬ, IL8 secretion was significantly increased at 546*** ±39 ng/ml. Concurrent treatment with RvD1 (100nM) resulted in significant inhibition of TNFŬ induced IL8 secretion (283**Ñ 44 ng/ml). (B & C) CuFi-1 bronchial epithelial cells were grown in monolayers and exposed to either vehicle control (NS), TNFŬ (80ng/ml) alone, or in combination with RvD1 (100nM) for 24 hours. IκB expression was detected by western blotting and quantified by band densitometry using Ŭ-Tubulin as a loading control. Results are presented as relative band intensities (RBI). TNF-Ŭ treatment resulted in a significant degradation of IκB**, which was significantly prevented* by concurrent treatment with RvD1 100nM. (Results are presented as Mean ±SEM, *P<0.05, **P<0.01, ***P<0.001, n=6-7 inserts for IL8 experiments, n= 4-5 epithelial preparations for IκB, ANOVA). **IκB antibody obtained from Dr Sarah Doyle.*

3.3.6 Baseline ALX/FPR2 receptor protein expression does not differ between NuLi-1 and CuFi-1 cells

Using Flow Cytometry, Al-Alawi et al. observed higher surface expression of the ALX/FPR2 receptor in NuLi-1 cells as compared with CuFi-1 cells, associated with a reciprocally higher intracellular localisation of ALX/FPR2 in CuFi-1 cells as compared with NuLi-1 cells (356). Those authors also reported that in CuFi-1 cells, LXA_4 treatment resulted in a very rapid (5 minutes) recruitment of ALX/FPR2 to the apical membrane (356). In light of these reports, an important component of cell sensitivity to ALX/FPR2 ligands remained outstanding; does the total available pool of ALX/FPR2 receptor differ between Nuli-1 and CuFi-1 cell lines?

Using a different approach (Western blotting); we measured whole cell ALX/FPR2 receptor protein expression by both cell lines. There was no significant difference in whole cell ALX/FPR2 receptor expression between NuLi-1 and CuFi-1 cell lines (0.92 ± 0.18; NuLi-1 vs. 1.22 ± 0.45; CuFi-1, $P>0.05$) (Figure 3-7).

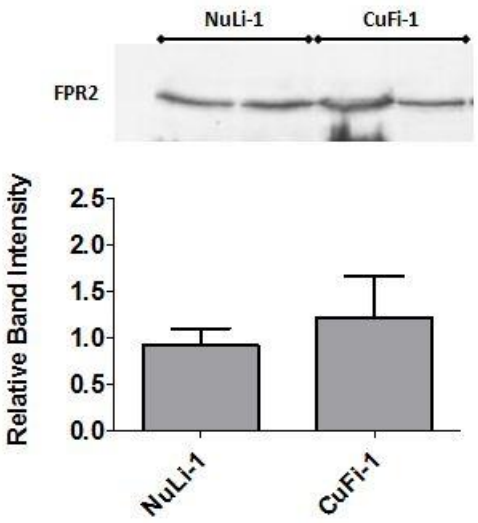

Figure 3-7 Baseline ALX/FPR2 receptor expression does not differ between NuLi-1 and CuFi-1 cells

NuLi-1 and CuFi-1 bronchial epithelia cells were grown in monolayers. ALX/FPR2 receptor expression was measured by Western Blotting using Ŭ-Tubulin as a loading control. Representative blots are shown above. Band densitometry was performed. The results were normalised by loading control and are presented as relative band intensities. No significant difference in baseline ALX/FPR2 receptor expression was observed between NuLi-1 and CuFi-1 cell lines (n=4 epithelial preparations quantified on at least 3 different occasions, Student t test).

3.3.7 Resolvin D1 does not affect whole cell ALX/FPR2 receptor protein expression in NuLi-1 or CuFi-1 cells

The ALX/FPR2 agonist LXA_4 is reported to enhance the activity of the ALX/FPR2 promoter (327). We investigated whether chronic exposure (24 hours) to RvD1 would result in alterations in ALX/FPR2 receptor expression. We found no significant effect of RvD1 (1nM or 100nM) on whole cell ALX/FPR2 receptor protein expression on NuLi-1 or CuFi-1 cells (P=NS) (Figure 3-8).

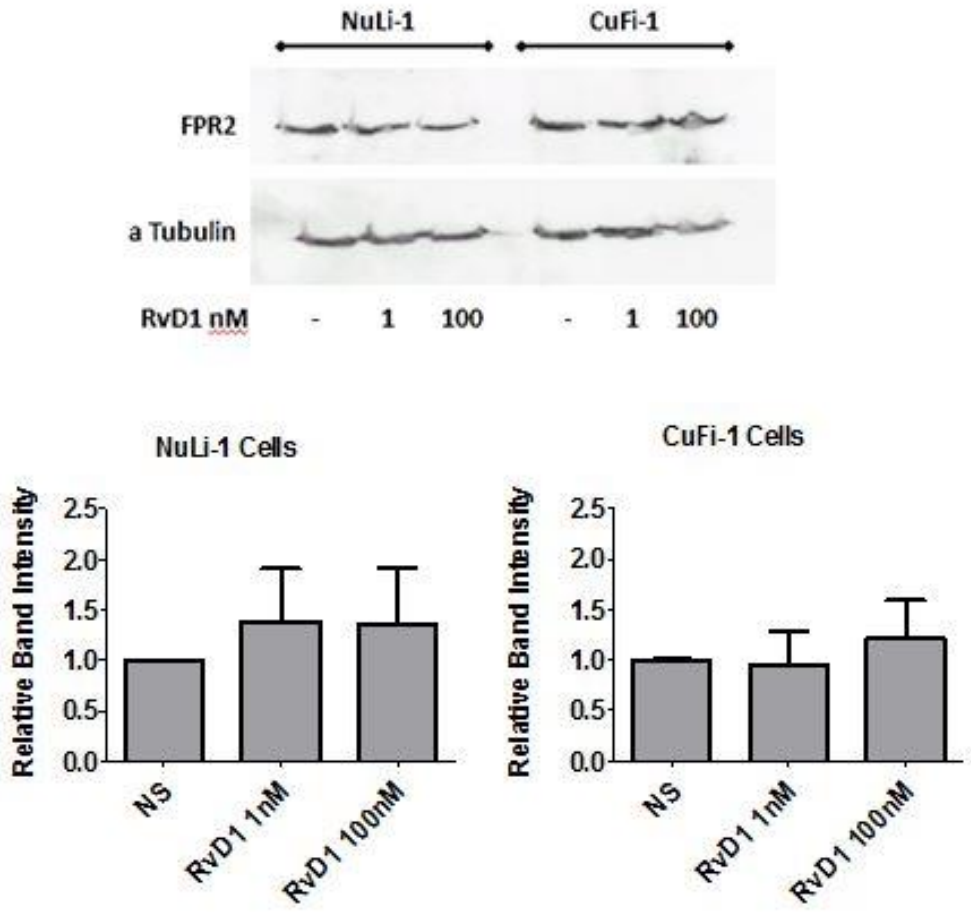

Figure 3-8 Resolvin D1 does not affect ALX/FPR2 receptor expression in NuLi-1 or CuFi-1 cells

NuLi-1 (left of panel) and CuFi-1 (right of panel) bronchial epithelia cells were grown in monolayers and exposed either to vehicle control (NS) or RvD1 (1 or 100nM) for 24 hours. ALX/FPR2 receptor expression was measured by Western Blotting using Ŭ-Tubulin as a loading control. Representative blots are shown above. Band densitometry was performed. The results were normalised by loading control and are presented as relative band intensities compared to the non-stimulated condition for the same cell line. No significant effect of RvD1 upon ALX/FPR2 receptor expression was observed in either cell line (n=3-4 epithelial preparations treated and quantified on at least 3 different occasions, ANOVA).

3.3.8 Resolvin D1 enhances the phagocytic capacity of Primary Alveolar Macrophages from children with CF

We hypothesised that RvD1would restore phagocytic capacity to CF alveolar macrophages. In order to investigate this hypothesis, we isolated alveolar macrophages from the bronchoalveolar lavage (BAL) of three female children under the age of 6 with CF. All three girls were homozygous for the Phe508del CFTR mutation. Isolated CF alveolar macrophages were treated with either vehicle control or RvD1 (100nM) and incubated with fluorescently labelled latex beads complexed with rabbit IgG. The fluorescence intensity from phagocytosed beads within alveolar macrophages was imaged, and separately quantified using a fluorescence plate reader.

**BAL fluid; the source material for primary alveolar macrophage isolation, was obtained via the SHIELD CF Study set up by Drs Paul McNally and Barry Linnane. Myself, Paul McNally, Des Cox, Ahmad Zaid, Sheila Javadpour, Peter Greally and Basil Elnazir performed bronchoscopy for the SHIELD CF study.*

A greater proportion of alveolar macrophages treated with RvD1 had associated fluorescence, and the intensity of fluorescence per cell was higher when compared with NS conditions (Figure 3-9 A-D). The fluorescence intensity measured from RvD1 treated macrophage samples was significantly higher than from vehicle control samples (7490 ± 950; RvD1 vs. 4420 ± 1020; NS, $P<0.01$, n=3 patient samples, performed in triplicate) (Figure 3-9 E).

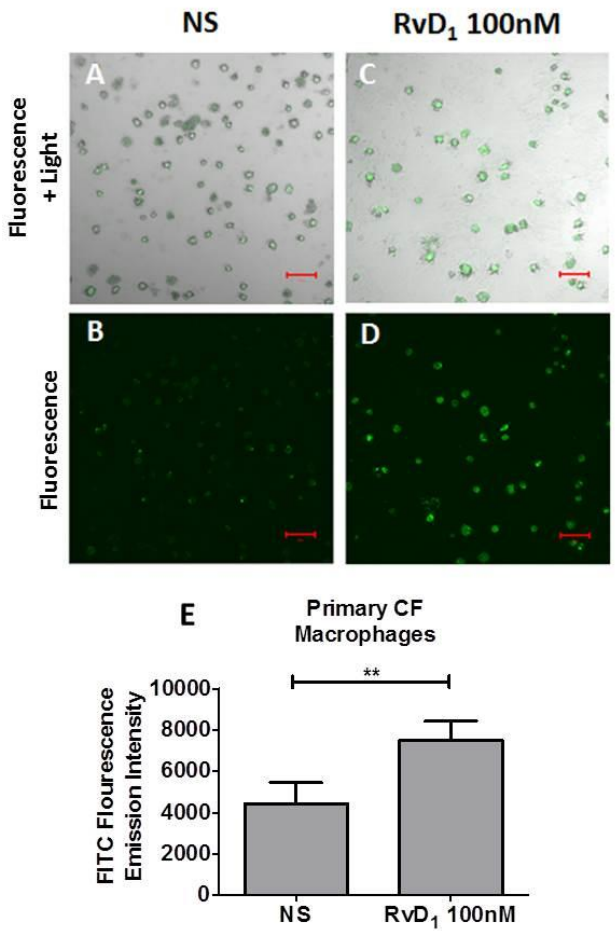

Figure 3-9 Resolvin D1 enhances the phagocytic capacity of Primary Alveolar Macrophages from children with CF (CFAM)

CFAM were isolated from BAL and pre-treated for 30mins with either vehicle control (A&B) or RvD1 100nM (C&D). CFAM were exposed to Latex Beads-rabbit IgG-FITC complex for 1 hour. Light microscopy (LM) images were captured concurrently with fluorescence images (B&D) from phagocytosed FITC-labelled beads (Panels A&C are LM / fluorescence composite). Representative images are presented (scale bar = 100µm). Engulfed bead fluorescence was more intense with RvD1 treatment (C&D) than under vehicle control conditions (A&B). Fluorescence intensity of phagocytosed FITC-labelled beads was quantified using a fluorescence plate reader (E). CFAM associated FITC fluorescence was higher in RvD1 treated versus control preparations (**P<0.01). (Results are presented as Mean ± SEM, experiments were performed in triplicate on n=3 patient samples, **P<0.01, paired Student t-test). * *Primary alveolar macrophages were obtained via the SHIELD CF Study.*

3.3.9 Resolvin D1 enhances intracellular killing of Pseudomonas aeruginosa (PAO1) by CF Alveolar Macrophages

Pseudomonas aeruginosa is a key CF pathogen, early acquisition of which predicts accelerated lung function decline (155). Impaired intracellular killing of *Pseudomonas aeruginosa* has been reported in CF alveolar macrophages (125-127, 228). We hypothesised that RvD1 would restore bactericidal capacity to CF alveolar macrophages (CFAM).

To evaluate this hypothesis, CFAM were isolated from BAL by adherence to tissue culture plastic. CFAM were treated with either vehicle control (NS) or RvD1 (100nM) for 3 hours and then exposed to *Pseudomonas aeruginosa* lab strain PAO1 (Optical Density=0.1, equivalent to 2x10^14 CFU/ml). After 30 minutes, non-engulfed bacteria were removed and the alveolar macrophages were treated with gentamicin to kill residual extracellular and membrane bound bacteria. CFAM were then lysed, and the bacterial load within the lysate was quantified.

**BAL fluid; the source material for primary alveolar macrophage isolation, was obtained via the SHIELD CF Study set up by Drs Paul McNally and Barry Linnane. FR, Paul McNally, Des Cox, Ahmad Zaid, Sheila Javadpour, Peter Greally and Basil Elnazir performed bronchoscopy for the SHIELD CF study. Dr Gerard Higgins taught me the technique to quantify bacteria and I conducted the quantification of PAO1 under his guidance.*

The intracellular viable bacterial load of PAO1 was significantly decreased in preparations treated with RvD1 than under vehicle control conditions (1.7±0.6 $x10^6$ CFU/ml; RvD1 100nM vs. 6.0±1.4 $x10^6$ CFU/ml; vehicle control, *$P<0.05$, n=4 preparations from 2 patients) (Figure 3-10).

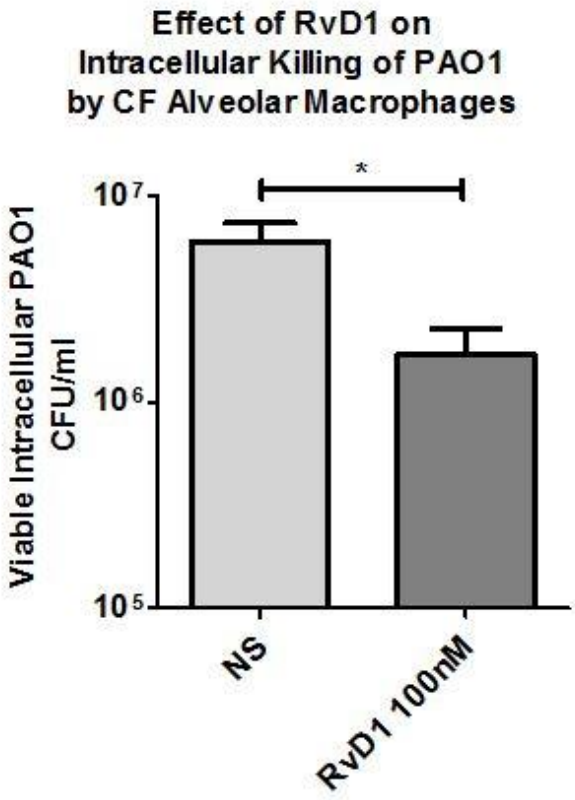

Figure 3-10 Resolvin D1 enhances intracellular killing of PAO1 by CF Alveolar Macrophages

CFAM were isolated from BAL by adherence to tissue culture plastic. CFAM were treated with either vehicle control (NS) or RvD1 (100nM) for 3 hours and then exposed to PAO1 bacteria. After 30 minutes non-engulfed bacteria were removed and CFAM were treated with gentamicin to kill residual extracellular and membrane bound bacteria. CFAM were then lysed and the bacterial load within the lysate was quantified. The intracellular viable bacterial load was significantly lower in preparations treated with RvD1 (1.7±0.6 x10^6 CFU/ml; RvD1 vs. 6.0±1.4 x10^6 CFU/ml; NS, Mean± SEM, n=4 preparations from 2 patients, *P<0.05, Studentôs t-test).

3.4 Discussion

3.4.1 Summary & Key Findings

We report here for the first time, that RvD1 ameliorates three key components of CF lung disease pathogenesis in CF models; ASL height loss, NFκB driven inflammation, and macrophage mediated killing of *P. aeruginosa*. We report that RvD1 elevated airway surface liquid height in Primary CF and CuFi-1 bronchial epithelial cell models. In CuFi-1 cells, this effect involved ALX/FPR2 receptor stimulation and was prevented by intracellular calcium chelation. We report that RvD1 had no net effect on ASL height in non-CF NuLi-1 cells, but rescued ASL height loss induced by CFTR inhibition. RvD1 attenuated IL8 secretion following TNFŬ mediated NFκB activation via preservation of IκB integrity in CuFi-1 bronchial epithelial cells. Furthermore, RvD1 enhanced the phagocytic capacity of primary CF alveolar macrophages and increased their ability to kill engulfed *P. aeruginosa*. These findings recommend the further development of RvD1 as a candidate for the treatment of CF lung disease.

3.4.2 Resolvin D1 in ASL Height Regulation

We hypothesised that RvD1 would increase ASL height in both normal and CF epithelial cell models. Our group had previously reported that the related SpM, LXA_4, restored ASL height in non-CF and CF airway epithelial cells (85, 318). The effect of LXA_4 on ASL was achieved via ALX/FPR2 receptor stimulation and raised intracellular calcium concentration, stimulating calcium-activated chloride secretion (85). Higgins et al. reported that LXA_4 induced an ALX/FPR2 dependent apical ATP release via Pannexin channels. Subsequently it was shown that P2RY11 purinoreceptor stimulation increased intracellular cAMP and calcium concentration (393). Al-Alawi et al. demonstrated that LXA_4 at physiological concentrations also mediated its effect on ASL hydration through ENaC inhibition (356). In the absence of functional CFTR, compensatory strategies proposed to overcome ASL dehydration and restore muco-ciliary function include; stimulating chloride secretion by Calcium Activated Chloride Channels (CaCC) via raised intracellular calcium; inhibition of ENaC mediated sodium hyper-absorption, and osmotic agents such as hypertonic saline and mannitol.

This is the first study to investigate the effect of RvD1 upon ASL height. We found that in the context of CFTR dysfunction; Primary CF bronchial epithelial cells, CuFi-1 cells, NuLi-1 cells in which CFTR was inhibited; RvD1 stimulated a significant increase in ASL height. In CuFi-1 cell preparations, this was paralleled by a more uniform appearance of the ASL layer. In contrast, in NuLi-1 cell preparations where CFTR function was intact, RvD1 had no net effect on ASL height.

In order to try to interpret these findings it is necessary to consider that three key ion transport pathways that contribute to the hydration of the ASL in airways epithelia; cAMP-dependent chloride secretion via CFTR; calcium-activated chloride secretion via CaCC (43); and, sodium absorption via ENaC (77).

In practice, ion transport processes affect each other via effects on the transmembrane electrochemical potential, and there is significant cross-talk between intracellular calcium and cAMP signalling cascades, and extracellular purine effects on the regulation of ASL hydration by both chloride secretion and sodium absorption (24, 38-40, 77, 103). In addition, CFTR exerts regulatory effects on both ENaC and CaCC (13, 20-43). Furthermore, the electrical driving force for chloride secretion and sodium absorption is provided by basolateral potassium channel activity (97). As such, any attempt to reduce RvD1ôs effects upon ASL height in the setting of CFTR dysfunction to an isolated effect on either chloride secretion via CaCC, or sodium absorption via ENaC, would be to oversimplify the biology of the system.

Having recognised that these ion transport processes are interdependent, there are cases to be argued that the effect of RvD1 on ASL height in the context of CFTR dysfunction is principally mediated either via CaCC activation or ENaC inhibition (illustrated in Figure 3-11).

Evidence in favour of the case that the principal effect of RvD1 is mediated via calcium activated chloride secretion (CaCC) comes from the observation that intracellular calcium chelation by BAPTA-AM prevented the RvD1 mediated ASL height increase in CuFi-1 cells. A precedent exists for LXA_4 as an ALX/FPR2 agonist stimulating intracellular calcium mobilisation and activating calcium

activated chloride currents in bronchial epithelial cells (85, 318). However, intracellular calcium chelation with BAPTA-AM also affects off-target cellular processes beyond calcium-activated chloride secretion, including tight junction stability, basolateral potassium transport, sodium absorption and calcium dependent protein kinase activation. The role of chloride secretion in the RvD1 mediated ASL height increase in CF cells could be further investigated by; using bumetanide to inhibit Na+/K+/2Cl- co-transport (85). The molecular identities of several types of calcium-activated Cl channel have been reported in recent years and the role of the CLCA family or TMEM16 (or Anoctamin 1) chloride channel in the response to RvD1 could be investigated by selective inhibition tools (88, 397).

There is also a case to be argued that the primary effect of RvD1 on ASL height is achieved via inhibition of ENaC mediated sodium hyper-absorption. Amiloride-sensitive sodium absorption (via ENaC) is elevated by 2- to 3- fold in CF (24), and in contrast to observations in non-CF airways epithelia, this channel accounts for a major component of the total trans-epithelial current in CF airways epithelia (78, 356, 398). If the primary effect of RvD1 on ASL height is achieved via ENaC inhibition, it is reasonable to expect that in the presence of functional CFTR, where the baseline current through ENaC is low, inhibition of ENaC would not impact ASL height significantly. This is consistent with the absence of an effect of RvD1 on ASL height in non-CF bronchial epithelial cells that we have observed. In the absence of functional CFTR, the ENaC current is higher (24, 356) and inhibition of this higher sodium current would have a more significant impact, and could give rise to the elevation in ASL height that we observed in Primary CF and CuFi-1 bronchial epithelial cells. Furthermore, intracellular calcium regulates ENaC channel activity in a variety of epithelia (394-396). The inhibition of the ASL height increase induced by RvD1 in CuFi-1 cells by BAPTA-AM would also be consistent with an inhibition of ENaC channel activity. Finally, acknowledging the recognised role of cAMP in ENaC regulation, a role played by cAMP in contributing the RvD1 mediated ASL height response cannot be excluded. The role of ENaC and sodium absorption in the RvD1 mediated ASL height increase in CF cells could be further investigated by; testing whether RvD1 can generate an additive (implying ENaC independent) ASL height increase when used in combination with amiloride (ENaC channel blocker); and, examining the contribution of RvD1 to amiloride-

sensitive ion transport. A candidate effect of RvD1 on basolateral potassium transport remains to be studied.

Taking into account the central role intracellular calcium plays in regulating both sodium absorption and chloride secretion, the effect of RvD1 on cytosolic calcium concentration could be also investigated using fluorescence microscopy (85).

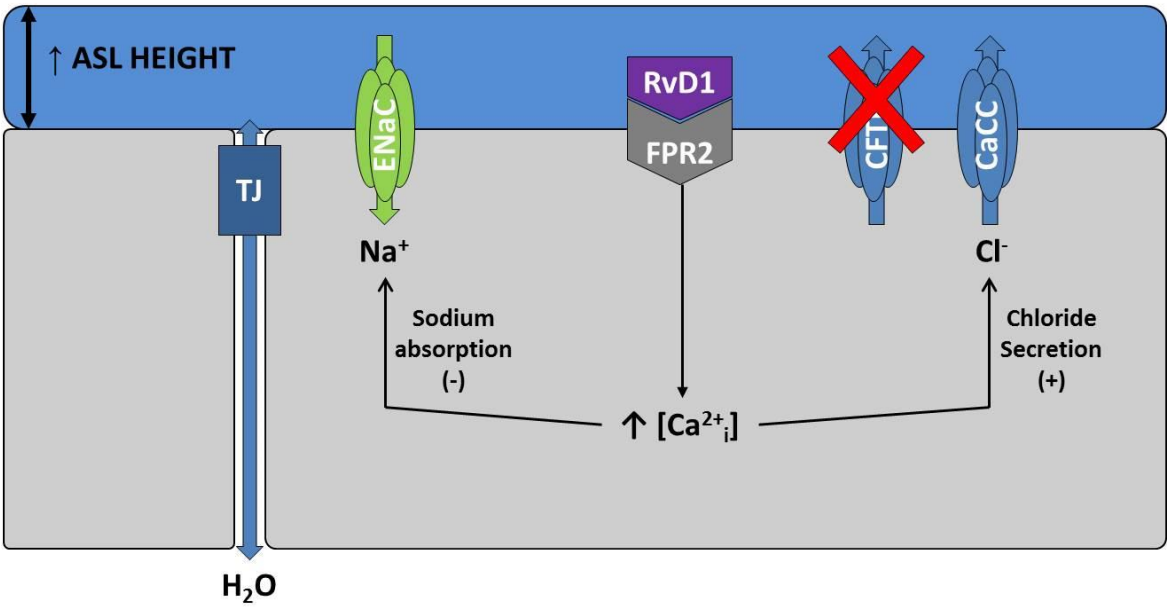

Figure 3-11 Proposed mechanisms by which RvD1 mediates the ASL height increase observed in Primary CF bronchial epithelial cells and CuFi-1 cells
RvD1 was shown to elevate ASL height in CF cells via ALX/FPR2 stimulation. This effect could occur via increased intracellular calcium concentration and subsequent calcium activated chloride secretion (CaCC), and/or inhibition of ENaC mediated sodium absorption.

In NuLi-1 cell preparations, where CFTR function was intact, RvD1 had no net effect on ASL height. In this respect RvD1ôs physiological effect on ASL regulation in NuLi-1 cells differed from the effect of LXA_4 on ASL height in NuLi-1 cells (85, 318). The absence of a net effect in NuLi-1 cells could reasonably be explained by the observation that CFTR functions to balance chloride secretion and sodium absorption, and thus, abrogates any unbalanced effect on one arm of ion transport via its regulatory function. This particular function of CFTR is a subject of controversy.

The ASL effect of ALX/FPR2 ligand LXA_4 was transduced via apical ATP release and purinoreceptor stimulation (393). The role of ATP and purinoreceptor stimulation in the RvD1 mediated increase in ASL height in primary CFBEõs and CuFi-1 cells could be tested using the published methods employed by Higgins et al., demonstrating ALX/FPR2 dependent apical ATP release and purinoreceptor stimulation in the ASL height effect of LXA_4 (393).

3.4.3 Impact of ASL Rehydration

Restoration of ASL height might reasonably be expected to give rise to improvement in muco-ciliary clearance and thus prevent mucous stasis and reduce airways infection. Rehydration of the ASL could potentially improve the ionic composition of the ASL and restore activity to innate anti-microbial peptides such as the beta-defensins (128, 129). During the active resolution of inflammation, RvD1 may be playing its part in tissue catabasis by elevating ASL height to encourage muco-ciliary clearance to flush inflammatory cells and debris trapped in mucous from the airway and restore airway homeostasis.

3.4.4 Resolvin D1 and the ALX/FPR2 Receptor

RvD1 was reported to signal through the GPR32 orphan receptor and ALX/FPR2 receptor (294, 295). GPR32 expression is reported in peripheral blood leukocytes, vascular tissue and small airway epithelial cells (295, 321). ALX/FPR2 is expressed in several types of cells including; neutrophils (306), alveolar macrophages (259, 322), human bronchial epithelial cells and human bronchial biopsy sections (323).

Evidence for a role played by the ALX/FPR2 receptor in the RvD1 mediated increase in ASL height in CFTR dysfunctional bronchial epithelial cells came from the demonstration that Boc-2 (pharmacological inhibitor of ALX/FPR2) completely abolished the ASL height increase. GPR32 expression has not been reported in bronchial epithelial cells and a pharmacological inhibitor is not yet commercially available. Further clarification of the involvement of ALX/FPR2 in the effect reported could be achieved by selective ALX/FPR2 receptor knockdown using siRNA (358).

The ALX/FPR2 agonist LXA_4 is reported to enhance the activity of the ALX/FPR2 promoter (327). Al-Alawi et al. reported differential cellular localisation of the ALX/FPR2 receptor between NuLi-1 and CuFi-1 cells, and an effect of LXA_4 in recruiting ALX/FPR2 to the apical membrane in CuFi-1 cells (356). We found no overall difference in the total protein concentration of ALX/FPR2 receptor between Nuli-1 and CuFi-1 cell lines, and no effect of RvD1 on whole cell ALX/FPR2 receptor protein expression in NuLi-1 or CuFi-1 cells. In resolving exudates, LXA_4 biosynthesis precedes RvD1 (253), and the effect of LXA_4 on ALX/FPR2 expression and trafficking might be important in sensitising the tissue to be able to receive and act upon the RvD1 signal.

3.4.5 Resolvin D1 Inhibited NFκB activation

Excessive NFκB activation has been observed in CF (65, 188, 189). At rest, NFκB dimers are bound to inhibitory IκB proteins which sequester NFκB complexes and maintain them in an inactive state in the cytoplasm (399). TNFŬ is a pro-inflammatory cytokine that interacts with its specific receptor to transduce a signal that results in phosphorylation, ubiquitination and degradation of IκB proteins (400, 401). Degradation of IκB releases NFκB and allows its translocation to the nucleus, where it binds to its site in the promoter regions of pro-inflammatory genes such as the IL-8 gene, rapidly up-regulating pro-inflammatory cytokine synthesis. RvD1 was shown to supress NFκB activation in a mouse model of LPS induced lung inflammation (335). In this study we demonstrate that RvD1 antagonises TNFŬ mediated IL8 secretion by preserving the integrity of IκB in CuFi-1 bronchial epithelial cells.

Zheng et al. recently reported that TAK1 (TGF-ß-activated kinase 1) is an indispensable signalling intermediate in the TNF signalling pathway (400, 402). TNF stimulation results in TAK1 phosphorylation and activation via the assembly of a TAK1 signalling complex, containing; TNF receptor-associated factor (TRAF), and TAK1-binding proteins, (TAB1ï TAB3) (402, 403). Activated TAK1 phosphorylates and activates the IκB kinase kinase complex, leading to the phosphorylation of IκB and release of NFκB (401). Consistent with our findings, Hsiao et al. recently identified the mechanism by which RvD1 disrupts pro-inflammatory signalling pathways. Although we did not specifically measure TAK1 phosphorylation, Hsiao identified TAK1 as the physiological target of RvD1 in

human small airways epithelial cells (321); RvD1 treatment largely blocked TAK1 phosphorylation, and the formation of the TAK1 / TAB1 / TRAF6 signalling complex (321) (illustrated in Figure 3-12). Furthermore, RvD1 is reported to regulate NFκB signalling via microRNA circuits, including miR-146b (255).

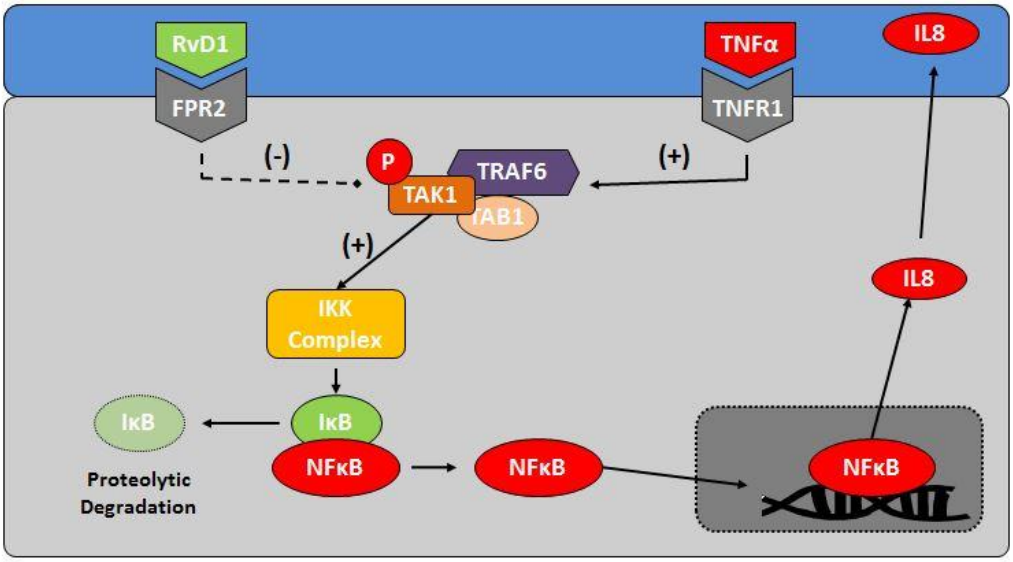

Figure 3-12 Proposed mechanism for RvD1 mediated antagonism of TNFŬ mediated IL8 secretion

TNF stimulation results in TAK1 phosphorylation and activation via the assembly of a TAK1 signalling complex with TNF receptor-associated factor 6 (TRAF6), and TAK1-binding protein 1, (TAB1). Activated TAK1 phosphorylates and activates the IκB kinase kinase complex (IKK Complex), leading to the phosphorylation of IκB and release of NFκB. RvD1 blocks TAK1 phosphorylation, and the formation of the TAK1 / TAB1 / TRAF6 signalling complex (321, 400).

Excessive NFκB activation (65, 188, 189) associated with elevated IL8 concentrations in the CF airway plays a role in the characteristic excess neutrophil recruitment into the airway (65, 187), which is attended by the release of tissue damaging Neutrophil Elastase (66). Favourable effects of RvD1 attenuating NFκB activation and IL8 secretion in CF airway epithelial cells might be expected to reduce neutrophil recruitment and dampen exaggerated pro-inflammatory cascades.

3.4.6 Resolvin D1 enhances alveolar macrophage mediated phagocytosis

Alveolar macrophages play diverse roles in airway innate immunity including; phagocytosis of dead cells and debris; phagocytosis and killing of bacteria; cytokine synthesis; tissue remodelling and repair (404). CFTR expression in macrophages has been reported (126, 224, 336) and in CF, macrophage defects reported include; impaired clearance of apoptotic cells (218, 219), and impaired intracellular killing of bacteria (125, 126).

Using primary CF alveolar macrophages and an in vitro phagocytosis assay based on the uptake of immunoglobulin labelled beads, we report that RvD1 improves the phagocytic capacity of primary CF alveolar macrophages.

Phagocytosis is an actin-dependent process whereby particulate targets are specifically internalised via diverse mechanisms. A major function of SPMôs is to enhance phagocyte function, especially macrophage-mediated efferocytosis (253, 259). Consistent with this role, RvD1 was reported to increase neutrophil efferocytosis in a mouse model of cigarette smoke induced lung inflammation (336). Pseudopod formation and particle engulfment depend on remodelling of the actin cytoskeleton. Notably, LXA_4 was reported to stimulate RhoA- and Rac-dependent cytoskeleton reorganization, enhancing apoptotic neutrophil uptake (405).

The in vitro phagocytosis assay used does not discriminate well between biological effects on different targets. Macrophage phagocytic targets may include micro-organisms, dead or dying cells or environmental debris (404). A phagocytic target of interest for CF research is neutrophil efferocytosis by macrophages, since it plays a key role in the restoration of tissue homeostasis, and initiates the synthesis of pro-resolution mediators (300, 301) in a virtuous cycle. This concept will be explored further in Chapter 8 (General Discussion).

3.4.7 Resolvin D1 enhances alveolar macrophage mediated intracellular killing of Pseudomonas aeruginosa

Micro-organisms are a target of macrophage mediated phagocytosis which is particularly relevant in CF. Impaired intracellular bacterial killing of *Pseudomonas aeruginosa* has been reported in several studies of human and mouse CF

macrophages (125-127). This has been mechanistically attributed to CFTRôs involvement in lysosomal acidification, though some debate surrounds this finding depending on the technique used by different investigators (126, 229). Altered ceramide concentration was linked to altered pH and the release of reactive oxygen species in CF macrophages (230). Defective endosome to late-endosome/lysosome maturation is a feature of LPS challenge (227) and impaired phagocytic capacity of human CF macrophages (or CFTR inhibited, non-CF macrophages), has been reported (127, 228).

Here we report that RvD1 improved primary CF alveolar macrophage intracellular killing of the PAO1 lab strain of *Pseudomonas aeruginosa*.

Following engulfment and maturation (406), microbicidal mechanisms operating within the phagolysosome lumen of a macrophage include; acidification via V-ATPase proton pumps (407), reactive oxygen and nitrogen species (ROS, RNS) generation by NADPH oxidase and inducible nitric oxide synthetase / isoform 2 (iNOS / NOS2) (406, 408), lactoferrin mediated scavenging of iron, defensin mediated antimicrobial effects, and the action of degradative enzymes (406) (illustrated in Figure 3-13).

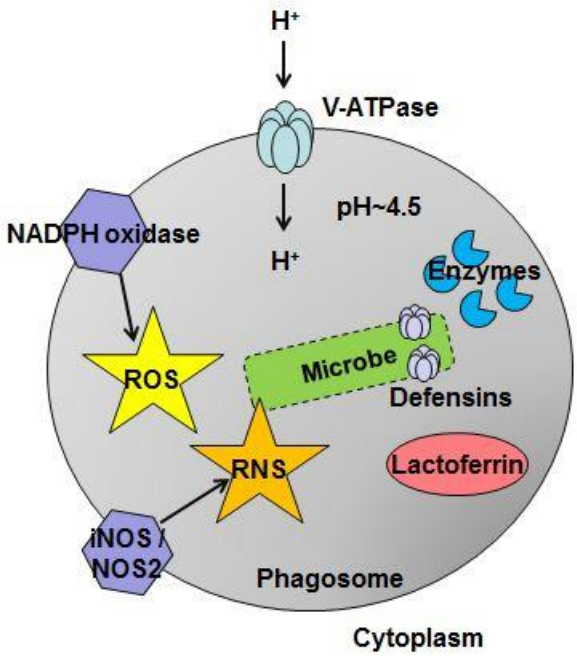

Figure 3-13 Bacterial killing mechanisms active within the lumen of a macrophage phagolysosome

Microbicidal mechanisms include; acidification via V-ATPase proton pumps, reactive oxygen and nitrogen species (ROS, RNS) generation by NADPH oxidase and inducible nitric oxide synthetase / isoform 2 (iNOS / NOS2), lactoferrin mediated scavenging of iron, defensin mediated antimicrobial effects, and the action of degradative enzymes (adapted from (406)).

The SPMõs as a family, are reported to improve bacterial killing in animal models of sepsis; LXA_4 administration resulted in a reduced bacterial load in a mouse model of *P. aeruginosa* infection (315); a panel of SPMõs, specifically, RvD1, Resolvin D5 and Protectin D1 improved the containment of a bacterial challenge in mouse models of peritoneal infection, and skin sepsis, acting in synergy with antibiotics (316). The specific cellular mechanisms by which SPMõs enhance bacterial killing have yet to be described.

In light of previous reports that LXA_4 stimulated cytoskeletal reorganisation, it is conceivable that RvD1 improves intracellular killing by accelerated recruitment of vesicles via the endocytic pathway to fuse with the phago-lysosome (406). We have observed effects of RvD1 on ion transport in epithelia and so we could

hypothesise that RvD1 affects phago-lysosome acidification. Alterations in cytosolic calcium concentration drive granule fusion in phagosomes in neutrophils (409, 410). Another hypothesis could be that RvD1 enhances vesicle recruitment to the phago-lysosome via effects on intracellular calcium mobilisation. Counter-intuitively, LXA_4 was described to inhibit peroxynitrite formation and block superoxide anion generation by neutrophils (262, 320). We do not know if this kind of physiological function of SPMs is ligand specific, or cell type specific, and perhaps the opposite effect of enhanced reactive oxygen and nitrogen species generation would be seen with RvD1 in macrophages.

RvD1 is synthesised from the parent essential fatty acid DHA which has been reported to be deficient in CF (346, 347). RvD1 biosynthesis involves two sequential lipoxygenation steps both catalysed by 15 Lipoxygenase. We have previously reported deficient 15LO expression in the CF airway (370). Therefore, it remains to be established whether RvD1 is adequately produced in the CF airway.

3.4.8 Limitations of this study

Whilst the effect of RvD1 on ASL height represents an exciting therapeutic angle this work is incomplete. It remains critical to the application of this work to further define which ASL regulatory pathways are involved in transducing this effect. Furthermore, demonstrating the effect of RvD1 on a more complex model of muco-ciliary clearance (e.g. radiolabelled marker clearance) could provide an important clinical research outcome and advance the case for clinical trials (411). The studies of alveolar macrophage function were conducted in relatively small numbers of patient samples. These samples were vulnerable to selection bias since the method to isolate these samples from BAL relied on a favourable neutrophil / macrophage cell differential and a lower burden of infection in BAL. A more clinically relevant strain of *Pseudomonas aeruginosa* could have been used in the study of RvD1 effect on intracellular killing of bacteria by CF alveolar macrophages.

3.5 Conclusion

We report that RvD1 elevates ASL height in CF epithelial cells, attenuates TNFŬ stimulated IL8 secretion by preserving intact IκB in CuFi-1 cells, enhances the

phagocytic capacity of primary CF alveolar macrophages, and, improves primary CF alveolar macrophage intracellular killing of *Pseudomonas aeruginosa*.

There are unmet needs in CF therapy for treatments to rehydrate the airway and improve muco-ciliary clearance, augment the effectiveness of existing antibiotics and strengthen the host immune response (252). This work suggests significant therapeutic potential for RvD1 in CF lung disease.

CHAPTER 4

ABNORMAL CLASS SWITCHING IN CF BAL: DEFECTIVE LIPOXIN A_4 SYNTHESIS

4 Abnormal Class Switching in CF BAL: Defective Lipoxin A_4 Synthesis

4.1 Introduction

A normal host response to bacterial infection involves neutrophil recruitment and inflammation that serves to contain and eliminate the bacterial insult. In CF, an excessive neutrophil burden, persistent inflammation and failure of tissue clearance of neutrophils by efferocytosis have been described (412).

This failure to resolve inflammation in CF could be due to abnormal function and / or production of the SPMs ò is central to the active resolution of inflammation. We recently published most of the data presented in this chapter in the European Respiratory Journal (370).

4.1.1 Eicosanoid Class Switching

In the normal regulation of inflammation, the temporal progression of acute inflammation from initiation to propagation and finally to active resolution is executed via the sequential biosynthesis of eicosanoid classes in inflammatory exudates in a process termed ñclass switchingò (264). Prostaglandins initiate the inflammatory response, followed by Leukotrienes, typified by Leukotriene B_4 (LTB_4) which amplifies and propagates inflammation (264). Lipoxin A_4 (LXA_4) biosynthesis initiates the active resolution phase of inflammation (257) and is followed by biosynthesis of the Resolvins, Protectins and Maresins.

4.1.2 Lipoxin A_4 Effector Functions

LXA_4 inhibits neutrophil effector functions (267), and counter-regulates the effector functions of LTB_4 (303, 360, 413). Furthermore, the tendency for neutrophils to migrate into tissues is regulated by the relative concentrations of LXA_4 and LTB_4 (Figure 4-1) (262, 304, 323, 360, 414). Mice treated with analogues of LXA_4 and subsequently challenged with *P. aeruginosa* contained the bacterial challenge more effectively (315).

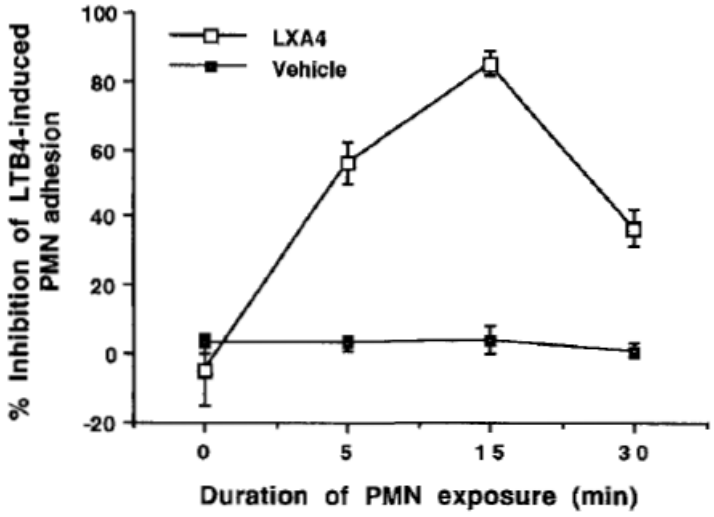

Figure 4-1 Reported inhibition of LTB_4 induced endothelial adhesion of neutrophils by LXA_4

LXA_4 caused a rapid inhibition of LTB_4 induced neutrophil adhesion to endothelium (reproduced from Papayianni et al (304)).

4.1.3 LXA_4 Biosynthesis

LXA_4 is biosynthesised in the respiratory tract by trans-cellular co-operation of neutrophils (275), eosinophils (276), alveolar macrophages (277) or airway epithelial cells (278), each expressing different Lipoxygenase (LO) enzymes (illustrated in Figure 4-2 (415, 416)). The 15-Lipoxygenase (15-LO) catalysis step is key to LXA_4 biosynthesis, and up-regulation of 15-LO activity favours LXA_4 biosynthesis at the expense of Leukotriene biosynthesis (264, 417). This occurs both as a result of 15-LO product 15-HETE competing for catalytic sites at the 5-LO enzyme and thereby reducing the formation of 5(S) HETE, and by competition for the common biosynthetic intermediate Leukotriene A_4 (264, 278, 416, 417).

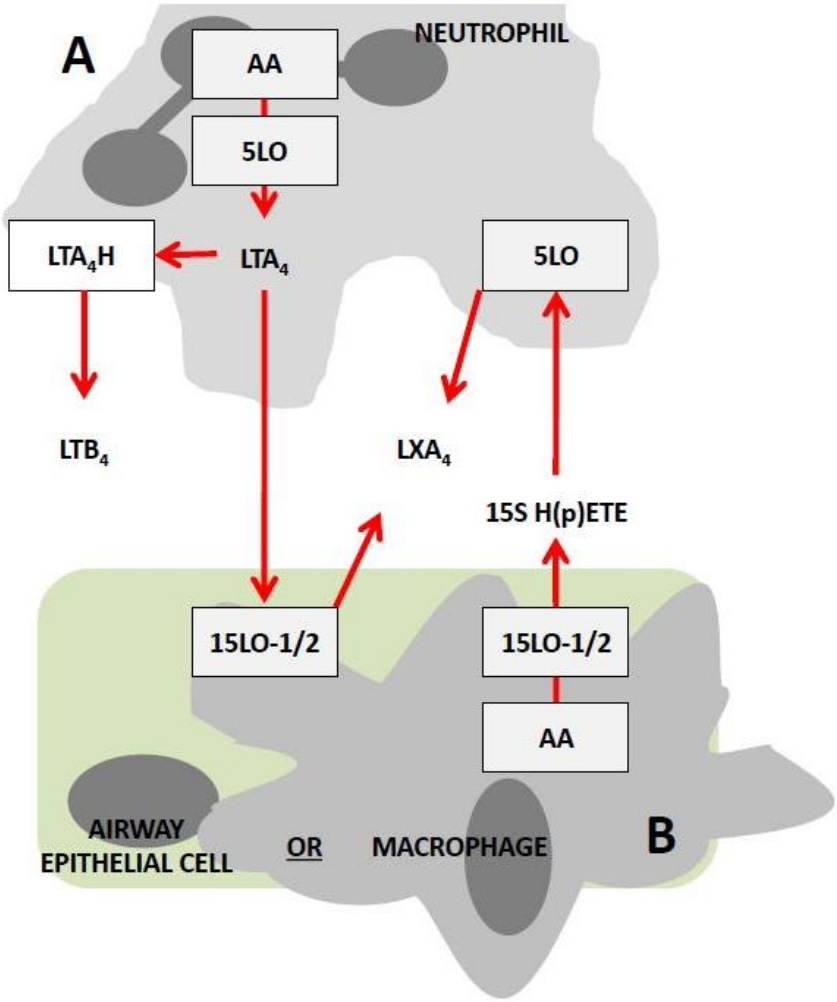

Figure 4-2 An illustration of LXA_4 biosynthesis pathways by trans-cellular co-operation in in the airways

(A) The neutrophil donates LTA_4 intermediate formed by the action of 5 Lipoxygenase on Arachidonic acid to the acceptor airway epithelial cell or alveolar macrophage whereby 15 Lipoxygenase catalyses LXA_4 formation. LTB_4 is also generated from the LTA_4 intermediate by the action of Leukotriene A_4 Hydrolase. (B) Airway epithelial cell or alveolar macrophage 15 Lipoxygenase activity catalyses the conversion of Arachidonic acid to 15S H(p)ETE which is donated to the acceptor neutrophil and converted to LXA_4 by 5 Lipoxygenase catalysis (reproduced from (370)).

4.1.4 LXA_4 in CF

In CF bronchial epithelial models, LXA_4 augments airway epithelial innate defence by stimulating tight junction formation (357), enhancing Calcium activated Chloride secretion (318), inhibiting sodium absorption (356), and restoring airway

surface liquid height (355). LXA_4 concentration in CF BAL has been variously reported as significantly suppressed or not significantly different from disease controls (315, 352).

4.2 Aims

The underlying basis for the failure to actively resolve inflammation in the CF airway remains incompletely understood. One primary function of SPMõs is to limit neutrophil recruitment and enhance tissue neutrophil clearance, thus enacting the active resolution of inflammation (253). In CF, neutrophil recruitment is excessive, the airway burden is high and inflammation is prolonged and ineffective and leads to tissue damage (221). LXA_4 is the first SPM to be synthesised in the acute resolution phase of inflammation and has key regulatory effects on tissue neutrophil burden. LXA_4 is generated following a õclass switchò in eicosanoid lipid mediator synthesis from leukotrienes to lipoxins via up-regulation of the synthetic enzyme, 15 Lipoxygenase (264).

It was our aim to examine eicosanoid mediator class switching (critical to the transition into active resolution of inflammation) in the CF airway, and to evaluate the impact of bacterial infection on this process.

4.3 Hypothesis

We hypothesised that;

- õEicosanoid mediator class switchingò is defective in CF and may play a mechanistic role in the failure to resolve inflammation in CF lung disease.

4.4 Results

In order to investigate the role of eicosanoid mediator class switching in the failure to actively resolve inflammation in the CF lung, we examined Bronchoalveolar lavage samples (BAL) from participants enrolled in the SHIELD CF study (described in more detail in Chapter 2).

The study population for this piece of work included children with CF, and, as a comparative, control children without CF who were undergoing bronchoscopy for clinical reasons. Assessments were undertaken when the children were in a stable clinical condition.

In order to further understand the influence of bacterial infection in the lung upon eicosanoid mediator profiles and synthesis, semi-quantitative conventional bacterial and viral studies were performed on BAL by the microbiology lab at OLCHC. We conducted subgroup analysis of our data based on the presence or absence of microbial pathogens detected in the BAL fluid. Samples were considered to have positive microbial culture if one or more pathogen was detected on viral studies or cultured at a concentration of $>10^4$ CFU/ml.

4.4.1 Population Characteristics

Fifty one BAL samples from children with CF (CF) and twenty two from Paediatric Control patients (control) were assessed for eligibility in this study. Five children with CF and three Control children were excluded because of Leukotriene Receptor antagonist treatment or recent use of treatment dose antibiotics. CF and control children did not differ significantly in age, sex or BAL neutrophil count (Table 4-1)(370).

Looking at the subgroup populations (based on the presence or absence of $>10^4$ CFU/ml pathogenic bacteria cultured from the BAL specimen) in this study; control children with positive BAL microbial culture were younger than control children with negative BAL microbial culture; whereas, children with CF and positive BAL microbial culture were older than children with CF and negative BAL microbial culture.

Infection in CF BAL samples was associated with a higher mean neutrophil count and more frequently detectable Neutrophil Elastase than was found in culture negative CF BAL samples (Table 4-1). The most common bacterial pathogens cultured were *H. influenza, S. aureus* and *S. pneumoniae. P. aeruginosa* >10^4 CFU/ml was identified in two CF and one Control BAL sample. Viral studies were negative for all 51 BAL samples examined. (370)

Table 4-1 Population Characteristics

Data are presented for the complete control and CF groups and separately for subgroups based on the presence or absence of >10^4 cfu/ml pathogenic bacteria cultured from the BAL specimen. (*P<0.05 / **P<0.01 within group unpaired comparison of subgroups; #P<0.05 between group unpaired comparison of subgroups; Studentôs t-test) (**P<0.01, within group comparison; Fisherôs Exact Test)(Reproduced from (370)).

	Non-CF Control			**CF**		
	No Growth Cult(-)	Pathogen Cultured Cult(+)	All Control	No Growth Cult(-)	Pathogen Cultured Cult(+)	All CF
Procedures	n=7	n=12	n=19	n=24	n=22	n=46
Subjects			n=19			n=34
Mean Age	8.2**	2.7**	4.5	2.7*	3.8*	3.2
(SD)	(4.3)	(1.6)	(3.6)	(1.5)	(1.4)	(1.6)
Male Sex	2	8	10	12	12	24
(%)	(28.6)	(66.7)	(52.6)	(50)	(54.5)	(52.2)
Neutrophils	0.8#	8.1	5.7	3.5**	12.8**/#	8.0
x10^5/ml (SEM)	(0.6)	(4.0)	(2.8)	(1.0)	(2.8)	(1.6)
Detectable NE	3	5	8	9#	17#	26
(%)	(42.9)	(41.7)	(42.1)	(37.5)	(77.3)	(56.5)

4.4.2 Baseline inflammatory parameters & their relationship to pulmonary infection

LTB_4 is associated with the propagation of inflammation and predominates before the ñeicosanoid class switch (264)òtowards resolution of inflammation. After the ñclass switchò LXA_4 is the sentinel pro-resolution mediator synthesised (264). LXA_4 directly counter-regulates LTB_4 effector functions (303, 360, 413). IL8 is a key neutrophil chemoattractant and pro-inflammatory cytokine which is suppressed by LXA_4 (262, 323, 414). We measured the concentration of LXA_4, LTB_4 and IL8 in BAL supernatant by ELISA and compared the results obtained between non-CF and CF samples, and those with either positive or negative bacterial culture results.

We found no significant differences between control (n=19) and CF samples (n=46) in mean LXA_4 or LTB_4 concentration (Figure 4-3A & C). Furthermore, comparison of culture negative and culture positive samples from control or CF BAL revealed no significant difference in LXA_4 concentration (Figure 4-3B).

LTB_4 concentration was significantly higher in infected BAL samples within a group (control/cult(+): 155 ±40pg/ml, n= 12 vs. control/cult(-): 26 ±12pg/ml, n=7, P<0.05 & CF/cult(+): 1383 ±454pg/ml, n=22 vs. CF/cult(-): 156 ±35pg/ml, n=24, P<0.01) (Figure 4-3D).

IL8 was higher in CF (n=46) than in control (n=19) BAL samples (1198 ± 149ng/ml vs. 379 ±110ng/ml, P<0.01) (Figure 4-3E). IL8 was higher in culture negative CF samples (n=n=24) than in culture negative control samples (n=7) (817 ±178ng/ml vs. 110 ±28ng/ml, P<0.05) (Figure 4-3F). Culture positive CF BAL samples had higher IL8 concentrations than either culture negative CF samples (1614 ±215ng/ml, n=22 vs. 817 ±178ng/ml, n=24, P<0.01) or culture positive control samples (1614 ±215ng/ml, n=22 vs. 536 ±159ng/ml, n=12, P<0.01).

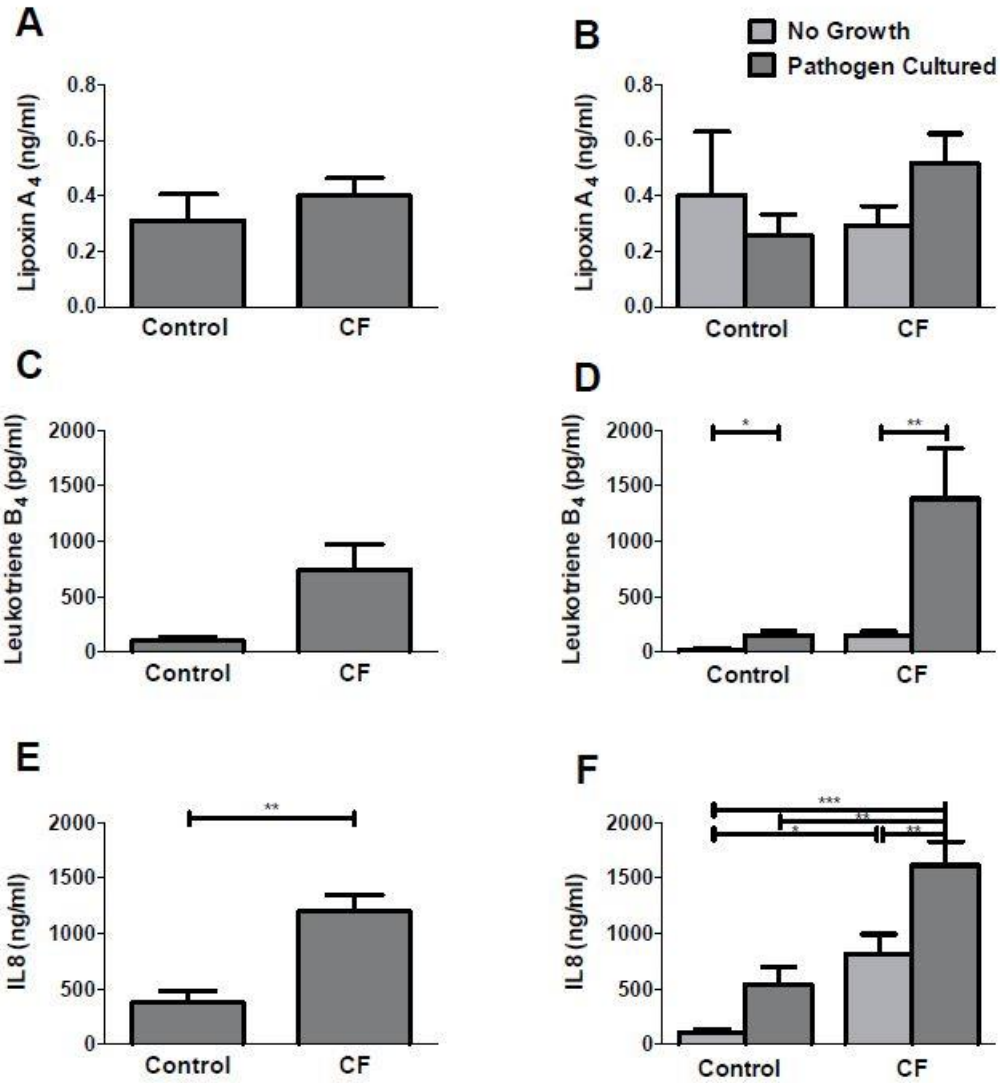

Figure 4-3 Baseline inflammatory parameters & their relationship to pulmonary infection

Left Panel: Comparison of mean (A) LXA_4 (ng/ml), (C) LTB_4 (pg/ml), and (E) Interleukin 8 (ng/ml) values measured in Control (n=19) and CF (n=46) BAL. Right Panel: Subgroups of the Control and CF cohorts based on the presence or absence of $>10^4$ CFU/ml pathogenic bacteria cultured from the BAL specimen. Mean (B) LXA_4 (ng/ml) (D) LTB_4 (pg/ml), and (F) Interleukin 8 (ng/ml). Lighter shaded bars represent BAL from which no pathogens were grown (n=7 control, n=24 CF samples) and darker shaded bars represent BAL from which pathogens were cultured (n=12 controls, n=22 CF samples). (Error bars represent SEM; *P<0.05, **P<0.01, ***P<0.001; Studentôs t test). (Reproduced from (370)). **BAL was obtained via the SHIELD CF study.*

4.4.3 LXA_4 relative to LTB_4 and IL8

Looking at relative LXA_4 and LTB_4 concentrations, expressed as a ratio, allows us to evaluate the extent of eicosanoid mediator transition from propagation phase mediators to early resolution mediators. Children with CF (n=46) had lower LXA_4 /LTB_4 ratios in BAL fluid compared with control children (n=19) (2.9 ±0.7 vs. 7.1 ±1.9, P<0.01) (Figure 4-4A). Among control samples, the LXA_4 /LTB_4 ratio in sterile samples (n=7) was significantly higher (3.7 ±1.31 vs. 3.1 ±3.6, P<0.01) than that measured in BAL samples from which pathogens were cultured (n=12) (Figure 4-4). In CF BAL (n=46), the LXA_4 /LTB_4 ratio was uniformly depressed, and did not vary with infection status (P=0.98). The LXA_4 /LTB_4 ratio measured in culture negative CF samples (n=24) was significantly depressed in comparison to culture negative control BAL samples (n=7) (CF/cult(-): 2.9 ±0.7 vs. Control/cult(-): 13.1 ±3.6, P<0.001). (370)

Furthermore, since LXA_4 counter-regulates IL8 synthesis and function LXA_4 /IL8 ratios were also calculated. The LXA_4 /IL8 ratio was significantly higher in control BAL than in CF BAL (3.5x10-3 ±1.7x10-3, n=19 vs. 0.5x10-3 ±0.1x10-3, n=46, P<0.01) (Figure 4-4C). The LXA_4 /IL8 ratio was significantly higher in culture negative control BAL (n=7) than culture positive control BAL (n=12) (7.9x10-3 ±4.2x10-3 vs. 0.9x10-3 ±0.2x10-3, P<0.05) (Figure 4-4D). The LXA_4 /IL8 ratio measured in culture negative CF BAL (n=24) was significantly low compared with culture negative control BAL (n=7) (0.6x10-3±0.2x10-3 vs. 7.9x10-3 ±4.2x10-3, P<0.01). In CF BAL, the LXA_4 /IL8 ratio was uniformly depressed and the correlation with infection seen in control BAL was absent. (370)

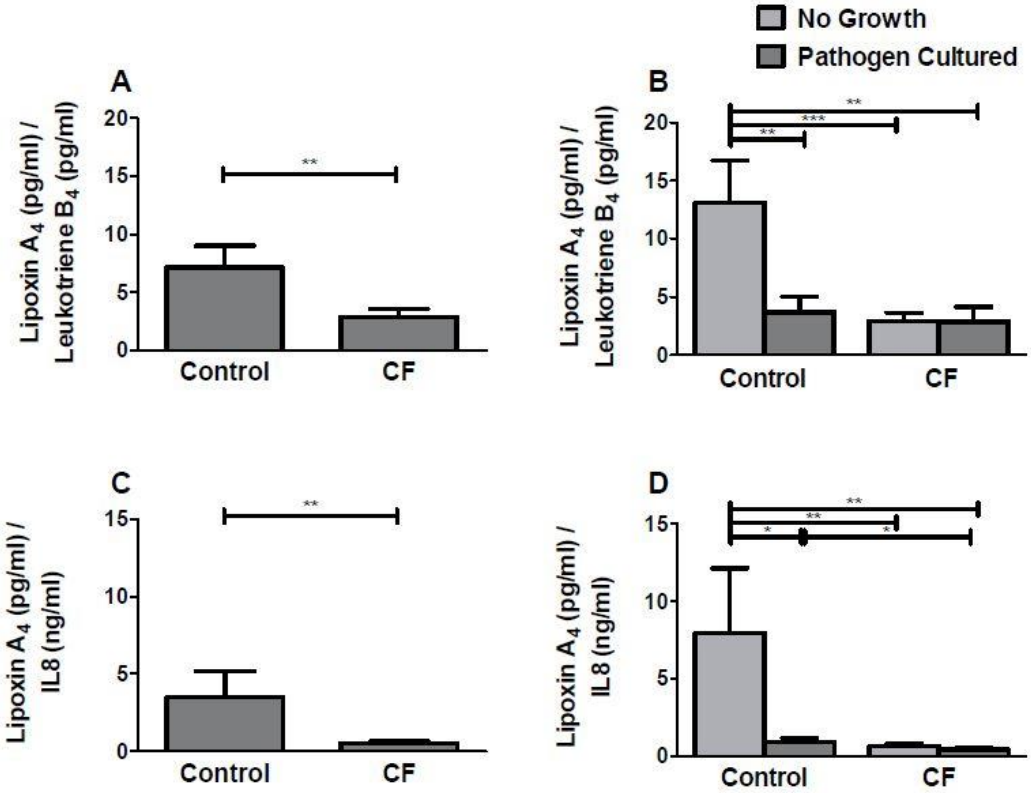

Figure 4-4 LXA_4 relative to LTB_4 and IL8

(A) Mean LXA_4 (pg/ml) / LTB_4 (pg/ml) ratio measured in Control (n=19) and CF BAL (n=46). (B) Mean LXA_4 (pg/ml) / LTB_4 (pg/ml) ratio compared between No Growth and Pathogen Cultured subgroups of the Control and CF cohorts. (C) Mean LXA_4 (pg/ml) / IL8 (ng/ml) ratio measured in Control and CF BAL. (D) Mean LXA_4 (pg/ml) / IL8 (ng/ml) ratio compared between No Growth and Pathogen Cultured subgroups of the Control and CF cohorts. Growth defined as $>10^4$ cfu/ml pathogenic bacteria cultured from the BAL specimen. Lighter shaded bars represent No Growth (n=7 Control, n=24 CF Samples) and darker shaded bars represent Pathogen Cultured BAL (n=12 Control, n=22 CF samples). (Error bars represent SEM; *P<0.05, **P<0.01, ***P<0.001; Studentôs t test). (Reproduced from (370)). **BAL was obtained via the SHIELD CF study.*

4.4.4 Abundance of transcripts for enzymes involved in eicosanoid synthesis quantified in the cellular phase of BAL

15-Lipoxygenase (15-LO) catalysis is key to LXA_4 biosynthesis, and up-regulation of 15-LO activity favours LXA_4 biosynthesis at the expense of Leukotriene biosynthesis (264, 417). In order to study the expression of genes involved in LXA_4 and LTB_4 biosynthesis in the airway lumen, we measured the relative expression of mRNA transcripts for; 5-LO, 12-LO, 15-LO 1/2 and Leukotriene A_4 Hydrolase (LTA_4H), in BAL cell pellets by qPCR. Samples from children with CF were compared to paediatric controls.

For this analysis, fourteen control samples (7 culture-negative control and 7 culture-positive control) and eleven CF samples (6 culture-negative CF and 5 culture-positive CF) with a similar distribution of infection, neutrophil (P=0.95) and macrophage (P=0.71) compositions were used (Figure 4-5E & F) (370).

The cellular fraction obtained from the airway lumen contained a full repertoire of transcripts required for LXA_4 biosynthesis (Figure 4-5). ALOX15B mRNA was significantly less abundant in CF samples (n=11) compared to control samples (n=14) (0.44 ± 0.13 vs. 2.63 ± 0.92 fold expression, $P<0.05$) (Figure 4-5A). A non-significant trend towards lower ALOX15 expression was observed in CF BAL (n=11) than in control BAL (n=14) (1.92 ± 0.49 vs. 5.51 ± 1.89 fold expressed, P=0.09) (Figure 4-5B).

ALOX5 and LTA_4H mRNA abundance did not significantly differ between control (n=14) and CF samples (n=11) (Figure 4-5C & D). ALOX12 mRNA was measurable in only 6/14 Control samples and in 4/11 of the CF samples. (370)

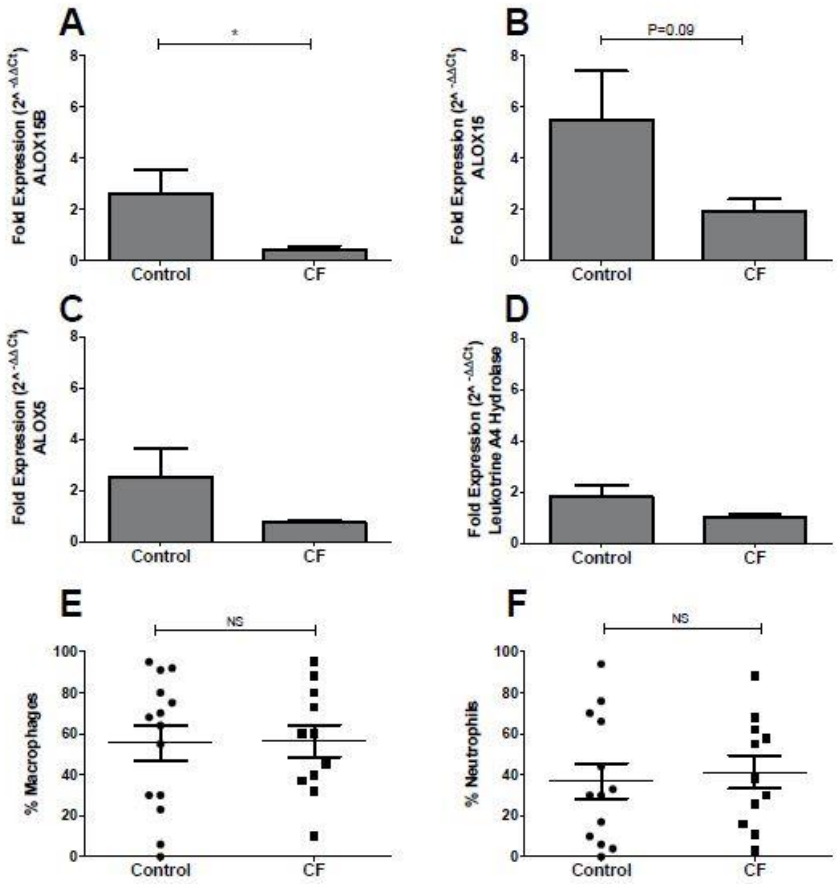

Figure 4-5 Abundance of transcripts for enzymes involved in eicosanoid synthesis quantified in the cellular phase of BAL

(A) ALOX15B, (B) ALOX15, (C) ALOX5 & (D) Leukotriene A_4 Hydrolase mRNA transcript relative abundances in the cellular component of the bronchoalveolar lavage fluid from a subset of the Control (n=14) and CF (n=11) samples. Results are expressed as Fold Expression = 2^(-ΔΔCt) compared to a reference patient sample. (E) Macrophage & (F) and Neutrophil composition of the Control and CF cell pellets expressed as % of nucleated cells assessed by light microscopy. (Error bars represent SEM; NS = Not Significant, *P<0.05; Student's t test)(Reproduced from (370)). **BAL differential cell counts in the SHIELD CF Study were performed by Dr Michael McDermott.*

4.4.5 ALOX15B Correlates

We further explored the relationships between ALOX15B mRNA expression in the airway lumen and the ratio of LXA_4 /LTB_4 in the sample.

In control BAL samples (n=14) there was a significant positive correlation between ALOX15B mRNA abundance in the cell pellet and LXA_4 /LTB_4 ratio (r = 0.66, P=0.01) (Figure 4-6A). In CF samples (n=11) this correlation was absent (P = 0.31) (Figure 4-6B).

Neutrophils and macrophages are the most abundant cell types in the BAL cell pellet. In order to understand which cell types were most responsible for ALOX15B mRNA expression in the airway we studied the correlation between % Neutrophil and % Macrophage composition of the BAL cell pellet and the expression of ALOX15B mRNA. In control samples (n=14) ALOX15B mRNA abundance correlates positively, strongly and significantly with macrophage percentage composition of the pellet (r= 0.82, P=0.0003) and, negatively with neutrophil percentage composition (r= -0.82, P=0.0003) (Figure 4-6C&D). However, in CF samples (n=11), despite variability in the macrophage composition of the samples, the correlation of ALOX15B abundance with macrophage / neutrophil composition was absent (P=0.20 & P= 0.17) (Figure 4-6E&F).

Variability in the abundance of ALOX5 mRNA, recognised to be expressed abundantly both by neutrophils and alveolar macrophages (418-420), did not significantly correlate with cell type for control (n=14) or CF (n=11) samples.

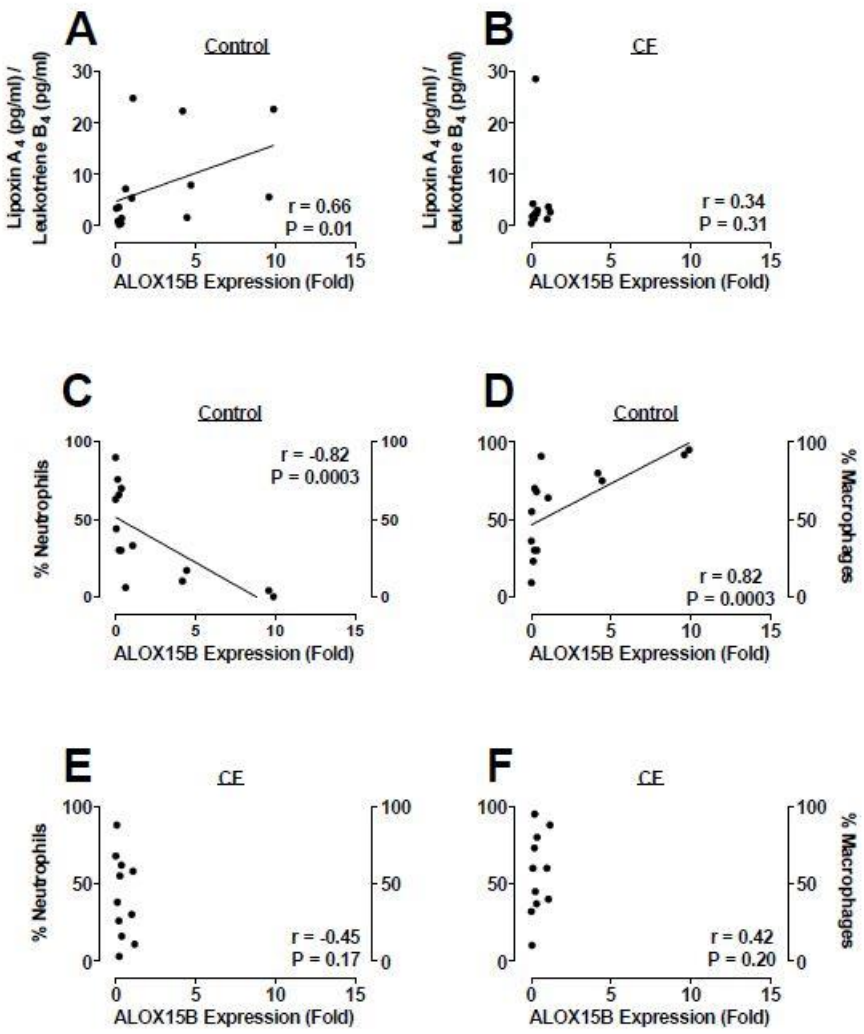

Figure 4-6 ALOX15B Correlates

Correlation between ALOX15B transcript relative abundance and; LXA_4 / LTB_4 ratio from (A) Control (n=14) and (B) CF (n=11) samples; BAL % Neutrophil from (C) Control and (E) CF samples; BAL % Macrophage composition from (D) Control and (F) CF samples. Fold Expression = 2^(-ΔΔCt) of ALOX15B mRNA versus reference patient sample. % Macrophages and Neutrophils represent % of nucleated cells in the cellular phase of the bronchoalveolar lavage fluid assessed by light microscopy. LXA_4 (pg/ml) / LTB_4 (pg/ml) ratio as measured in the BAL supernatant. (Spearman correlation coefficient (r) and associated P value given for each analysis). (Reproduced from (370)). * *BAL differential cell counts in the SHIELD CF Study were performed by Dr Michael McDermott.*

4.5 Discussion

Under normal circumstances, lung inflammation serves to eliminate the original threat, and then actively resolve and return the tissue to homeostasis (253). In CF, however, the neutrophil predominant response is severe, sustained and persistent even if the original inflammatory stimulus is removed (221). The underlying basis for this failure of active resolution remains incompletely understood.

In this study we demonstrated defective eicosanoid class switching, a key step in initiating the active resolution of inflammation, in the lower airways of young children with CF, despite the absence of recognised pathogens. We have presented *in vivo* evidence of impoverished 15 Lipoxygenase-2 gene expression associated with a depressed LXA_4 / LTB_4 ratio in the lower airways of children with CF. Furthermore, we found that the correlations between LXA_4 /LTB_4 ratio, infection, abundance of 15-LO2 transcripts and macrophages observed in control BAL were lost in CF BAL. This report provides new and important insights into the failure to resolve inflammation in CF lung disease (370).

In order to test the hypothesis that eicosanoid class switching is defective in CF giving rise to a failure to resolve acute inflammation, we looked at the *in vivo* expression of eicosanoids and their synthetic enzymes. We compared eicosanoids temporally associated with the ñswitchò from propagation to resolution; LTB_4 and LXA_4, respectively. Additionally, with the expectation that the phase of inflammation would be affected by the infectious context (354), we conducted subgroup analysis based on the presence or absence of microbial pathogens detected in the BAL fluid. By comparing relative mediator concentrations expressed as a LXA_4 /LTB_4 ratio, we demonstrate that the balance in eicosanoid biosynthesis weighs heavily in favour of LXA_4 production over LTB_4 production in control BAL when compared to CF BAL (Figure 4-4). This finding indicates an abnormality in the active resolution of inflammation in CF and is consistent with the reduced LXA_4 /IL8 ratio in CF compared to control BAL also reported here (Figure 4-4) (370).

Consistent with a previous report (352), we did not find significant differences when comparing control and CF BAL, directly or in sub-population analyses

(based on microbial culture result), as regards the absolute content of LXA_4 (Figure 4-3). One other study suggested there is a failure to resolve acute inflammation in CF and reported a significant suppression in the ratio of LXA_4 /Neutrophils in CF (315). In our study, we did not find any significant difference in LXA_4 /Neutrophils ratio between control and CF BAL (data not illustrated). This could potentially be explained by differences in the control population between that study and ours, especially with regards to allergic airways disease and infection. Although LTB_4 concentration did not significantly differ between control and CF BAL (Figure 4-3), we found that LTB_4 concentration was always higher in infected BAL sub-groups consistent with its role in neutrophil recruitment and host defence. It is noteworthy that a negative correlation has been described between LTB_4 and pulmonary function outcome in CF (214, 370).

LXA_4 and its synthetic analogues have been shown, in vitro, to suppress IL8 production by leukocytes and bronchial epithelial cells (262, 323). As such, the LXA_4 /IL8 ratio represents a compound outcome incorporating both the local concentration of each mediator, and the effectiveness of LXA_4 in antagonising IL8 production. In control samples the LXA_4 /IL8 ratio was significantly lower in the presence of infection in BAL, consistent with an appropriate pro-inflammatory response to infection. In contrast, the higher LXA_4 /IL8 ratio seen in uninfected control BAL, in favour of the resolution of inflammation, was absent in uninfected CF BAL where the ratio remained low independent of infection.

In the absence of airway infection in control children, the balance between LXA_4 and LTB_4 biosynthesis was skewed in favour of LXA_4 production, reflecting on-going active resolution of inflammation. Furthermore, the results presented in this Chapter provide *in vivo* evidence that LXA_4 /LTB_4 balance in control childrenôs lower airways correlates strongly with the abundance of 15-LO2 transcripts in the cellular phase of BAL. This finding is consistent with in vitro reports that 15-LO enzyme activity promotes LXA_4 biosynthesis at the expense of Leukotriene biosynthesis (264, 417). In the control population, we also find a positive correlation between 15-LO2 transcript abundance and the percentage macrophage composition of the BAL cell pellet, and reciprocally, a negative correlation with the percentage neutrophils (Figure 4-6). This is consistent with the recognised expression of 15-LO, and 15(S)-HETE and LXA_4 generation by human

alveolar macrophages (277). Although both neutrophils and macrophages in induced sputum both expressed 15-LO, the majority has been attributed to macrophages (421). Furthermore, 15-LO 2 (ALOX15B) is the predominantly expressed Lipoxygenase in human macrophages (280). (370)

Taken together our observations suggest that macrophages, by expressing 15-LO2 and participating in trans-cellular co-operation with neutrophils expressing 5-LO, make a significant contribution to LXA_4 synthesis in the bronchial lumen and regulate eicosanoid balance *in vivo* in the airway (370). This idea will be further explored mechanistically in Chapter 5.

In CF, in contrast to our findings in control childrenôs BAL, the bias in favour of LXA_4 production in uninfected BAL was absent. LXA_4 /LTB_4 and LXA_4 /IL8 ratios were not significantly different between non-infected and infected CF BAL subgroups (Figure 4-4). In order to further our understanding of impaired eicosanoid class switching in CF we considered the roles played by various enzymes involved in eicosanoid biosynthesis (12-LO, LTA_4H, 5-LO, 15-LO1 and 15-LO2). The activity of 12-LO in platelets from CF patients has been reported to be reduced (351). In our study, 12-LO transcripts were detected at very low copy numbers in only ten of twenty five airway samples tested. 12-LO is characteristically expressed within platelets and therefore this finding might possibly represent blood cell contamination from a friable and inflamed airway wall. Over-expression of Leukotriene A_4 Hydrolase (LTA_4H) could result in a steal phenomenon, routing the common immediate Leukotriene A_4 away from LXA_4 synthesis towards LTB_4 synthesis giving rise to the reduced LXA_4 /LTB_4 ratio observed, however expression was not significantly different in CF BAL than in control BAL. We observed non-significant trends towards lower abundance of 15-LO1 and 5LO transcripts in CF airway (Figure 4-5) (370).

At the same time as the publication of the results presented in this Chapter, Fredman et al. (422) reported that the balance between arachidonic acid-derived mediators in leukocytes was regulated by the cellular localization of 5-LO; with nuclear 5-LO localisation favouring LTB_4 biosynthesis, whereas, cytoplasmic 5-LO localisation favours the biosynthesis of LXA_4 (422). They also found that RvD1 promotes nuclear exclusion of 5-LO and thereby suppresses LTB_4 and enhances

LXA_4 in macrophages. Whilst this mechanism could potentially impact LXA_4 /LTB_4 balance in the CF airway, our archived samples were not preserved in a manner such as to be able to evaluate the intracellular localisation of 5-LO, however this remains an interesting line of investigation.

Importantly, we found reduced 15-LO2 gene expression in CF BAL samples (Figure 4-5). Furthermore, in BAL from children with CF there was a breakdown in the relationships between 15-LO2 transcript abundance, LXA_4 /LTB_4 ratio, and percentage macrophage composition of the BAL. The macrophage and neutrophil content of the CF BAL cell pellets analysed had a wide range of composition and a similar distribution to the control samples, thus 15-LO2 reduced expression is not attributable simply to the neutrophil cell population simply overwhelming the macrophage population numerically. In fact, reduced 15-LO activity in the face of preserved 5-LO activity provides a reasonable explanation for the excess of LTB_4 over LXA_4 biosynthesis seen in CF subjects free from infection in this study.

These findings, describing altered eicosanoid mediator balance even in the absence of infection in CF, might contribute to the persistence of neutrophil mediated inflammation in lieu of its active resolution in CF lung disease. (370)

15-LO expression is a hallmark feature of the alternative activation phenotype (M2) in macrophages, induced by the action of interleukins 4 & 13 (produced by T-helper 2 cells) (423, 424). Deficient expression of IL4 / 13, delayed or impaired macrophage differentiation (such as that seen in bronchial epithelial cells in CF (425)) or polarisation classical activation (M1) over alternative activation could all contribute to the observed reduction in the expression of 15-LO reported here. In an example of feed-forward amplification, 15-LO product RvD1 promotes M2 macrophage differentiation, and thus, 15-LO deficiency could lead to reduced RvD1 biosynthesis and consequently to reduced M2 macrophage differentiation, further compounding the deficiency in 15-LO expression (336). These ideas will be further explored in Chapter 5.

Hostile environmental factors could play a role in the failure to actively resolve inflammation observed here. Whilst we examined the impact of conventional microbial infection upon eicosanoid balance we cannot exclude the possibility that

sterile inflammatory stimuli (e.g. pulmonary aspiration (384), adherent mucus plaques), or non-culturable microbes (426) affected eicosanoid expression and synthesis. (370)

Although there were differences between the mean ages of children with infection in their BAL within control and CF subgroups, we found no significant correlation between LXA_4 /LTB_4 ratio and age among control children (P=0.31, n=19) or children with CF (P=0.49, n=46).

The measured neutrophil count in our CF cohort is consistently lower than that recently reported for another large study of early lung disease in CF, the AREST CF cohort (66). There are differences in lavage sampling protocol between these two studies. In our study lavage fluid is obtained from two instillations into the right middle lobe and two into the lingula, with the entire lavage then being pooled and analysed as a homogenous fluid. In the AREST study the right middle lobe is lavaged thrice, followed by either the lingula or the ñmost affected lobe on CTò and different lavage fractions are used for microbiology and inflammation studies (66). These protocol differences may give rise both to dilutional variation and differences arising from the selection bias introduced by the selection of lavage zone by CT in the AREST study.

In our study the proportion of children, stratified by age, with infection cultured from BAL was lower than that reported for the AREST cohort at similar ages; 14.2% and 33.3% of children in the second and third years of life versus 21.1% and 40.2% at the same ages (66). The definition of infection we have employed differs from that used by AREST CF ï with a more stringent criterion of 10^4 CFU/ml versus 10^5 CFU/ml for positive culture of pathogenic bacteria, whereas the AREST study classified ñanyò *P. aeruginosa* cultured as infection. The overall prevalence of *P. aeruginosa* was low in this cohort with 2 children with CF culturing more than 10^4 CFU/ml and only one further child with CF culturing ñanyò at 900 CFU/ml. Repeat analysis was performed classifying ñanyò *P. aeruginosa* culture as ñinfectionò but did not significantly affect any outcomes reported. Whilst the ELISA method has good sensitivity and specificity and is widely accepted as a method to measure lipid mediators, it is acknowledged that the mass spectrometry method yields better specificity. (370)

In light of reports that LXA_4 /LTB_4 balance is disturbed across the spectrum of asthma severity (334, 427-429), and in scleroderma lung disease (430) and, given that the D-Series Resolvins (431), Protectin D1 (270) and 15(S) HETE (an agonist of the anti-inflammatory nuclear receptor PPARɔ) (432), are also 15-LO products, these insights may have wider relevance in the pathophysiology of inflammatory respiratory disease (370).

4.5.1 Limitations of this study

Conceptually, ñeicosanoid class switchingò is relatively new and has only been described by a small group of investigators. This concept has relevance for the sequential production of lipid type bioactive mediators, and interactions between bioactive lipids and protein based mediators of inflammation have been described. However, the place of ñeicosanoid mediator class switchingò in the hierarchical regulation of the inflammatory cascade remains controversial. This work is based around a calculated ratio of LXA_4 to LTB_4. Whilst we are not the first group to make this comparison (334, 427-429), its mechanistic or disease modifying relevance has not been demonstrated in any *in vivo* animal or human context.

4.6 Conclusions

This study provides *in vivo* evidence of impoverished 15 Lipoxygenase-2 gene expression associated with a depressed LXA_4 / LTB_4 ratio in the lower airways of children with CF. We provide new insights into the failure to resolve inflammation in early CF lung disease, even in the absence of infection.

4.6.1 Perspectives

Macrophages play a central role in the regulation the active resolution of inflammation in the airway. In Chapter 5 we describe our attempts to optimize a method to explore further the mechanisms operating within alveolar macrophages that may underlie aberrant 15 Lipoxygenase 2 expression and LXA_4 / LTB_4 imbalance in the CF airway.

CHAPTER 5

DEVELOPING METHODS TO INVESTIGATE 15-LO REGULATION IN CF ALVEOLAR MACROPHAGES AND THEIR PHENOTYPIC POLARISATION

5 Developing methods to investigate 15-LO regulation in CF Alveolar Macrophages and their phenotypic polarisation

5.1 Introduction

Macrophages play key regulatory roles in the active resolution of inflammation. They receive, interpret and transmit signals to communicate tissue injury or invasion, and conversely, containment, resolution, homeostasis and repair. By phagocytosis they remove pathogens, inflammatory and cellular debris(433).

In Chapter 4 we learned that impoverished 15-LO2 gene expression was associated with a depressed LXA_4 / LTB_4 ratio in the lower airways of children with CF (370). In this chapter we explore how (a) 15-LO expression in alveolar macrophages (AM), and (b) macrophage polarisation (15-LO is a hallmark of M2 polarisation / alternative activation) are mechanistically controlled and affected by CF. We develop a method to characterise AM 15-LO expression and AM polarisation in airway samples from children with CF and controls *ex vivo* (pilot data is also presented). Finally, we evaluate methods to perform functional studies on AM from people with CF.

5.1.1 Macrophage Polarisation

Macrophage functional phenotypes have been characterised (434). These activation states exist along a spectrum, and are coupled with plasticity to allow them to switch phenotype in response to micro-environmental signals in the local milieu (433, 434). Macrophage phenotype affects the balance between pro-inflammatory processes and the active resolution of inflammation. Classically activated Macrophages (M1) mediate host defence against micro-organisms, produce large amounts of pro-inflammatory cytokines, ROS and nitric oxide, and have accentuated anti-microbial activity (433). They are polarized by cytokines characteristic of a T-Helper 1 response, associated with exposure to pathogens, e.g. LPS, interferon and TNFŬ (435). Alternatively Activated Macrophages (M2), are anti-inflammatory in their function and regulate wound healing. They are polarised by cytokines characteristic of a T-Helper 2 response e.g. IL4 & IL13. M2 macrophages have higher efferocytic capacity and greater capacity to synthesise SPMĝs, as illustrated by the distinct pro-resolution lipid mediator profile secreted by M2 macrophages (313). 15-LO and PPARɔ expression characterise M2 differentiation in macrophages (433).

5.1.2 15-Lipoxygenase in Alveolar Macrophages

15-LO expression is a hallmark feature of M2 differentiation in macrophages (433). Human AM possess 15-LO activity and produce SPMôs including LXA_4 (277, 280). 15-LO 2 (ALOX15B) is the predominantly expressed Lipoxygenase in human macrophages where it is regulated by IL4, LPS and hypoxia (280). Although both IL4 and IL13 induce 15-LO type 1 (ALOX15) in human macrophages, the effect is most pronounced with IL4 (280). 15-LO activity plays a key role in the biosynthesis of the SPMôs; LXA_4 (257), ResolvinsôD1, D2 and D5 (262) and Protectin D1 (270). Interestingly, RvD1 and the Maresin 1 precursor 13, 14-epoxy-Maresin, are also reported to promote M2 macrophage polarisation (298, 336). 15-LO, in addition to SPM biosynthesis, catalyses the production of PPARɔ agonists, such as 15-HETE (274), 9-HODE and 13-HODE (271-273).

5.1.3 PPARɔ and macrophage polarisation

PPARɔ is a ligand-activated transcription factor and master regulator of polarisation in macrophages. PPARɔ expression is up-regulated when monocytes differentiate into macrophages, especially M2 polarised macrophages (329) and in the resolution phase of wound repair (330). PPARɔ-deficient macrophages are resistant to M2 polarization (436). Additionally, PPARɔ inhibits pro-inflammatory gene expression through several mechanisms, including the trans-repression of NFHB (331). PPARɔ can be activated by a range of lipid mediators including the 15-LO products mentioned, and prostaglandin type pro-resolving lipid mediators such as 15-d-PGJ2 (332). Furthermore, PPARɔ agonists 9-HODE and 13-HODE were reported to up-regulate CD36 expression in monocytes, associated with M2 polarisation via PPARɔ activation (436, 437).

5.1.4 Alveolar macrophages in CF

Macrophages express CFTR (126, 224). There are conflicting reports on the subject of alveolar macrophage polarisation in CF (225). Endotoxin tolerance was observed in circulating monocytes isolated from cystic fibrosis patients and this was taken as evidence of M2 skewing (438). However, a distinct subpopulation of macrophages has been subsequently described called ñSatiated macrophagesò These arise after ingestion of apoptotic neutrophils and are poorly responsive to TLR ligands such as endotoxin (439). Another study looked at differences

between alveolar macrophages isolated from *P. aeruginosa* non-infected, versus infected CF subjects. They found that subjects infected with *P. aeruginosa*, and who were treated with azithromycin, had elevated M2 markers (440). However, an effect of azithromycin promoting M2 polarisation has also been independently reported (441). In contrast, Krysko et al. examined nasal tissue from patients with cystic fibrosis, and observed by immunohistochemistry and cytokine examination, findings in favour of the M1 phenotype of macrophages (442). Reduced expression of PPARɔ in CFTR knockout mice might be taken as indirect evidence in favour of M1 polarisation (361).

5.1.5 15-LO-2 regulation in the CF alveolar macrophages

A range of explanations for reduced 15-LO 2 transcript abundance in CF macrophages (370) are proposed (illustrated in Figure 5-1).

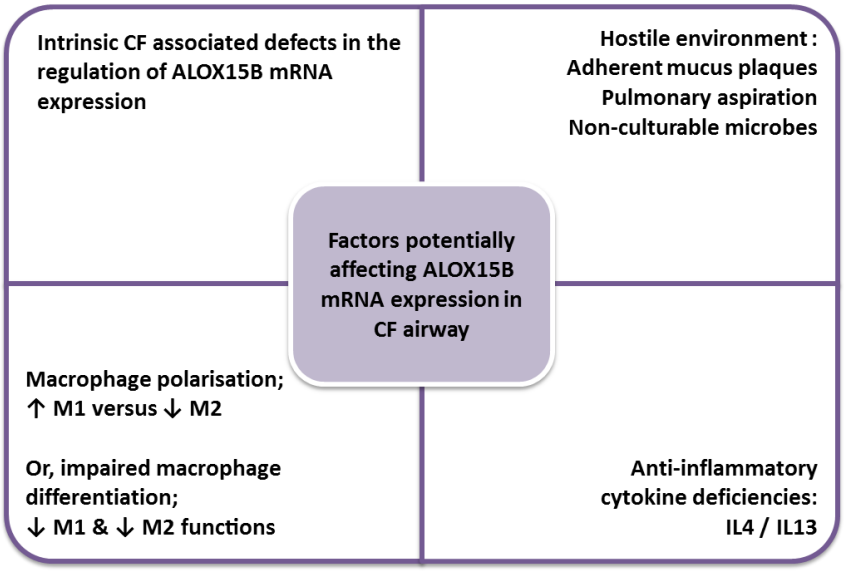

Figure 5-1 Potential explanations for the finding of reduced ALOX15B mRNA expression observed in the CF airway

5.1.6 Interleukin 4 is not depressed in CF BAL

One explanation for depressed 15LO-2 in the CF airway could be reduced airway IL4 concentration, since 15LO-2 is up-regulated by IL4 (280). IL4 is produced by T-helper 2 cells (423, 424), and a bronchial mucosal lymphocytic infiltrate has been identified in the CF airway (64). Unpublished data from the SHIELD CF study comparing IL4 concentration in BAL between control children and children

with CF demonstrates that IL4 concentration is not depressed in the airways of children with CF (Control children; 0.5±0.8 pg /ml, versus Children with CF; 2.2±2.7pg/ml, *P<0.05) (Figure 5-2). Conversely, IL4 is elevated when compared with controls, which would be expected to yield both an elevation in 15-LO expression and favour M2 polarisation in CF alveolar macrophages.

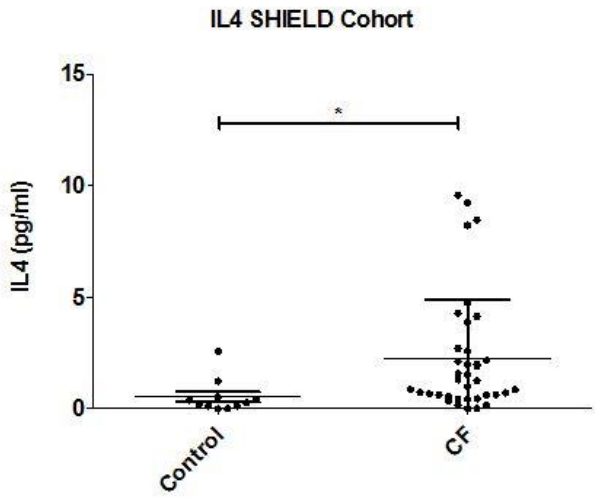

Figure 5-2 Interleukin 4 measured in the BAL of control children and children with CF

Unpublished data from the SHIELD CF study. IL4 concentration compared between control children (n=11) and children with CF (n=37) (Results are presented as Mean ÑSEM, *P<0.05, Studentôs t-test).

5.2 Aims

We sought to develop the methodology to explore the underlying mechanisms regulating the resolution of inflammation in CF alveolar macrophages. We aim to specifically examine; 15-LO regulation and phenotypic polarisation in CF alveolar macrophages.

5.3 Hypotheses

We hypothesised that;

- ALOX15B mRNA expression is reduced in a pure population of alveolar macrophages from children with CF.

- That CF alveolar macrophage differentiation is skewed towards an M1 phenotype.
- That CF alveolar macrophages do not adequately up-regulate 15-LO expression following IL4 stimulation when compared with controls.

5.4 Results

5.4.1 Developing a method to evaluate native 15-LO expression and polarisation phenotype of CF alveolar macrophages

We attempted three different methodologies to examine 15-LO expression and the polarisation phenotype of *ex vivo* alveolar macrophage samples from the BAL of children with CF and controls.

Immunohistochemistry on Historical Cytospin Preparations

We had a large repository of historical BAL cytospin preparations from the SHIELD CF cohort stored in a bio-bank. We attempted to evaluate the phenotype of macrophages within these samples by immunohistochemistry. The method involved using antibodies against 15-LO, and epitopes that are differentially expressed between M1 (CD68+/CD80+) and M2 (CD68+/CD163+) polarised macrophages. This method had the advantage that we could both differentiate macrophages from neutrophils morphologically, and evaluate double-staining with 15-LO and either M1 or M2 markers. Unfortunately, when we attempted to use the archived slides we discovered that the method of preservation was inadequate, and the samples had deteriorated to the extent that the epitopes could no longer be recognised by this method.

Prospective flow cytometry on new BAL cell pellets

We attempted to perform flow cytometry upon newly obtained BAL cell pellets using antibodies against 15-LO and the same differentially expressed epitopes; M1 (CD68+/CD80+) versus M2 (CD68+/CD163+) polarisation. Using adherence to tissue culture plastic as the method to enrich the cell population for macrophages, the quantitative cell yield from our paediatric samples was too low to achieve the minimum reliable event threshold (1000 events) considered technically appropriate for a valid flow cytometry result (Mean of 5 million cells from whole BAL pellet, Mean of 0.6 million isolated macrophages). Technical limitations affecting the yield of isolated macrophages included; infrequent access

to small volumes of BAL fluid; inflammatory milieu affecting pellet cell differential, macrophage yield & cell viability; heavily infected samples didnâ survive overnight selection in medium containing antibiotics.

Assessment of 15-LO and M1/M2 Differential Gene Expression by PCR

In view of the sensitivity of the PCR method and the possibility to obtain results with a smaller amount of cellular material (Mean RNA yield of 3360ng from BAL cell pellets enriched for macrophages by adherence to tissue culture plastic) we discovered that we could successfully evaluate 15-LO expression and macrophage polarisation profiles by measuring the expression of genes characteristic of M1 (IL-12 p35 & IDO1) and M2 phenotype markers (CCL22 & PPARɔ)(329).

5.4.2 Pilot data; 15-LO1 and 15-LO2 expression in a pure alveolar macrophage population

Alveolar macrophages were enriched from the BAL cell pellets of control children and children with CF enrolled in the SHIELD CF study by adherence to tissue culture plastic. We extracted total RNA from the enriched fraction and quantified ALOX15 and ALOX15B mRNA transcripts by qPCR, using 18S as a housekeeping gene. ALOX15 mRNA relative abundance ranged from 0.1 ï 396 in control BAL samples, and from 0.2 ï 12.7 in CF BAL samples. ALOX15B mRNA transcript relative abundance ranged from 0.1 ï 59.1 in control BAL samples, and from 0.1 ï 2.7 in CF BAL samples (Figure 5-3).

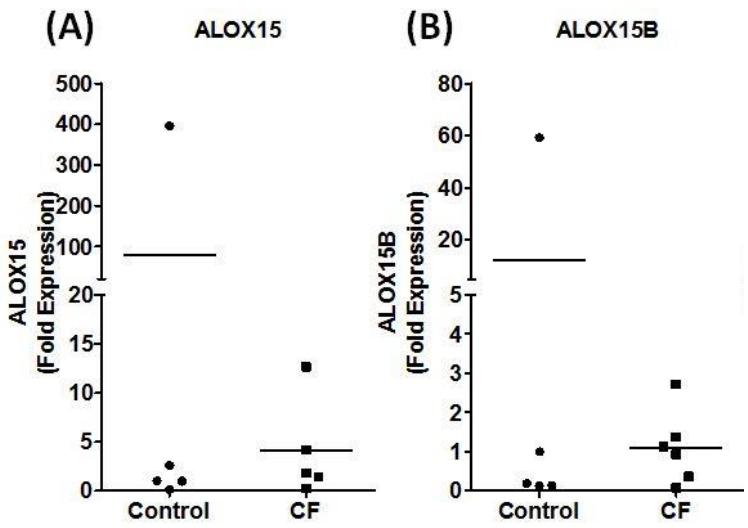

Figure 5-3 Abundance of 15 Lipoxygenase type 1 & 2 transcripts quantified in alveolar macrophages isolated from the BAL of control children and children with CF (pilot data)

(A) ALOX15 and (B) ALOX15B mRNA transcript relative abundances in alveolar macrophages, isolated from bronchoalveolar lavage fluid from Control and CF samples by adherence to tissue culture plastic. Results are expressed as Fold Expression = 2^(-qpqpCt) compared to a reference patient sample (n=5 control samples & 5-6 CF samples).

5.4.3 Pilot data; Macrophage phenotype polarisation

Macrophage polarisation phenotype affects 15-LO expression and the capacity of a macrophage to synthesise pro-resolution mediators (280, 313). In order to evaluate macrophage polarisation phenotype, CF alveolar macrophages were enriched from the BAL cell pellets of children with CF and controls by adherence to tissue culture plastic. We extracted total RNA and quantified the expression of M1 markers IL-12 p35 & IDO1, and M2 phenotype markers CCL22 & PPARɔ by qPCR (329).

High IL12p35 expression is associated with m1 polarisation (329). In control BAL samples mean IL12p35 relative expression measured 1.5 (± 0.3) as compared with 3.3 (± 2.4) in CF samples. High IDO1 expression is also associated with M1 polarisation (329). In control BAL samples mean relative IDO1 expression

measured 1.5 (± 0.8) compared with 0.7 (± 0.5) in CF BAL samples (Figure 5-4A&B).

High CCL22 and PPAR expression are associated with M2 polarisation (329). In control BAL samples mean CCL22 relative expression measured 0.6 (± 0.2) compared with 2.1 (± 1.6) in CF BAL samples. Mean PPARɔ relative expression measured 1.9 (± 0.5) in control BAL samples, and 5.8 (± 4.1) in CF BAL samples (Figure 5-4 C&D).

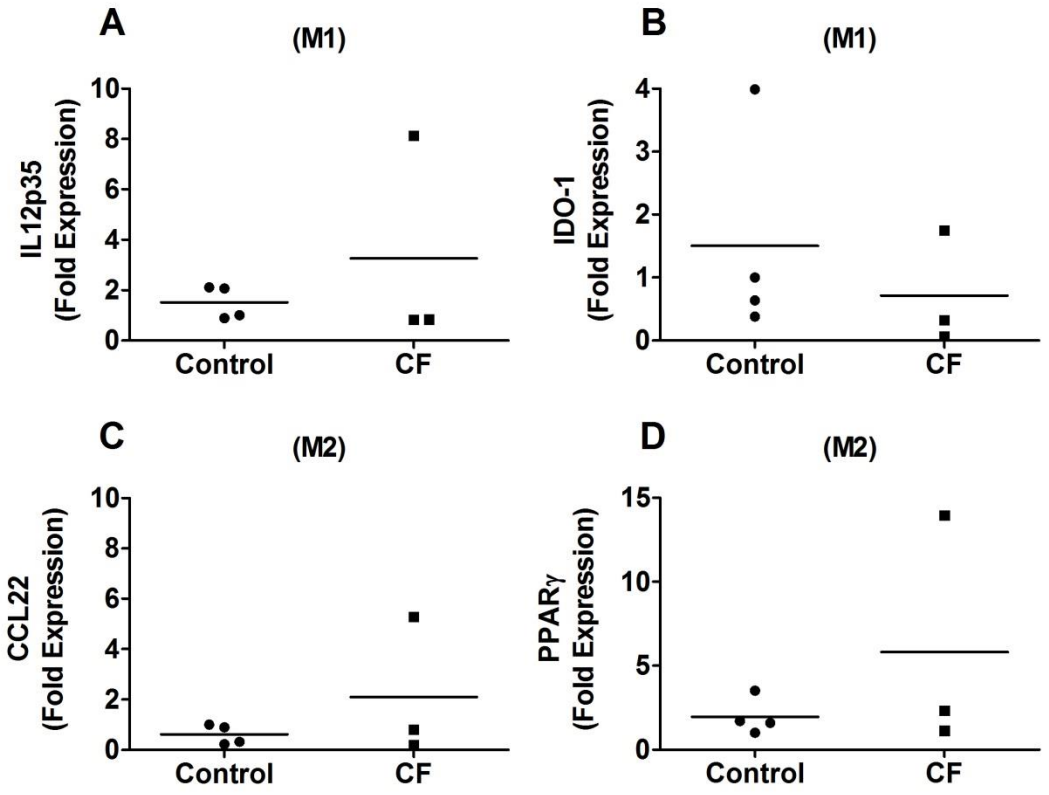

Figure 5-4 Pilot data on the abundance of transcripts characteristic of M1 and M2 polarisation phenotypes in isolated alveolar macrophages from BAL samples obtained from control children and children with CF

M1 phenotype markers; (A) IL-12 p35, and (B) IDO1, and M2 phenotype markers; (C) CCL22, and (D) PPARɔ mRNA. Results represent transcript relative abundances in isolated alveolar macrophages from Control and CF samples. Results are expressed as Fold Expression = 2^(-qpqCt) compared to a reference patient sample (n=4 control, n=3 CF samples, Lines represent Mean).

5.4.4 Developing a method for functional studies on CF alveolar macrophages

To understand the defect underlying impoverished ALOX15B mRNA expression in CF alveolar macrophages we sought to evaluate whether CF alveolar macrophages can effectively up-regulate the expression of ALOX15B mRNA following stimulation with IL4 (280).

In order to conduct this investigation we needed to obtain control and CF macrophages upon which to experiment. It has proved challenging to obtain sufficiently large quantities of primary human CF alveolar macrophages to conduct these functional studies.

We evaluated the cellular yield of PBMC derived macrophage culture from 10mls of paediatric blood obtained through the SHIELD CF Study. From 10mls of a child with CFôs blood we obtained an average of 4 million Peripheral Blood Mononuclear Cells (PBMCôs) by the Ficoll density gradient separation method (443, 444). We were successfully able to select Monocytes by adherence to tissue culture plastic, and, following culture in Complete Macrophage medium for 7-11 days, the cells had the morphological appearance of differentiated macrophages. Unfortunately, however, this method (in our hands), produced an insufficient cell yield for functional studies (on average 25,000 - 50,000 cultured macrophages and 225ng RNA).

The alveolar macrophage cell yield (quantified in 5.4.1) from BAL would be sufficient for a very limited number of experimental conditions to be evaluated by qPCR. Limitations that we encountered in using BAL derived primary CF alveolar macrophages as a source for functional studies included; infrequent access to sufficiently large volumes of uninfected BAL, and baseline variation due to inflammatory exposures *in vivo*.

5.5 Discussion

Our aim was to develop methodology to explore the underlying mechanisms regulating the resolution of inflammation in CF alveolar macrophages. We have optimised a method to perform *ex vivo* analysis of alveolar macrophage 15-LO expression and polarisation phenotype in primary alveolar macrophages obtained from BAL obtained from children with CF and controls. We have also produced pilot data to begin to quantify ALOX15BmRNA expression in an enriched alveolar macrophage population and evaluate polarisation phenotype in CF.

Furthermore, we evaluated two methods to obtain primary CF alveolar macrophages in sufficient quantities for functional studies of 15-LO regulation (particularly in response to IL4 stimulation). To this end, we concluded that PBMC derived macrophage culture from paediatric blood samples provided an insufficient quantity of cellular material. BAL derived alveolar macrophages would be sufficient in quantity, but pragmatically speaking, this is not an ideal cellular source due to the infrequent rate of access to sufficiently large volumes of uninfected BAL and baseline variation due to inflammatory exposures *in vivo*.

New strategies may be necessary to circumvent the technical hurdles posed by critically limited access to CFTR deficient alveolar macrophages. Moving forward we could consider; collaborating with our colleagues at the adult CF centres and applying for ethical permission to approach adults with CF to obtain larger volumes of blood (compared with paediatric volumes) or BAL fluid. Alternatively, we could consider using undifferentiated PBMCôs for functional study, however this strategy is limited by the recognition that ALOX15B mRNA expression is characteristic of differentiation in macrophage populations.

Another approach would be to knock down or pharmacologically inhibit CFTR in macrophages differentiated from healthy donor PBMCôs or a human monocyte cell line. Finally, CFTR deficient alveolar macrophages could be sourced by lavage from an animal model of CF. This approach could be limited by the subtle but relevant differences in animal Lipoxygenase repertoires and their regulation (445).

5.5.1 Limitations of this study

This chapter presents pilot data from a small number of childrenôs airways samples. No conclusions can be drawn from this data until the target number of samples have been analysed. We have calculated a target sample size of 15 children with CF and 15 controls for the 15-LO expression experiment based on the 15-LO differential expression results reported in Chapter 4. For the evaluation of macrophage polarisation, Murphy et al. found skewing of phenotype in a study of 48 people with CF (440), and we would need to achieve a similar sample size to be able to draw conclusions. Methodological difficulties with isolating macrophages from children with CFôs airways samples were explored in this piece of work. In recommending a viable method, a compromise was struck between cellular yield and sampling bias, however the characteristics of the sampling bias must be acknowledged. Samples obtained by adherence to tissue culture plastic represent those with a high percentage of macrophages in the differential cell count, and those relatively free from bacterial infection, since they survive and retain functionality *ex vivo*. Such samples are more likely to come from a healthier, younger cohort of people with CF. Observational and functional studies performed in this way are therefore subject to sampling bias.

5.6 Conclusion

We have optimised a method evaluate ALOX15B mRNA expression and macrophage polarisation phenotype in an *ex vivo* pure population of alveolar macrophages isolated from the BAL of children with CF and controls.

Neither PBMC derived macrophage culture, nor alveolar macrophage isolation from BAL yielded reliable access to sufficient quantities of cellular material to perform extended functional studies on CF alveolar macrophages.

In the airway 15 Lipoxygenase (LO) expression can be found in; neutrophils (275), eosinophils (276), alveolar macrophages (277) and airway epithelial cells (278). In Chapter 6, we describe our investigations of 15-LO activity in bronchial epithelia in CF.

CHAPTER 6

15 LIPOXYGENASE ACTIVITY IN CF BRONCHIAL EPITHELIUM

6 15 Lipoxygenase in CF Bronchial Epithelium

6.1 Introduction

6.1.1 15 Lipoxygenase enzyme plays a key role in the biosynthesis of Lipoxins, D- Series Resolvins and the Protectins

The activity of the 15 Lipoxygenase enzyme plays a key role in the biosynthesis of the Lipoxins (257) D- Series Resolvins (262) Protectins (270) and the PPARɔ agonists, 15-HETE, 9 & 13-HODE (271-274). SPM biosynthesis occurs by trans-cellular co-operation of two cell types, each cell contributing a separate enzymatic catalysis step. In the airway 15 Lipoxygenase (LO) expression can be found in neutrophils (275), eosinophils (276), alveolar macrophages (277) and airway epithelial cells (278). In this chapter we examine how 15-LO expression and activity in airway epithelial cells is affected by CFTR deficiency.

6.1.2 15-LO is subject to translational and allosteric regulation

Pathways for the transcriptional regulation of 15-LO expression associated with IL4/IL13, STAT6 signalling, PPARɔ and M2 polarisation in macrophages were described in Chapter 5. Beyond these, 15-LO is additionally subject to translational and allosteric regulation. 15-LO messenger RNA transcripts contain a Differentiation Control Element (DICE element) which allows HNRNPE1 and HNRNPK to repress 15-LO mRNA translation by binding to this DICE element, and preventing the recruitment of the 60S ribosomal subunit to the 48S pre-initiation complex (282). Intracellular calcium concentration regulates 15-LO catalytic activity by affecting the degree of membrane insertion, which increases its catalytic activity and is necessary for the production of specific products (283).

6.1.3 Lipoxygenase activity, protein expression and intracellular calcium regulation are affected by CFTR dysfunction

In an example of a direct effect of CFTR function upon Lipoxygenase Mattoscio et al. reported that by an effect on 12-LO activity, platelets from patients with CF generated 40% less LXA_4 than platelets from healthy subjects (351). They reported that platelets express a biologically active CFTR and CFTR blockade results in a 50% reduction in LXA_4 formation during platelet / neutrophil co-incubations (351). Furthermore, in CF, calcium signalling is amplified and protein synthesis is disturbed. More specifically, intracellular calcium stores are increased, and release from the endoplasmic reticulum and entry through the

plasma membrane are amplified in CF airway epithelial cells (192, 193, 206, 209, 210). Accumulation of mutant CFTR in the ER is thought to lead to ER stress and the activation of the unfolded protein response which leads to a reduction of protein synthesis, as an adaptive mechanism to cope with ER stress (196, 446).

6.2 Aim

The underlying basis for the failure of active resolution of inflammation in CF remains incompletely understood. Airway eicosanoid balance is disturbed in CF (370). 15-LO is expressed by bronchial epithelial cells (278) and its activity is important in the biosynthesis of several pro-resolution mediators (257, 262, 270-274). Furthermore, 15-LO is subject to transcriptional, allosteric and post-translational regulation.

We sought to investigate the impact of CFTR dysfunction upon 15-LO expression and activity in airway epithelial cells.

6.3 Hypothesis

We hypothesised that CF bronchial epithelial cells would have an impoverished ability to produce LXA_4 (the best characterised example of a 15-LO derived SPM) due to reduced 15 Lipoxygenase expression or activity.

6.4 Results

6.4.1 Transcription of 15-LO1&2 did not differ significantly between NuLi-1 and CuFi-1 cells

In order to investigate the hypothesis that CF bronchial epithelial cells have reduced 15 Lipoxygenase expression, we quantified the expression of 15-LO-1&2 enzymes (ALOX15 & ALOX15B) in well differentiated NuLi-1 and CuFi-1 epithelial cell cultures by qPCR. There was no difference in the relative expression of ALOX15 (4.3 ± 2.7; NuLi-1, n=7 vs. 9.8 ± 5.4; CuFi-1, n=9, P=NS) or ALOX15B mRNA between NuLi-1 and CuFi-1 differentiated bronchial epithelial cells (1.3 ± 0.3; NuLi-1, n=5, vs. 1.2 ± 0.3; CuFi-1, n=7, P=NS) (Figure 6-1).

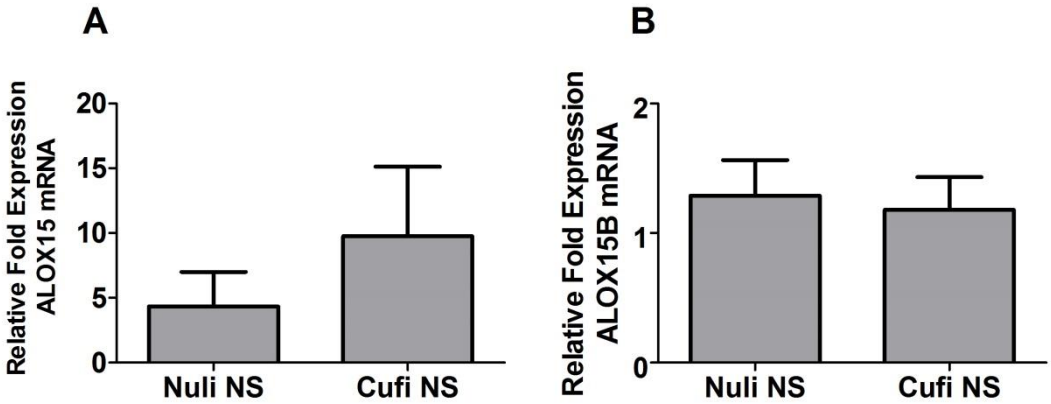

Figure 6-1 The relative quantity of mRNA transcripts for 15 Lipoxygenase-1 and 15 Lipoxygenase-2 enzymes did not differ significantly between NuLi-1 and CuFi-1 cells

(A) ALOX15, and (B) ALOX15B mRNA transcripts expressed by differentiated NuLi-1 and CuFi-1 bronchial epithelial cells were quantified by qPCR (n=5-9 epithelial preparations, normalised to a reference NuLi-1 sample, P=NS, Studentôs t-test).

6.4.2 15-LO1&2 protein expression did not differ between NuLi-1 and CuFi-1 cells

In order to investigate the hypothesis that CF bronchial epithelial cells would have reduced 15 LO expression, we quantified the expression of 15-LO-1&2 enzymes in well differentiated NuLi-1 and CuFi-1 epithelial cell cultures by Western Blotting. We found no difference in 15-LO 1 (0.9 ± 0.1; NuLi-1, n=7, vs. 0.7 ± 0.2; CuFi-1, n=5, P=NS), or 15-LO 2 protein expression (1.1 ± 0.1; NuLi-1, n=3, vs. 1.6 ± 0.4; CuFi-1, n=3, P=NS) between NuLi-1 and CuFi-1 cells (Figure 6-2).

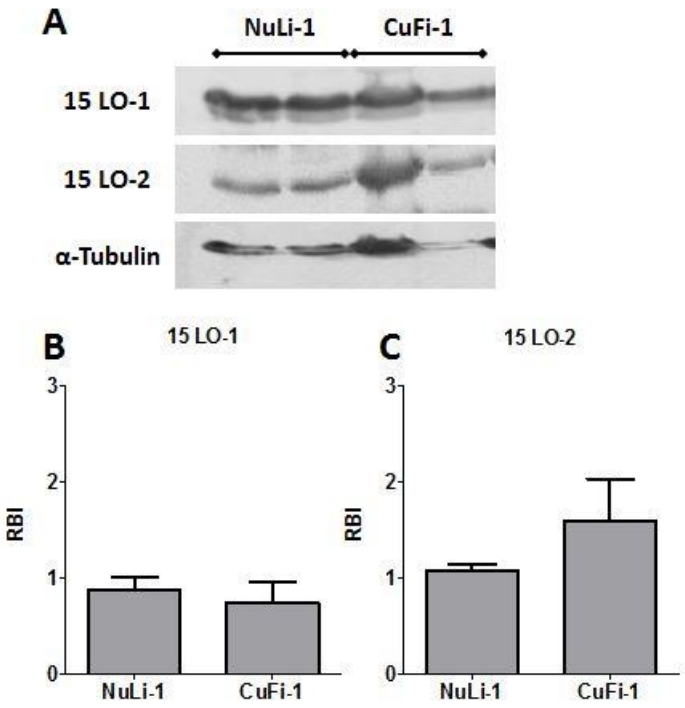

Figure 6-2 15-Lipoxygenase 1 and 15-Lipoxygenase 2 protein expression did not differ significantly between NuLi-1 and CuFi-1 cells

(A&B) 15-LO1 and (A&C) 15-LO2 protein expression by NuLi-1 and CuFi-1 bronchial epithelial cells was quantified by Western Blotting. (B&C) Band densitometry was performed and results are presented as relative band intensities normalised by Ŭ-Tubulin. Representative blots are presented (upper part of panel), (n=3-7 epithelial preparations, P=NS, Studentôs t-test).

6.4.3 Total 15 LO activity did not differ between NuLi-1 and CuFi-1 cells

In order to investigate the hypothesis that CF bronchial epithelial cells would have an impoverished ability to produce LXA_4 due to reduced 15 Lipoxygenase activity, we measured the ability of tissue expressed 15-LO to synthesise LXA_4 from its synthetic precursor 5(S),6(R) Di-HETE. LXA_4 thus produced, was measured by ELISA. There was a non-significant trend towards lower 15-LO tissue activity in CuFi-1 cells as reflected by a lower mean LXA_4 generated (4.3 ± 0.4ng/ml LXA_4; NuLi-1 cell preparation (n=21), vs. 3.7 ± 0.24ng/ml LXA_4; CuFi-1 cell preparation (n=18), P=0.2 (NS)) (Figure 6-3).

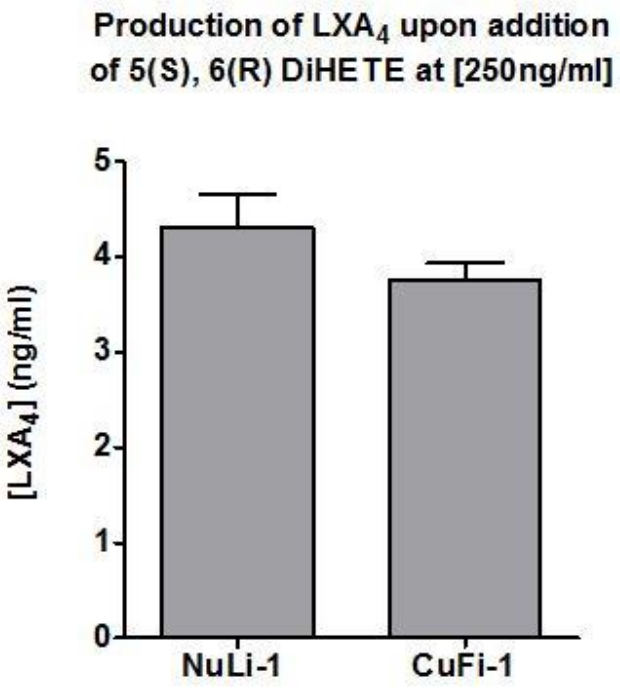

Figure 6-3 Total 15 Lipoxygenase activity did not differ significantly between NuLi-1 and CuFi-1 cells

Total 15-LO activity was measured by exposure of NuLi-1 and CuFi-1 epithelial cells to exogenous 5(S),6(R)-DiHETE and LXA_4 production was quantified in the medium by ELISA. There was no significant difference between NuLi-1 and CuFi-1 cells with respect to LXA_4 generated (n=18-21 epithelial preparations, P=NS, Studentôs t-test).

6.4.4 LXA_4 did not affect the transcription of 15 LO in NuLi-1 or CuFi-1 differentiated bronchial epithelial cells

Examination of the kinetics of LXA_4 biosynthesis in tissues (reported in mouse models by Levy et al.) revealed a pattern whereby a very small amount is generated initially, followed by a rapid amplification in its concentration (264). Those authors also reported that PGE2 up-regulated 15-LO expression in neutrophils. This rapid amplification of LXA_4 biosynthesis reported gave rise to the hypothesis that LXA_4 might stimulate feed forward amplification of its own synthetic capacity by up-regulating 15-LO expression. This hypothesis did not bear out in NuLi-1 or CuFi-1 bronchial epithelial cells; LXA_4 administration at a low concentration (1nM) had no significant effect on ALOX15 (n=7 NuLi-1 preparations & n=9 CuFi-1 preparations) or ALOX15B mRNA transcript abundance (n=5 NuLi-1 preparations & n=7 CuFi-1 preparations) in NuLi-1 or CuFi-1 cells (Figure 6-4). Furthermore, PGE2 (4 hours exposure, 100nM) administration did not significantly affect ALOX15 or ALOX15B mRNA transcript abundance in NuLi-1 differentiated bronchial epithelial cells (n=3, data not presented).

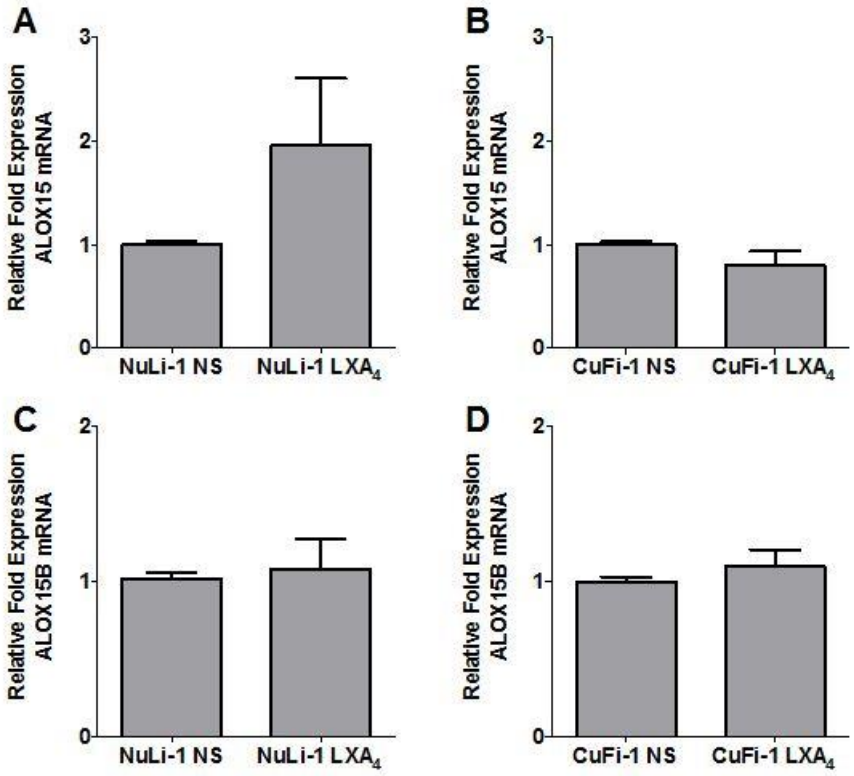

Figure 6-4 LXA_4 did not affect 15-LO transcription in NuLi-1 or CuFi-1 differentiated bronchial epithelial cells

(A&B) ALOX15 mRNA and (C&D) ALOX15B mRNA transcripts were quantified by qPCR in (A&C) NuLi-1 and (B&D) CuFi-1 cells exposed to either vehicle control (NS) or LXA_4 (1nM) overnight (n=5-9, P=NS, Studentôs t-test).

6.5 Discussion

In this chapter we report that airway epithelial 15-Lipoxygenase transcription, expression and activity is broadly unaffected by CFTR deficiency. We found no evidence of 15-LO transcriptional regulation by LXA_4 or by Prostaglandin E2 in airways epithelia.

We found no difference in 15-LO type 1 or 2 transcription, protein expression or activity between NuLi-1 and CuFi-1 cells. Our findings are in agreement with the findings of Bickford et al. (447) who found no difference in unstimulated ALOX15 or ALOX15B expression between IB3.1 cells (bronchial epithelial cell line from a CF patient (qF508/W1282X)) and S9 cells (CFTR-corrected cell line).

Whilst regulation of 15-LO transcription by PGE2 has been described in neutrophils (264), we found no evidence for PGE2 regulation of 15-LO in bronchial epithelia. This is consistent with cell type specific regulation of 15-LO transcriptional regulation. Whilst one group reported that 15-LO2 was subject to feedback regulation by a related 15-LO product, 15-HETE (448), we found no evidence of direct feedback regulation of 15-LO by LXA_4.

6.5.1 Limitations of this study

The method used to evaluate 15-LO activity by an epithelial monolayer has limitations. The ELISA antibody used to detect LXA_4 has some crossover affinity for the 5(S),6(R)-DiHETE substrate used by 15-LO to produce LXA_4. Therefore, it is possible that a true difference in 15-LO activity between CuFi-1 and NuLi-1 cell preparations has been masked by the contaminating effect of 5(S),6(R)-DiHETE substrate on the method of LXA_4 quantification. This could be further tested by measuring each of these moieties more specifically by LC-MS/MS.

6.6 Conclusions

In agreement with the findings of Bickford et al. (447) in CF cell bronchial epithelial cell lines we found that airway epithelial 15-LO transcription, expression and activity are broadly normal in this CF bronchial epithelial cell line.

CHAPTER 7

PRO-RESOLUTION MEDIATOR CHARACTERISATION DURING PULMONARY EXACERBATION OF CYSTIC FIBROSIS IN CHILDREN

7 Pro-Resolution Mediator characterisation during Pulmonary Exacerbation of Cystic Fibrosis in children

7.1 Exploring the relationship between pro-resolution lipid mediators and clinical outcomes of CF Pulmonary Exacerbation

In this chapter we tested the hypothesis that endogenous Pro-resolution mediator production in the airway affects the course of recovery from Cystic Fibrosis Pulmonary Exacerbation (CFPE).

The Epidemiologic Study of Cystic Fibrosis, reported that 23% of children under 6, 32% of 6-12 year olds, and 48% of 13-17 year olds with CF require antibiotic treatment for CFPE annually (449). Pulmonary exacerbations are associated with reduced overall survival (450), deterioration in lung function (369) and increased bronchiectasis (451, 452) and have a profoundly negative impact on health related quality of life (453).

Despite current best practice management, patients may not completely recover their baseline lung function after CFPE (368). Children with CF accumulate clinical management problems over time associated with the heavy reliance on conventional antibiotic use, such as antibiotic side effects and the emergence of multi-drug resistant bacterial species. In this landscape there is an unmet need for new adjuvant therapies in the management of CFPE. The ñidealò antibiotic adjuvant might; hasten the resolution of infection and inflammation; shorten the required duration, or minimise the required doses of antibiotic treatment; and / or, limit the extent of structural pulmonary damage incurred during exacerbation.

7.1.1 European consensus definition of CFPE

CFPE is defined as the clinical need for intravenous antibiotics, as indicated by presence of at least 4 of 12 possible signs or symptoms (Fuchs criteria (115, 372));

- Change in sputum volume or colour
- New or increased haemoptysis
- Increased cough
- Increased dyspnoea
- Increased malaise, fatigue or lethargy

- Temperature over 38°C
- Anorexia or weight loss
- Sinus pain or tenderness
- Change in sinus discharge
- Change in physical ýndings on examination of the chest
- Decrease in pulmonary function by 10% or more
- Radiographic changes

7.1.2 Pathophysiology of CFPE

Very little is known about the pathophysiology of CFPE (454). Exacerbations are thought to be related to perturbations in the complex relationship between host defence and airway microbiology that impacts on sputum production and airflow obstruction (454). Microbiological factors implicated in the pathogenesis of CFPE include; viral infection such as RSV (455, 456); acquisition of new bacterial pathogens (454); clonal expansion of colonizing bacteria, especially in those chronically infected with *P. aeruginosa* (457-459). The inflammatory response to bacterial changes in the airway during CFPE is characterised by; increased neutrophil abundance, IL8, TNFŬ, and free Neutrophil Elastase (460, 461). These inflammatory mediators decrease in response to antibiotic treatment of CFPE (461).

7.1.3 Current Treatment of CFPE

Patients with pulmonary exacerbations are frequently hospitalized and treated with intravenous antibiotics, augmented airway clearance therapy and nutritional support (462). Consistent with a significant role played by bacterial infection in CFPE, intravenous antibiotics have been shown to improve both clinical symptoms and lung function (457, 463). There is debate and active study concerning the optimum duration of intravenous antibiotic treatment for CFPE (464). It is widely recognised that the optimal duration of antibiotic therapy may be shorter than is current practice (368).

7.1.4 SPMôs as antibiotic adjuvants

SPMôs including the Lipoxins, D & E Series Resolvins, Protectins and Maresins, are differentially and temporally regulated during infection and have both pro-

resolution and anti-microbial properties (253, 308, 316). In animal models of infection, SPMõs enhance bacterial containment and reduce antibiotic requirements for bacterial clearance (316). In a previous study, Chiron et al reported that after antibiotic therapy for CFPE, the levels of LXA_4 measured in sputum were significantly increased compared to before treatment and this was inversely correlated to IL8 content (354). Mice treated with analogues of LXA_4 and subsequently challenged with *P. aeruginosa* were better able to contain the bacterial challenge resulting in decreased pulmonary bacterial burden (315). In mouse models of peritoneal *E. Coli* infection, RvD1 and Resolvin D5 reduced bacterial titres in blood and exudates, reduced hypothermia and increased survival (316). Adjuvant treatment with ciprofloxacin and a panel of SPMõs (RvD1, Resolvin D5, Protectin D1) heightened host antimicrobial responses, accelerated resolution, and shortened resolution intervals in E-Coli infected mice compared to antibiotics alone (316). In mouse skin infection models, the same panel of SPMõs enhanced vancomycin clearance of *Staphylococcus aureus* (316). In a mouse peritoneal sepsis model, Resolvin D2; reduced local and systemic bacterial burden, excessive cytokine production (IL6, IL1ß, IL23, PGE2, LTB4 and TNFŬ), and neutrophil recruitment; and increased peritoneal mononuclear cells and macrophage phagocytosis (308) resulting in improved survival of the mice from sepsis (308). These lipid mediators are thought to be non-redundant in their function; however, the tissue and disease specific functional roles of individual mediators within this class remain open questions.

7.1.5 SPMõs in clinical drug development

Several SPMõs are in clinical drug development programmes including Protectin D1 (for neurodegenerative diseases), and Maresin 1 (253). Resolvin E1 and synthetic analogues have been formulated for oral, IV and subcutaneous routes of administration, and Phase I-II clinical studies have demonstrated efficacy across a range of inflammatory disease models, including; asthma, colitis, rheumatoid arthritis, atherosclerosis, dry eye and retinopathy (366, 367).

7.1.6 PPARɔ ligands as lipid Pro-Resolution Mediators

PPARɔ agonists have also been proposed for therapeutic application in CF (331). PPARɔ is a ligand-activated transcription factor that mediates the anti-inflammatory effects of a number of essential fatty acid derived lipid mediators.

PPARɔ expression and activation is up-regulated during the resolution of inflammation where it plays roles in macrophage directed active resolution and wound repair (329, 330).

Potent endogenously produced PPARɔ ligands include;

- Prostaglandin type pro-resolving lipid 15-d-Prostaglandin J2 (15-d-PGJ2) (331, 332)
- 12/15 Lipoxygenase metabolites, 12-HETE and 15-HETE (274, 331, 332)
- SPM parent compounds EPA & DHA (332)
- Oxidation products of linoleic acid formed either non-enzymatically, or via the action of 15 Lipoxygenase, 9-HODE & 13-HODE (332)

7.1.7 PPARɔ agonists currently in clinical practice

The thiazolidinediones are a class of PPARɔ agonists which are approved for use in diabetes mellitus therapy. These include; Rosiglitazone, Pioglitazone (restricted) and Lobeglitazone.

7.1.8 SPMôs and PPARɔ agonists as pro-resolution candidate antibiotic adjuvants for CFPE

Despite accumulating in vitro experimental evidence to suggest that SPMôs and PPARɔ agonists offer therapeutic opportunities for CF (85, 331, 356-358, 465), to our knowledge, neither has been evaluated as an adjuvant to antibiotic therapy in CFPE.

7.2 Aims

Pro-Resolution lipid mediators could represent promising antibiotic adjuvants for CFPE therapy. In order to establish the basis for Pro-Resolution lipid mediator application in therapy it was first necessary to characterise their native *in vivo* production during CFPE, and to understand how that relates to clinical resolution of CFPE. Thus, we designed a paediatric observational cohort study (the Pro-Resolution mediators in CF Exacerbation (PRINCE) study) with the following specific aims:

- To characterise the temporal evolution of Pro-Resolution lipid mediators in CFPE in childhood.

- Compare variations in the Pro-Resolution lipid mediator profile in CFPE to clinical outcome measures of the resolution of exacerbation.

7.3 Hypothesis

The kinetics and amplitude of Pro-Resolution lipid mediator production in the airway correlates with the time course of clinical resolution in CFPE.

Primary End-Point

A description of the relationship between airway concentrations of Pro-Resolution lipid mediators and the extent of FEV_1 recovery.

7.4 Results

7.4.1 Population characteristics of PRINCE study participants

Ten episodes of CFPE were captured in this study from 8 children (Table 7-1). Two female children with CF were enrolled twice for distinct episodes separated by at least 3 months. At the time of study conduct there were no reported studies describing the normal airway concentrations of pro-resolution lipid mediators in children. In order to characterise pro-resolution mediator production in a non-CF airway context, five inpatients (at Our Ladyôs Childrenôs Hospital, Crumlin) that did not have CF, but were productive of sputum were recruited as a disease control group.

Four children in the control group had tracheostomies in situ; two for severe tracheobronchomalacia, one for subglottic stenosis, and a fourth for tracheal stenosis. All of the children with tracheostomies in situ were long term hospital inpatients and were stable at the time of sputum sampling. One child in the control group had primary ciliary dyskinesia and was afebrile, but more productive than usual at the time of sampling.

All participants contributed at least one sputum sample per episode. At least three sputum samples were captured for eight episodes of CFPE. One child with CF had a nasogastric tube inserted after the first time-point and did not contribute further sputum samples due to discomfort and another child with CF was transferred to another hospital to complete her treatment after the first time-point. During four episodes of CFPE, the child became ñdryò of sputum before Day 11

and could not contribute a fourth sample by spontaneous expectoration. A fifth child completed treatment and was discharged prior to the last sampling time point. Sputum samples were captured at all four time-points for three episodes of CFPE.

Nine out of the ten episodes of CFPE occurred in female children, whereas all of the control children were boys. The mean age of children with CF at the time of exacerbation was significantly higher than the mean age of children participating in the control group (12.7 ± 2.6 years; CF group, n=10 episodes in 8 children, vs. 2.4 ± 2.7 years; control group, n=5 episodes in 5 children, $P<0.001$).

Seven episodes of CFPE occurred in 5 children who were homozygous for the Phe508del CFTR mutation. Both of the children who were enrolled twice were homozygous for the Phe508del CFTR mutation. Three children were heterozygous for the Phe508del CFTR mutation.

Table 7-1 Population characteristics of PRINCE participants

Data is presented for CFPE episodes and the Control cohort. (***P<0.001 between group unpaired comparison, Studentôs t-test).

	CF Cohort	**Control Cohort**
Inpatient Episodes Captured (n) **Children**	10 8	5 5
Samples Contributed per Episode	Ó1 Time-point (10/10) Ó2 Time-points (8/10) Ó3 Time-points (8/10) Ó4 Time-points (3/10)	1 Time-point (5/5)
Sex	1/7 (M/F)	5/0 (M/F)
Age **(Range)** **(Mean & SD)**	8 ï 17 years 12.7 ± 2.6 years***	4 months ï 7 years 2.4 ± 2.7 years***
CFTR Mutations **(Phe508del / Phe508del)** **(Phe508del / Other CF Mutation)**	n=7 episodes in 5 children n=3 episodes in 3 children	-
Relevant Medical Diagnoses	-	Tracheostomy in situ (4) Primary Ciliary Dyskinesia (1)

7.4.2 Clinical characteristics CFPE episodes

The baseline lung function of participating children with CF was in the range of mild - moderate lung disease (8) (Table 7-2).

**I was trained in spirometry by the staff in the pulmonary function lab at OLCHC, and I performed the spirometry for the PRINCE Study. Baseline spirometry measurements were obtained from the medical records.*

In five out of ten episodes of CFPE among 5 children, the childôs peak post exacerbation FEV_1 (best FEV_1 within a month of the initiation of treatment) recovered to at least the pre-exacerbation baseline level. In five out of ten episodes of CFPE among 5 children, the childôs peak post exacerbation FEV_1 did not recover fully to the pre-exacerbation baseline level. Both children who were re-enrolled had one episode of full recovery and one episode where their peak post exacerbation FEV_1 did not recover to the pre-exacerbation baseline level. There was no significant difference in baseline FEV_1 between these two groups of children (77.8 ± 17.1% predicted, n=5 episodes in 5 children; Baseline FEV_1 recovered, vs. 84.2 ± 5.2% predicted, n=5 episodes in 5 children; Peak FEV_1 < Baseline group, Mean ± SD, P=NS, Student t-test) (Table 7-2).

Among the episodes for which the childôs peak post exacerbation FEV_1 did not recover to the pre-exacerbation baseline level, the mean loss was 6.6% of predicted FEV_1, with a range from 2% loss to 13% loss (n=5 episodes in 5 children) (Table 7-2).

During most episodes of CFPE children had two pathogens isolated by conventional culture from their sputum (n=8 episodes). The most common organisms isolated were *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Between 9 and 13 days of antibiotic treatment were required for four episodes of CFPE among 3 children. 14 or more days of antibiotic treatment (up to a maximum of 20 days) were required for six of the CFPE episodes among 5 children.

Table 7-2 Clinical characteristics of CFPE Episodes

Data is presented on baseline and post exacerbation FEV_1 measurements, sputum microbiology and duration of antibiotic treatment. *Baseline FEV1 defined as the best reliable FEV_1 recorded in the 3 months prior to exacerbation. #Peak FEV_1 Post exacerbation was defined as the best reliable FEV_1 recorded for that child within a month following the initiation of treatment.

	Characteristics of CFPE Episodes
***Baseline FEV_1** **(% Predicted)**	51 ï 96 % (Range) 81.0 ± 12.4 % (Mean & SD)
#Peak Post Exacerbation FEV1: **ÓBaseline FEV_1** **< Baseline FEV_1** **(Number of CFPE Episodes)**	 5 / 10 among 5 children 5 / 10 among 5 children
œFEV$_1$ loss (% Predicted) **Peak Post Exacerbation FEV_1** **< Baseline FEV_1 sub-group**	 -6.6 ± 1.8% (Mean & SD) -2% Ÿ -13% (Range)
Sputum Microbiology Characteristics **(No. CFPE Episodes)**	1 pathogenic species (1/10) 2 pathogenic species (8/10) Ó3 pathogenic species (1/10) *Pseudomonas aeruginosa* (7) *Staphylococcus aureus* (5) *Candida spp.* (3) *Haemophilus influenzae* (2) *Aspergillus spp.* (2) *Stenotrophomonas maltophilia* (1)
Duration of Antibiotic Rx **No. CFPE Episodes**	Range: 9 Ÿ 20 Days Mode: 14 Days <14 Days (4/10 episodes, 3 children) Ó14 Days (6/10 episodes, 5 children)

7.4.3 Evolution of symptom scores during CFPE

In order to quantify perceived changes in the childõs respiratory and systemic symptoms during treatment for CFPE, a Respiratory and Systemic Symptoms Questionnaire© was administered at three time-points (Day 1-2, Day 3-5, Day 6-12). Positive symptom scores represent a symptom that is õmore than normalò, zero represents a symptom that is õas normalò, and negative scores represent a symptom that is õless than normalò.

Childrenõs symptom scores improved during the course of treatment for CFPE (Mean scores ± SEM are reported) (Figure 7-1). Sputum colour improved from the first time-point to the third time-point (1.2 ± 0.2; Day 1-2, n=5 episodes in 5 children, vs. 0.2 ± 0.4; Day 6-12, n=5 episodes in 5 children) (Figure 7-1A). Sputum thickness (1.4 ± 0.3; Day 1-2, n=5 episodes in 5 children, vs. 0.2 ± 0.4; Day 6-12, n=5 episodes in 5 children) (Figure 7-1B) and production improved over the course of CFPE (1.2 ± 0.4; Day 1-2, n=5 episodes in 5 children, vs. 0.2 ± 0.4; Day 6-12, n=5 episodes in 5 children) (Figure 7-1C).

Increased cough frequency was reported at Day 1-2 and normalised by Day 6-12 (1.0 ± 0.3; Day 1-2, n=5 episodes in 5 children, vs. 0.0 ± 0.4; Day 6-12, n=5 episodes in 5 children) (Figure 7-1D). Cough intensity peaked at Day 3-5 and normalised by Day 6-12 (0.8 ± 0.3; Day 3-5, n=6 episodes in 6 children, & 0.0 ± 0.3; Day 6-12, n=5 episodes in 5 children) (Figure 7-1E). Chest congestion improved somewhat over the course of treatment (1.0 ± 0.4; Day 1-2, n=5 episodes in 5 children, vs. 0.4 ± 0.2; Day 6-12, n=5 episodes in 5 children) (Figure 7-1F).

Symptom scores for fatigue (1.2 ± 0.4; Day 1-2, n=5 episodes in 5 children, vs. 0.8 ± 0.4; Day 6-12, n=5 episodes in 5 children) (Figure 7-1G) and dyspnoea on exertion (0.8 ± 0.4; Day 1-2, n=5 episodes in 5 children, vs. 0.4 ± 0.2; Day 6-12, n=5 episodes in 5 children) (Figure 7-1H) did not improve greatly over the time course. Reduced exercise tolerance was not a prominent complaint and changed little over the time course (0.2 ± 0.7; Day 1-2, n=5 episodes in 5 children, vs. 0.4 ± 0.2; Day 6-12, n=5 episodes in 5 children) (Figure 7-1I).

Although subjective weight scores increased to a level ñbetter than normalò (-0.8 ± 0.4; Day 1-2, n=5 episodes in 5 children, vs. 0.4 ± 0.5, Day 6-12, n=5 episodes in 5 children) (Figure 7-1J), childrenôs appetites did not improve over the time-course (-0.8 ± 0.4; Day 1-2, n=5 episodes in 5 children, vs. -1.0 ± 0.4; Day 6-12, n=5 episodes in 5 children) (Figure 7-1K).

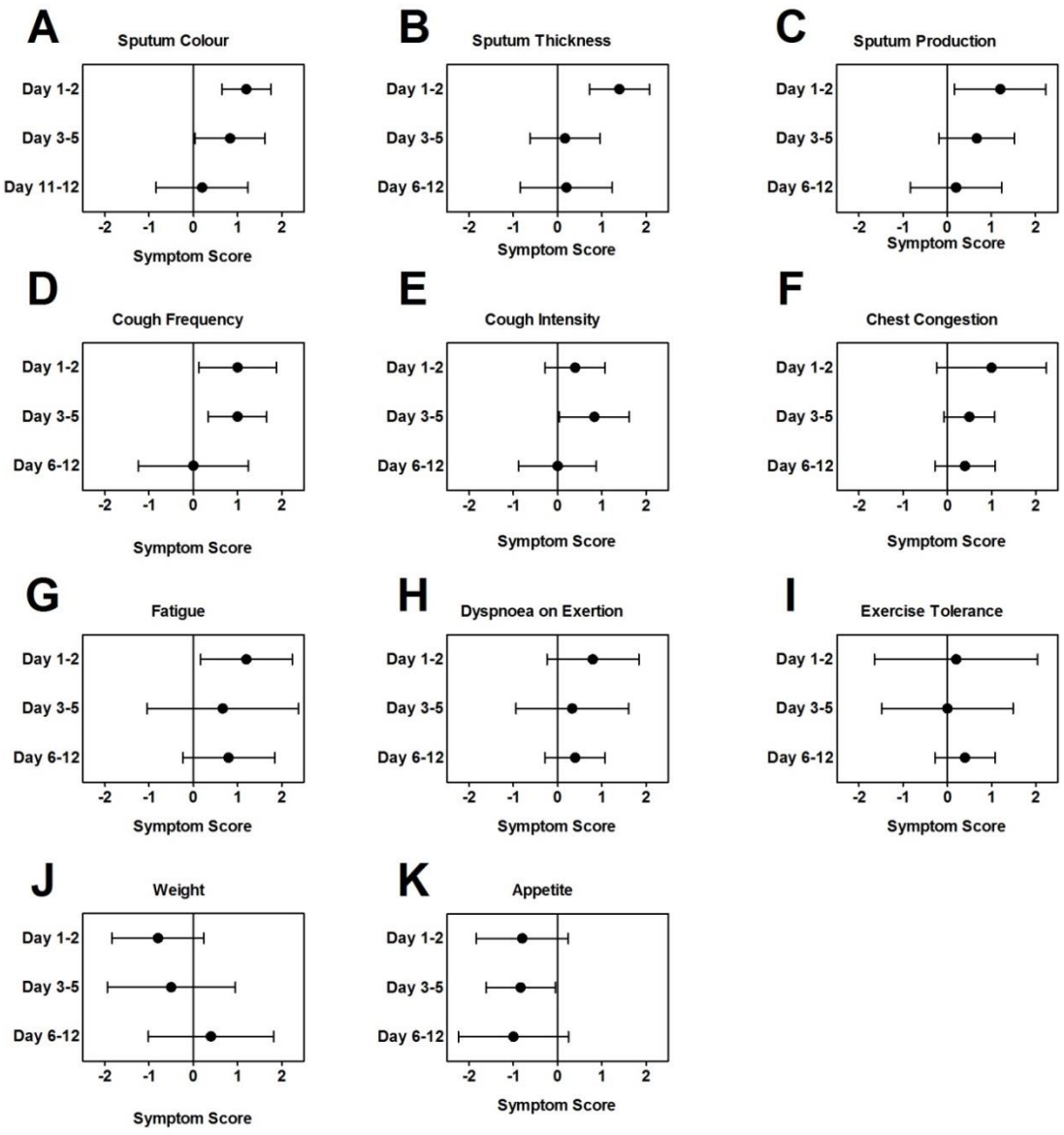

Figure 7-1 Evolution of symptom scores during CFPE

Respiratory and Systemic Symptoms Questionnaire© was administered at three time-points (Day 1-2, Day 3-5, Day 6-12 (y-axis)) to children during CFPE. Symptom scores are presented; positive scores represent ñmore than normalò, zero is ñas normalò, and negative scores represent ñless than normalò, (A) Sputum colour, (B) Sputum Thickness, (C) Sputum Production, (D) Cough Frequency, (E) Cough Intensity, (F) Chest Congestion, (G) Fatigue, (H) Dyspnoea on Exertion, (I) Exercise Tolerance, (J) Weight, and (K) Appetite (Results are presented as Mean and 95% Confidence Intervals, childrenôs responses on Day 1-2 (n=5 episodes in 5 children), Day 3-5 (n=6 episodes in 6 children), Day 6-12 (n=5 episodes in 5 children).

7.4.4 Total cell count and IL8 concentration in sputum during CFPE

The inflammatory response during CFPE involves an increase in sputum neutrophils and IL8 concentration, which reduce following antibiotic treatment (460, 461). We obtained spontaneously expectorated sputum samples from children over the time course of CFPE. Sputum was processed to obtain a cell pellet and supernatant. A cell count was performed on the cell pellet by trypan blue exclusion and IL8 was measured in the supernatant by ELISA. Cytospin slides were prepared from the processed cell pellet and stained with Haematoxylin and Eosin.

Examination of cellular morphology revealed the vast majority (>90%) of cells found in sputum across the time course of CFPE to be neutrophils with a small quantity of squamous epithelial cell contamination. In agreement with previous reports, there was a trend (non-significant) towards a reduction in the total cellularity of sputum across the time course of CFPE (71,350 ± 21,940 cells/mg sputum; Day 1-2 (n=7 episodes in 6 children), vs. 25,590 ± 8,490 cells/mg sputum; Day 6-8 (n=7 episodes in 6 children), Mean ± SEM, P=0.07, ANOVA) (Figure 7-2A) (460, 461).

Mean IL8 concentration in sputum did not change significantly over the course of CFPE (960 ± 306ng/ml; Day 1-2 (n=7 episodes in 6 children), vs. 1080 ± 510ng/ml; Day 6-8 (n=6 episodes in 5 children), Mean ± SEM, P=NS, ANOVA) (Figure 7-2B). A non-significant trend towards reducing IL8 concentration over time may have been masked by one childôs measurements which were particularly high and rose over the course of CFPE. That patient did not recover baseline FEV_1 post exacerbation.

There was no significant difference in the total sputum cell count or IL8 concentration measured from sputum at all time-points between full baseline FEV_1 recovery episodes, and incomplete FEV_1 recovery episodes (n=5 episodes in 5 children, data not illustrated, P=NS, Student t-test).

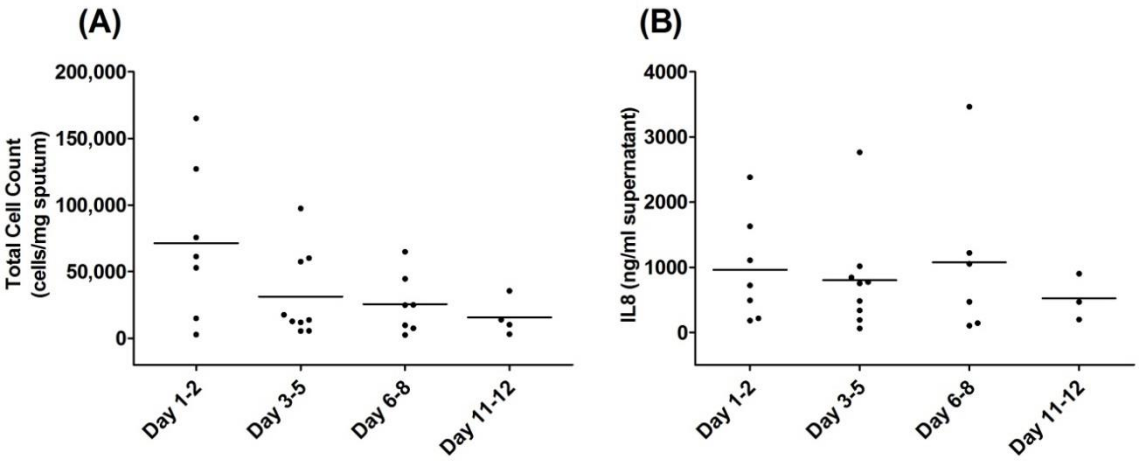

Figure 7-2 Total cell count and IL8 concentration measured from sputum of children with CF at various time points during CFPE

Sputum was processed to obtain a cell pellet and supernatant. A total cell count was obtained by the trypan blue exclusion method on the cell pellet and normalised by the weight of the original unprocessed sputum sample. IL8 in the processed sputum supernatant was measured by ELISA. (A) Sputum Total cell count (cells/mg sputum) at 4 time points during CFPE. (B) Sputum IL8 at 4 time points during CFPE (Day 1-2, n= 7 episodes in 6 children, Day 3-5, n=9 episodes in 8 children, Day 6-8, n=6 episodes in 5 children, Day 11-12, n=3-4 episodes in 3-4 children) (Lines represent Mean, P=NS, ANOVA).

7.4.5 SPMâs found in Control and CF Sputum

In order to characterise the sputum levels of Pro-Resolution lipid mediators during CFPE, we obtained spontaneously expectorated sputum samples from children over the time course of CFPE. Specific lipid mediators were quantified in unprocessed sputum plugs by the LC ï MS/MS method by our collaborator, Dr Justine Bertrand-Michel at the Lipidomic Platform in Toulouse. Spontaneously expectorated sputum samples were obtained from control inpatient children and analysed by the same method as a baseline comparison.

Fifteen SPMâs have been described in the literature to date (253). At the time of analysis, internal standards were commercially available for only six SPMâs and one SPM pathway intermediate. Consequently, the following were quantified by LC ï MS/MS; LXA_4, LXB_4, RvD1, Resolvin D2, 18-HEPE, Protectin Dx and 7-Maresin 1.

LXB4, RvD1 and 7-Maresin 1 were examined, but not detected in any control or CF sputum sample analysed.

LXA_4, 18-HEPE and Protectin Dx were identified in a small number of samples. LXA_4 was detected in one control sample, and three CFPE sputum samples from 3 children (Figure 7-3A).

18-HEPE is a precursor of the E-Series Resolvins. It is not itself bioactive, but is rapidly converted to RvE1 or E2 via trans-cellular biosynthesis (261). 18-HEPE was detected in one control and one CFPE sample on Day 1-2 (Figure 7-3C).

Protectin DX is a bioactive stereoisomer of Protectin D1 (296). Protectin Dx was reported to inhibit platelet activation and block neutrophil infiltration in a mouse model of peritonitis (296, 466). Protectin Dx was detected in one CFPE sample (Figure 7-3D).

Resolvin D2 (RvD2) was not detected in any control sample, but was detected in 11 out of 28 CF samples (Figure 7-3B). As such, RvD2 was detected in sputum with sufficient frequency to allow us to attempt further analysis.

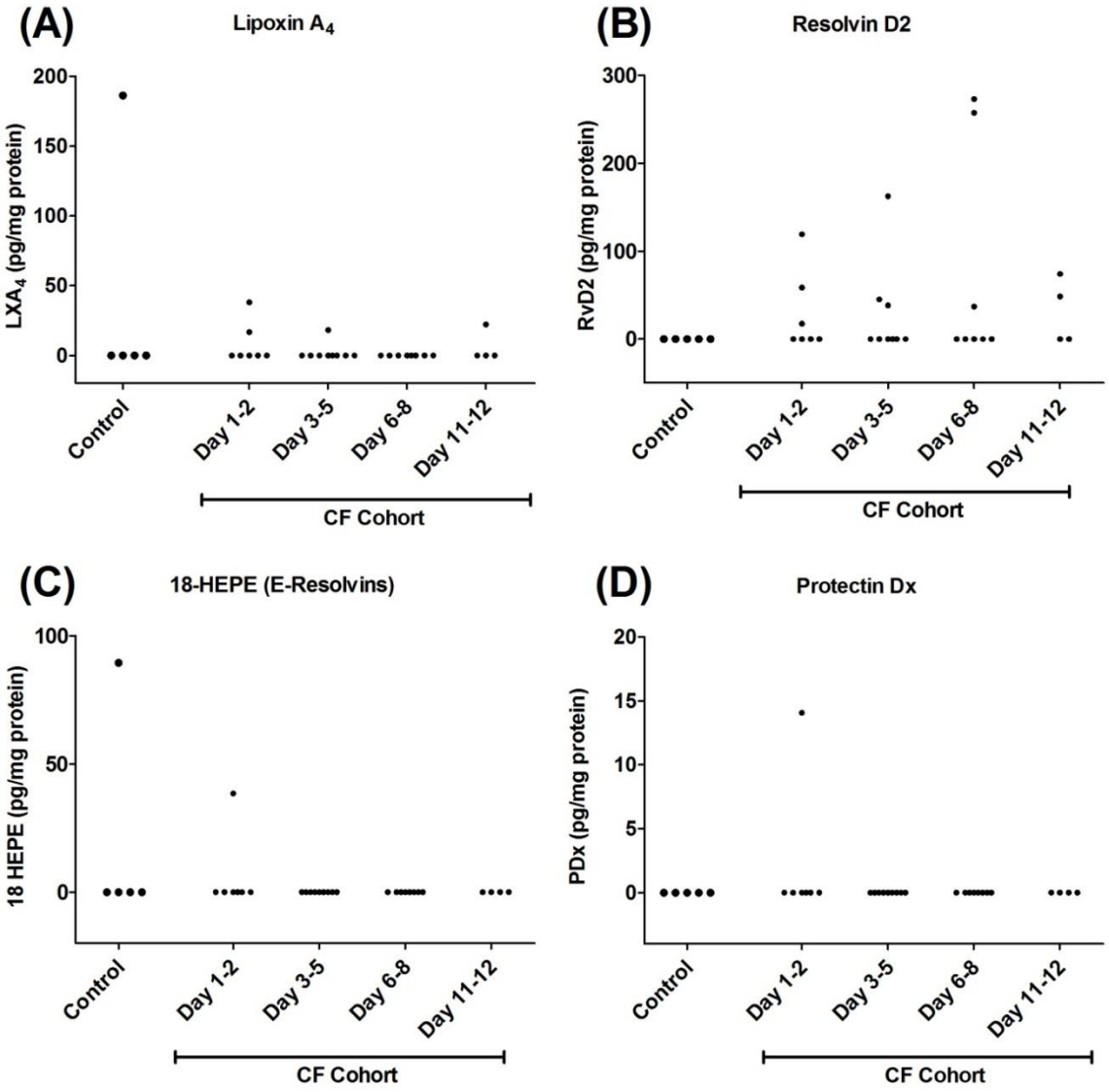

Figure 7-3 SPMõs detected in sputum

Spontaneously expectorated sputum samples were obtained from control inpatient children (first column) and children being treated for CFPE at four time-points (Day 1-2 (n=7 samples), Day 3-5 (n=9 samples), Day 6-8 (n=8 samples) and Day 11-12 (n=4 samples)). Specific lipid mediators were quantified in unprocessed sputum plugs by the LC ï MS/MS method; (A) LXA_4 (pg/mg protein), (B) Resolvin D2 (pg/mg protein), (C) Precursor of the E-Series Resolvins; 18-HEPE (pg/mg protein), (D) Protectin Dx (pg/mg protein). **LC-MS/MS performed by Dr Justine Bertrand-Michel at the Lipidomic Platform in Toulouse.*

7.4.6 Higher Resolvin D2 in sputum was associated with full recovery of pre-exacerbation baseline FEV_1

The aim of this study was to relate pro-resolution mediator production during CFPE to indicators of the clinical resolution of CFPE. The primary outcome was pre-defined as the extent of FEV_1 recovery. Therefore, we conducted a sub-group analysis comparing pro-resolution mediators detected in sputum with FEV_1 recovery post CFPE.

CFPE episodes were divided into two sub-groups based on whether the peak post-exacerbation FEV_1 was less than, or equal to/greater than the pre-exacerbation baseline measurement. Five episodes of CFPE occurring in 5 children were thus designated ñfull baseline FEV_1 recoveryò episodes, and five episodes of CFPE occurring in 5 children were designated as ñincomplete baseline FEV_1 recoveryò episodes.

Sputum RvD2 concentration was compared between sub-groups at all time-points (Figure 7-4A) and over the time course of CFPE (Figure 7-4B). In samples from the ñincomplete baseline FEV_1 recoveryò CFPE episodes (n=14 measurements during 5 episodes in 5 children), RvD2 was detected only once. 10 of the 14 sputum samples from ñfull baseline FEV_1 recoveryò episodes (n=14 measurements during 5 episodes in 5 children) had a measurable amount of RvD2 detected. The mean sputum concentration of RvD2 at all time-points in the ñfull baseline FEV_1 recoveryò subgroup was significantly higher than among the ñincomplete baseline FEV_1 recoveryò subgroup (78 Ñ 25pg/mg protein; FEV_1 ÓBaseline (n=14 measurements during 5 episodes in 5 children), vs. 3 ± 3pg/mg protein; FEV_1 < Baseline (n=14 measurements during 5 episodes in 5 children), P<0.01, Student t-test) (Figure 7-4A).

Mean sputum RvD2 examined over the time course of CFPE was significantly higher among the ñfull baseline FEV_1 recoveryò subgroup than among the ñincomplete baseline FEV_1 recoveryò subgroup (n=14, P<0.05, 2 way ANOVA), and higher at Day 6-8 than on any other time-point among children who recovered their baseline FEV_1 (141.9 ± 71.7pg/mg protein; Day 6-8 (n=4 measurements from 3 children in each subgroup), baseline FEV_1 recovered group, P<0.05, 2-way ANOVA, Bonferroni post-test) (Figure 7-4B).

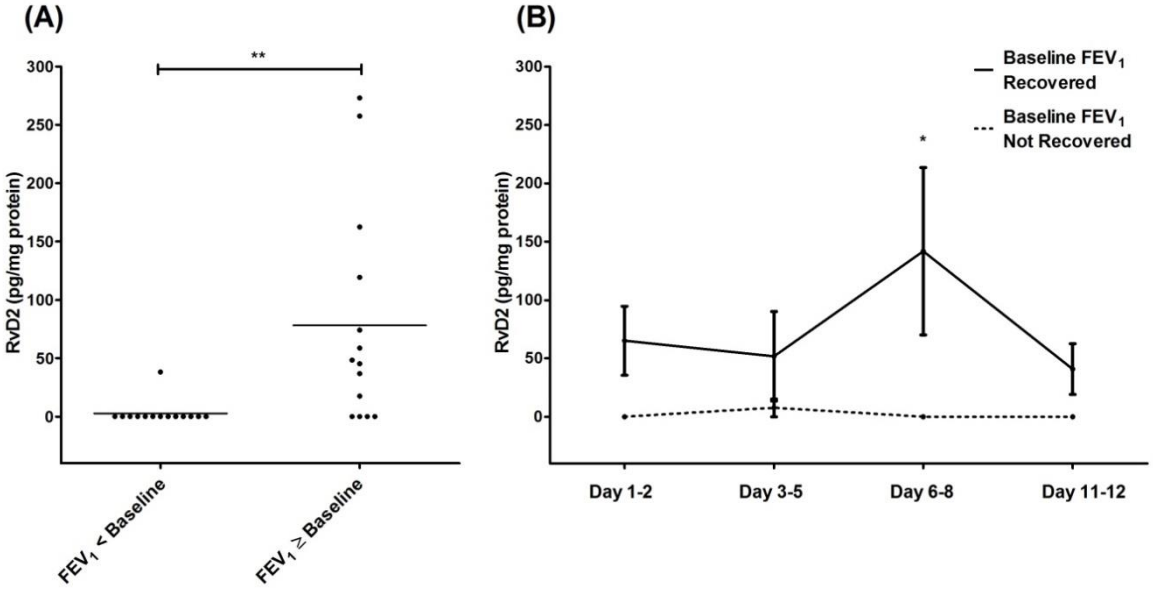

Figure 7-4 Relationship between sputum Resolvin D2 concentration during CFPE and extent of recovery of pre-exacerbation baseline FEV_1

Spontaneously expectorated sputum samples were obtained from children during CFPE at four time-points; Day 1-2, Day 3-5, Day 6-8 and Day 11-12. Resolvin D2 was measured in sputum by the LC ï MS/MS method. Sub-group analysis was conducted to compare children with ñincomplete baseline FEV_1 recoveryò(left hand column (A) and dashed line (B) (n=14 samples from 5 children)) with those with ñfull baseline FEV_1 recoveryò(right hand column (A) and continuous line (B)(n=14 samples from 5 children)). (A) Sputum Resolvin D2 at all time-points (Line represents Mean, **P<0.01, Student t-test). (B) Sputum Resolvin D2 over the time course of CFPE (Day 1-2, n=7 samples, Day 3-5, n=9 samples, Day 6-8, n=8 samples, and Day 11-12, n=4 samples) (Mean plotted, error bars represent SEM, *P<0.05, 2 way ANOVA, Bonferroni post-test). **LC-MS/MS performed by Dr Justine Bertrand-Michel at the Lipidomic Platform in Toulouse. FR performed the spirometry for the PRINCE Study. Baseline spirometry measurements were obtained from the medical records.*

7.4.7 PPARɔ agonists detected in sputum

PPARɔ ligands are lipid anti-inflammatory and Pro-Resolution lipid mediators. They play roles in macrophage directed active resolution of inflammation and wound repair and have been proposed for therapeutic application in CF (329-331).

We examined the production of five endogenously produced PPARɔ ligands that were reported to play pro-resolution roles in the active resolution phase of inflammation in unprocessed sputum plugs by the LC ï MS/MS method, namely; 12-HETE, 15-HETE, 9-HODE, 13-HODE, and 15-d-Prostaglandin J2. **LC-MS/MS was performed by Dr Justine Bertrand-Michel at the Lipidomic Platform in Toulouse.*

All five specific PPARɔ agonists examined were detected (Figure 7-5).

12-HETE is produced from arachidonic acid by platelet type 12 Lipoxygenase and is reported to be an agonist of the anti-inflammatory nuclear receptor PPARɔ (274, 332, 351). 12 Lipoxygenase enzymatic function in platelets was reported to be affected by CFTR dysfunction (351). The mean 12-HETE concentration measured in CFPE sputum samples (n=28 measurements during 10 episodes among 8 children) was not significantly higher than in control samples (n=5 measurements among 5 children) (2547 ± 1712 pg/mg protein; Control, vs. 9034 ± 2252; CFPE samples) (Figure 7-5A). Whilst the mean concentration of 12-HETE appeared highest in sputum during CFPE at Day 3-5 this was not significant (n=9 measurements from 8 children, P=NS, ANOVA) (Figure 7-5A), nor did sputum 12-HETE concentration differ significantly between FEV_1 recovery outcome sub-groups (n=14 measurements from 5 children in each subgroup, not illustrated, P=NS, Student t-test).

In humans, 15-HETE is produced from arachidonic acid by 15 Lipoxygenase. 15-HETE is both bioactive as a PPARɔ agonist and a major pathway intermediate in the formation of the lipoxins (274, 277). Mean 15-HETE concentration measured from sputum did not differ between control (n=5 measurements among 5 children) and CFPE samples (n=28 measurements during 10 episodes among 8 children) (1600 ± 590pg/mg protein; Controls, vs. 2000 ± 660pg/mg protein; CF samples, P=NS, Student t-test). 15-HETE did not change significantly over time during

CFPE (Figure 7-5B) (P=NS, ANOVA) or differ significantly between the ñfull baseline FEV_1 recoveryòCFPE subgroup and the ñincomplete baseline FEV_1 recoveryòCFPE subgroup (n=14 measurements from 5 children in each subgroup, not illustrated, P=NS, Student t-test).

9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) are both oxidation products of linoleic acid and potent agonists of PPARɔ (271).

Whilst the mean 9-HODE concentration measured in sputum during CFPE (n=28 measurements during 10 episodes among 8 children) was lower than that found in control sputum (n=5 measurements among 5 children), the difference was not significant (3425 ± 1063 pg/mg protein; controls, vs. 1655 ± 375 pg/mg protein; CFPE samples, P=NS, Student t-test). A non-significant trend towards increasing 9-HODE concentration over the time-course of CFPE was seen (P=NS, ANOVA) (Figure 7-5C). 9-HODE concentration did not differ between ñfull baseline FEV_1 recoveryòCFPE episodes and ñincomplete baseline FEV_1 recoveryòCFPE episodes (n=14 measurements from 5 children in each subgroup, not illustrated, P=NS, Student t-test).

There was no difference in the mean concentration of 13-HODE detected from control (n=5 measurements among 5 children) and CFPE samples (n=28 measurements during 10 episodes among 8 children) (4800 ± 1200pg/mg protein; Controls, vs. 4900 ± 890pg/mg protein; CF samples, P=NS, Student t-test). Mean overall 13-HODE concentration in sputum did not change significantly over the time-course of CFPE (Figure 7-5D). Among CFPE samples, there was a wide distribution in 13-HODE concentration measured at each time point.

15-deoxy-Prostaglandin J2 (15d-PGJ2) is a prostaglandin type pro-resolving lipid mediator and a high affinity ligand of PPARɔ formed from PGD2 by the enzymatic activity of prostaglandin D2 synthase (PGD2S) (467). 15d-PGJ2 concentration in control samples (n=5 measurements among 5 children) was significantly higher than that measured from CFPE samples overall (n=28 measurements during 10 episodes among 8 children) (218 ± 120pg/mg protein; Controls, vs. 14 ± 4pg/mg

protein; CF samples P<0.001, Student t-test), as well as at any individual time-point in CFPE samples (P<0.05-0.01, ANOVA) (Figure 7-5E).

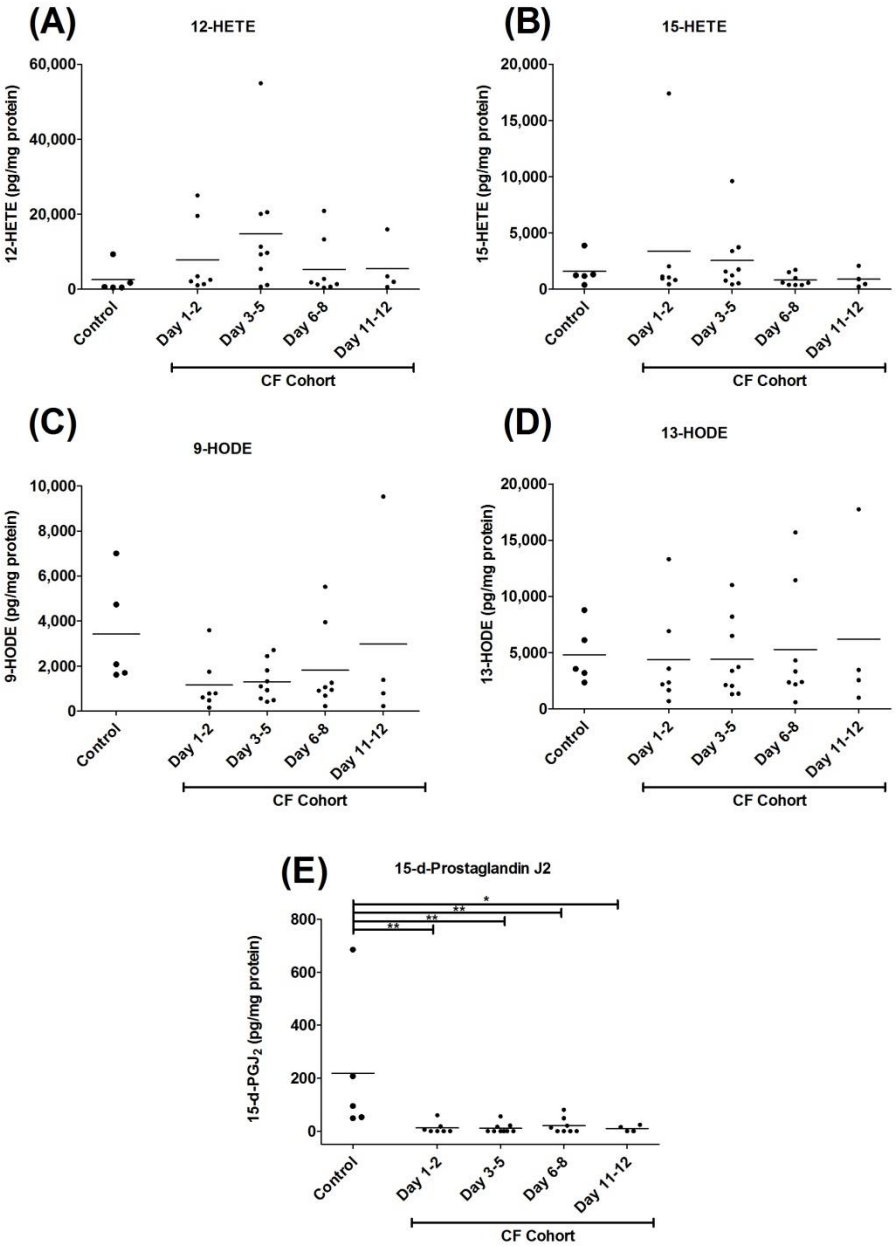

Figure 7-5 PPARɔ agonists detected in sputum

Spontaneously expectorated sputum samples were obtained from control inpatient children (first column) and children being treated for CFPE at four time-points (Day 1-2, Day 3-5, Day 6-8 and Day 11-12). Specific PPARɔ agonist lipid mediators were quantified in unprocessed sputum plugs by the LC ï MS/MS method; (A) 12-HETE (pg/mg protein), (B) 15-HETE (pg/mg protein), (C) 9-HODE (pg/mg protein), (D) 13-HODE (pg/mg protein), & (E) 15-d-Prostaglandin J2 (pg/mg protein) (Control, n=5 samples, Day 1-2, n=7 samples, Day 3-5, n=9 samples, Day 6-8, n=8 samples, and Day 11-12, n=4 samples, Line represents Mean, *P<0.05, **P<0.01, ANOVA, Newman Keuls Post Test). **LC-MS/MS performed by Dr Justine Bertrand-Michel at the Lipidomic Platform in Toulouse.*

7.4.8 PPARɔ agonists 13-HODE & 15-d-Prostaglandin J2 and FEV_1 Recovery post exacerbation

The aim of this study was to relate sputum levels of pro-resolution mediators during CFPE to the extent of FEV_1 recovery.

Two PPARɔ agonists examined tended to be differentially expressed between ñfull baseline FEV_1 recoveryò episodes and ñincomplete baseline FEV_1 recoveryò CFPE episodes; 15-d-PGJ2 and 13-HODE.

Sputum 15-d-PGJ2 concentration was compared between FEV_1 recovery sub-groups at all time-points (Figure 7-6A) and over the time course of CFPE (Figure 7-6B). 15d-PGJ2 concentration from ñfull baseline FEV_1 recoveryò episodes (n=14 measurements during 5 episodes in 5 children) was significantly higher than 15-d-PGJ2 concentration measured from ñincomplete baseline FEV_1 recoveryò CFPE episodes (n=14 measurements during 5 episodes in 5 children) at all time-points (22 ± 7pg/mg protein; FEV_1 ÓBaseline, vs. 5 Ñ2pg/mg protein; FEV_1 < Baseline, P<0.05, Student t-test) (Figure 7-6A). Further analysis of 15-d-PGJ2 concentration, compared between these two sub-groups over the time-course of CFPE revealed a higher measured 15-d-PGJ2 concentration among ñfull baseline FEV_1 recoveryò episodes over time that approached statistical significance (P=0.06, 2 way ANOVA) and was highest in ñfull baseline FEV_1 recoveryò episodes at Day 6-8 (n=4 measurements from 3 children in each subgroup) (Figure 7-6B).

Sputum 13-HODE concentration was compared between FEV_1 recovery sub-groups at all time-points (Figure 7-6C) and over the time course of CFPE (Figure 7-6D). There was a non-significant trend towards higher sputum 13-HODE in the ñfull baseline FEV_1 recoveryò subgroup (6036 Ñ1459pg/mg protein; FEV_1 Ó Baseline (n=14 measurements during 5 episodes in 5 children), vs. 2863 ± 408pg/mg protein; FEV_1 < Baseline (n=14 measurements during 5 episodes in 5 children), P=0.05, Student t-test) (Figure 7-6C). Despite a non-significant trend towards higher 13-HODE sputum concentration at all time-points in the ñfull baseline FEV_1 recoveryò subgroup, 13-HODE concentration did not change significantly over the time course of CFPE (P=NS, 2 way ANOVA) (Figure 7-6D).

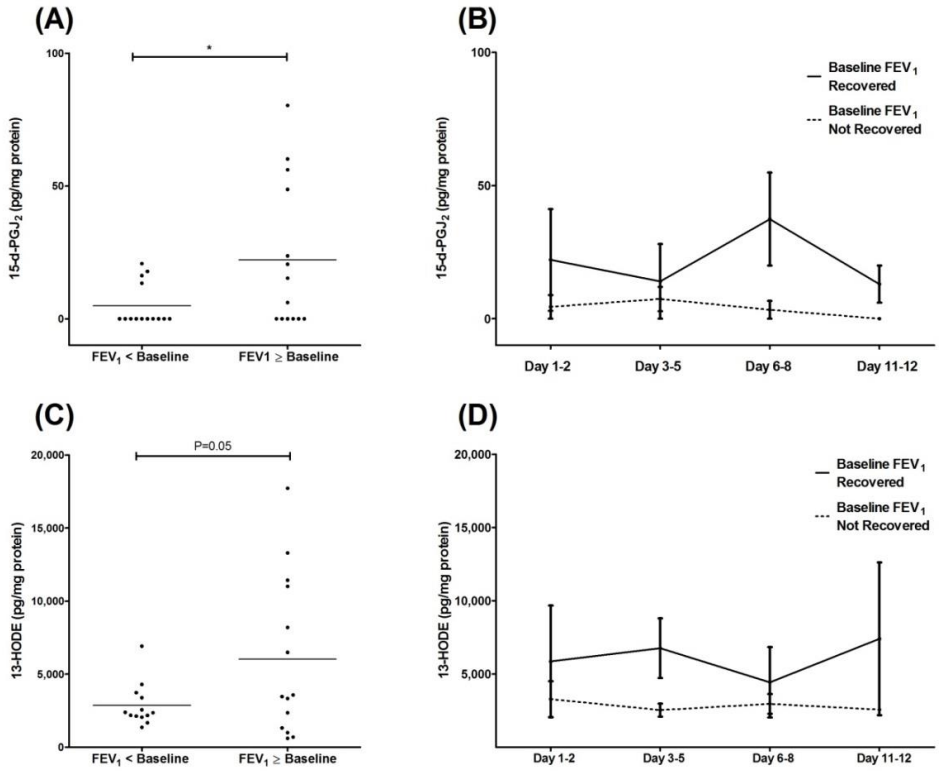

Figure 7-6 Relationship between sputum 15-d-Prostaglandin J2 and 13-HODE concentration during CFPE and extent of recovery of pre-exacerbation baseline FEV_1.

Spontaneously expectorated sputum samples were obtained from children during CFPE at four time-points. 15-d-Prostaglandin J2 (15-d-PGJ2) and 13-HODE were measured in sputum by the LC ï MS/MS method. Sub-group analysis compared children with ñincomplete baseline FEV1 recoveryò(left hand column (A&C) and dashed line (B&D)) with those with ñfull baseline FEV1 recoveryò(right hand column (A&C) and continuous line (B&D)) (n=14 samples from 5 children in each subgroup). (A) Sputum 15-d-PGJ2 at all time-points (Line represents Mean, *P<0.05, Student t-test). (B) Sputum 15-d-PGJ2 over the time course of CFPE (Mean plotted, error bars represent SEM, P=0.06, 2 way ANOVA). (C) Sputum 13-HODE at all time-points (Line represents Mean, P=0.05, Student t-test). (D) Sputum 13-HODE over the time course of CFPE. (Mean plotted, error bars represent SEM, P=0.11(NS), 2 way ANOVA). (Day 1-2, n=7 samples, Day 3-5, n=9 samples, Day 6-8, n=8 samples, and Day 11-12, n=4 samples). **LC-MS/MS was performed by Dr Justine Bertrand-Michel at the Lipidomic Platform in Toulouse. FR performed the spirometry for the PRINCE Study. Baseline spirometry measurements were obtained from the medical records.*

7.5 Discussion

7.5.1 Resolvin D2 and 15-d-PGJ2 significantly associate with full recovery of pre-exacerbation FEV_1 following CFPE

We set out to investigate whether pro-resolution mediator production in the airways played a relevant role in the natural course of clinical resolution during treatment for CFPE. The rationale being, that, if pro-resolution mediator production was found to impact recovery, or to be comparatively sub-optimal, adjuvant administration could represent an effective strategy to boost recovery from CFPE. We report for the first time positive relationships between two pro-resolution mediators that significantly associate with full recovery of pre-exacerbation FEV_1 following CFPE, namely; RvD2, and 15-d-PGJ2.

The degree of loss of lung function among those who failed to regain their baseline FEV_1 in this small study was at a clinically important level (mean loss of 6.6% predicted). Whilst it is not completely understood why some patients fail to recover baseline lung function following exacerbation, it is agreed that to some degree treatment factors may play a part, whereas in other cases, irreversible lung damage may have occurred (468). Given that half of all the long-term lung function decline in CF has been attributed to pulmonary exacerbations (469), this is an important area to study in greater detail, with a significant unmet clinical need for therapeutic options.

In the field of cytokine research there is a concept called ñredundancyò, meaning that in some cases the functions of cytokines overlap (redundant), whereas, in other cases, functions are cytokine-specific (non-redundant)(470). Although pro-resolution lipid mediators, as a class, are claimed to be non-redundant in their function - the specific roles played by individual mediators in health and disease remain open questions. This was the first study of its kind to look at the levels and timing of pro-resolution mediator expression in childrenôs airways during CFPE and to understand the relationships between individual mediators and lung function recovery.

7.5.2 Resolvin D2 & FEV_1

We found a significant positive association between the detection, and higher concentration of RvD2 in sputum and the full recovery of pre-exacerbation

baseline FEV_1. RvD2 concentration peaked at Day 6-8 of treatment during ñfull baseline FEV_1 recoveryò CFPE episodes.

Only a small body of literature has been published on Resolvin D2 (18 citations on Pubmed). In a mouse model of sepsis, RvD2 was reported to reduce neutrophil infiltration, promote phagocyte dependent bacterial clearance and reduce pro-inflammatory cytokine release (308). Although evidence suggests that RvD2 signals via GPCR(s), the specific details have not yet been reported (288). To further evaluate RvD2 in an antibiotic adjuvant role for CFPE, we could consider (a) studying RvD2ôs anti-microbial effects in CF cell culture models, or (b) an interventional study in a CF animal model. In the latter approach, RvD2 (or a stable analogue) could be co-administered (orally, or by inhalation) with an antibiotic to a CF animal model faced with an infectious airway challenge and the animal could be monitored for the timing and extent of recovery. Since we found that RvD2 was higher among ñfull baseline FEV_1 recoveryò episodes (even from Day 1-2 and peaking at Day 6-8), co-administration could be initiated at the same time as commencing antibiotic treatment.

7.5.3 15-d-PGJ2, 13-HODE & FEV_1

We observed positive relationships between PPARɔ ligand concentration in sputum during CFPE and the extent of recovery of pre-exacerbation baseline FEV_1. In particular, 15-d-PGJ2 was significantly higher among ñfull baseline FEV_1 recoveryò CFPE episodes, and a trend towards higher 13-HODE concentration associated with ñfull baseline FEV_1 recoveryò CFPE episodes approached significance.

Expression of PPARɔ itself in CFTR knockout mice was reported to be down-regulated at the RNA and protein levels, and associated with reduced nuclear localisation (361). Notably, PPARɔ agonist pioglitazone was reported to normalize pulmonary infiltration of neutrophils and pro-inflammatory cytokine levels in the lungs of CFTR-deficient mice challenged with Pseudomonas (362). Furthermore, ion transport effects of PPARɔ agonists have been reported (inhibition of chloride secretion (471) and intracellular calcium increases (472)). Recent studies also demonstrated a role for PPARɔ in the expression of carbonic anhydrases and bicarbonate transport in the lung (363). Moreover, pro-proliferative effects

reported on airway epithelial cells could potentially benefit repair (472). Our findings support the previously described therapeutic potential for PPARɔ agonists in CF (331).

7.5.4 Patient Cohorts

The Control and CFPE cohorts differed significantly with respect to sex and age. Control children were younger and male, in contrast to the older CFPE cohort with a majority of female children. The control participants that we able to recruit were clinically stable at the time of sampling. Whilst their participation provided some data to attempt to answer the question ï ñare pro-resolution mediators found in detectable quantities in sputum at clinical baseline?òthe cohort did not constitute the ideal control group sought. We actively attempted to recruit children undergoing treatment for exacerbation of non-CF bronchiectasis, and able to expectorate sputum, however, this presentation is not common in a hospital inpatient or outpatient population in childhood and no suitable candidates were identified over the study period.

In the CFPE cohort sampling at the fourth time-point (Day 11-12) was sub-optimal. A sputum sample was obtained for only three CFPE episodes at Day 11-12. By Day 11-12, one child had transferred to another treatment centre, one child had completed therapy and been discharged home and one child withdrew their assent to further sputum sampling due to discomfort independent of the study conduct. Children were much less productive of sputum by Day 11-12 of treatment and therefore found it difficult to generate a spontaneously expectorated sample. An increased rate of late CFPE sampling could have been achieved if we had designed the study to obtain induced sputum samples from the outset.

The microbiological characteristics of the cohort were similar to those widely reported (8, 473). Children reported overall improvement in their symptoms during the time course of CFPE. Sputum and cough related symptoms normalised over the time period, however, appetite and fatigue symptoms persisted throughout the time course.

There was an over-representation of female children in the CF cohort. Unselected, sequentially presenting eligible children presenting with CFPE were

approached for consent to participate in the study. There was no active selection procedure, however, given the average age of participants (mean 12.7 years) this female over-representation may have reflected the increased rate of presentation for exacerbation of CF seen in adolescent girls (the ñGender Gapò) (474). The 50% rate of failure to regain baseline FEV_1 in this study was higher than the 23.1% reported by Sanders et al in a study of 104 children in Seattle (473). Our sample size was small and this 50% failure to regain baseline FEV_1 rate likely reflects a chance over-representation of more severe CFPE episodes. Due account must be taken of this in the interpretation of the results.

7.5.5 Limitations of the LC-MS/MS Analysis of SpMôs

We set out to quantify a wider range of pro-resolution mediators in sputum than was ultimately achieved. The first problem pertained to the lack of commercially available internal standards. Specifically, our collaborators at Toulouse were not able to quantify; Resolvin E1, Resolvin E2, Protectin D1, Maresin 2, or Resolvins D3, D4, D5 & D6. This could potentially have been addressed by engaging with Dr Serhanôs lab at Boston where they have in house internal standards for the listed compounds. Whilst we cannot comment on the SpMôs that could not be examined for technical reasons, it is important to recognise that Resolvin E1 (353) and Protectin D1 (338) have been reported to play relevant roles in the airways. A study of 16 stable adult patients with CF conducted at the University of California found that the detectable presence of Resolvin E1 (9 samples) was associated with a higher FEV_1 (353). Protectin D1 was reported in exhaled breath condensate from healthy volunteers (mean 2.2ng/ml) by Levy et al. who found it to be undetectable in asthmatics during exacerbation (n=4) (338). As such these mediators should not be discarded from our ongoing consideration of candidates for CFPE therapy but borne in mind for future investigation when this becomes technically feasible.

The second limitation related to the low rate of SpM detection in sputum samples. LXB4, RvD1 and 7-Maresin 1 were not detected in any control or CF sputum sample, and, LXA_4, 18-HEPE and Protectin Dx were detected so infrequently such that further analysis could not reliably be performed.

We have previously reported measurable quantities of LXA_4 in BAL from stable children with CF, and in sputum from adults with CF during CFPE (354, 370). Those studies were conducted on site by ELISA and found mean concentrations of 4pg/ml of LXA_4 in BAL, and 15.9ng/ml of LXA_4 at the beginning of exacerbation rising to 45.5ng/ml at the close of treatment (354). This LC-MS/MS study of children during CFPE failed to reliably detect LXA_4 with a lower limit of detection for the method reported at 2.5pg/ml (379). However, LXA_4 in healthy serum was reported to deteriorate at a rate of 5.9% loss over 5 days in serum samples (380). In light of this, it is conceivable that the combination of transport on dry ice and the inflammatory milieu of sputum might result in degradation rendering it difficult to detect.

Ours is not the first study to report difficulty in recovering SPMõs from human samples (380, 381). Whilst investigators at the University of California found Resolvin E1 in sputum, they failed to detect LXA_4 in any sputum sample analysed (353). Conversely, whereas we did not have access to Resolvin E1 analysis itself, we found the pre-cursor 18-HEPE in only 2 samples. Although Protectin D1 has been reported in airways secretions, we found the stereoisomer Protectin Dx in only one sample (338). Even the human serum metabolome reference project reported significant variation in the recovery of SPMõs from two different blood serum sources examined by the same assay at the same site (381). For example LXB_4 and 7-Maresin 1 (we failed to detect either in sputum) in serum were not detectable in one serum set and measured 49 / 21 pg/ml respectively in another set. We also failed to detect RvD1 which is found in healthy human serum at a concentration of between 3 & 31 pg/ml (381). The limit of detection for our assay was 2.5pg. I have found no other report in the literature of the detection or quantification of RvD1 in human airways secretions.

Storage conditions can have a very significant impact upon degradation (25-85% loss over 4 weeks of storage) and in this knowledge, our sputum samples were preserved in methanol and stored at minus 80 degrees prior to transport (381). Regarding those SpMõs which were infrequently or not detected in sputum in this study, to establish whether they are truly synthesised in childrenõs airways during CFPE, protocol modifications could be considered including; sampling of sputum on a site which is co-located with the facilities to rapidly perform LC-MS/MS to

eliminate decay during storage & transportation; alternative airways sampling methods such as Exhaled Breath Condensate to avoid the degradative milieu of sputum; the preservation of unstable SpMõs might be improved by storing the samples in liquid nitrogen.

7.5.6 Exploring the relationships between Resolvin D2, 15dPGJ2, 13 HODE and FEV_1 and the resolution of inflammation

This study was an observational study and as such cannot distinguish between association and causation in the relationships between RvD2, 15-d-PGJ2, 13-HODE and FEV_1 and the resolution of inflammation. Nonetheless, identification of these significant relationships in the native setting of CFPE in children lends a weight of relevance to further studies attempting to; (a) confirm these findings in a larger cohort; (b) to investigate the underlying pathophysiological mechanisms operating differentially between full and sub-optimal recovery episodes and the role of RvD2, 15-d-PGJ2 and 13-HODE in the active resolution of inflammation in CFPE; and, (c) intervene to administer RvD2, an upstream target or stable analogue, or a PPARɔ agonist in a CFPE model or clinical study.

7.5.7 Overall Limitations of this study

This study was underpowered due to the failure to achieve the target sample size. Spontaneously produced sputum was used to sample the airway. A higher rate of sampling might have been achieved via sputum induction. The study was designed to capture a convenience sample of inpatients, however, recruitment might have been bolstered by capturing children with CFPE undergoing home IV antibiotic therapy. A higher rate of control recruitment could have been achieved by targetting a cohort of age-matched well children consenting to sputum induction. In this observational study we cannot outrule differences in treatment regimen contributing to differences in outcome. The study population may also represent a more severe clinical presentation of CFPE inherent to the recruitment bias associated with inpatient children versus ñallò children with CFPE, since candidates for home IV therapy may have had a less severe presentation.

We did not specifically exclude children who had been previously enrolled and therefore there is potential for re-enrollment bias. Given the large yield of data from this type of study there is potential for Type I statistical error due to multiple

comparision / multiple analyses. Whilst the FEV_1 subgroup analysis was pre-determined as an endpoint in the original study design, the risk of Type I errors could be reduced by having a more stringent P value to determine statistical significance.

7.6 Conclusions

Recurrent pulmonary exacerbations are associated with long-term decline in lung function and reduced survival (450). There is an unmet need for adjuvant therapies in this landscape to prevent CFPE associated decline in lung function.

We have reported significant positive associations between Resolvin D2 and 15-d-PGJ2 measured in sputum during CFPE and the full recovery of FEV_1 post exacerbation. These findings are novel and require validation in a larger population of both children and adults with CF, with a view to determining which specific mediators have greatest relevance for CF pulmonary exacerbations. If our data can be replicated in a larger patient population it may lead to a potentially exciting avenue for development of antibiotic adjuvant therapies in CF.

In the General Discussion we will explore the question of how RvD2, 15-d-PGJ2, 13-HODE might influence the resolution of inflammation in greater detail.

CHAPTER 8
GENERAL DISCUSSION

8 General Discussion

8.1 Summary and Key findings

In this thesis we have demonstrated an ALX/FPR2 dependent RvD1 mediated increase in ASL height in primary CF and CuFi-1 bronchial epithelial cell models. We showed that RvD1 attenuates TNFŬ stimulated IL8 secretion via IŦB preservation in the CuFi-1 bronchial epithelial cell line. We show that RvD1 increases the phagocytic capacity of primary CF alveolar macrophages and increases their bactericidal capacity against *Pseudomonas aeruginosa*. These physiological actions of RvD1 in CF bronchial epithelial and alveolar macrophage models serve to redress several of the key deficits implicated directly in the pathogenesis of CF.

We presented *in vivo* evidence of deficient 15 Lipoxygenase expression in association with LXA_4 / LTB_4 imbalance in the airways of children with CF, indicating the failure to actively resolve inflammation (370).

We reported significant positive associations between the concentrations of Resolvin D2 and the PPARɔ agonist 15-d-Prostaglandin-J2 measured in sputum during CFPE and the full recovery of FEV_1 post exacerbation.

These findings; advance the proof of principle that RvD1 could be considered for application as an adjuvant in CF therapy; improve our understanding of the failure to actively resolve inflammation in the CF airway; and suggest exciting and novel approaches to antibiotic adjuvant therapy including Resolvin D2 and PPARɔ agonists for application in CFPE.

Furthermore, we optimised a method to isolate a purified alveolar macrophage population from the BAL of children with CF and controls in order to further investigate how defective 15-LO expression in alveolar macrophages could result in the failure to synthesise LXA_4. Pilot data were presented on 15-LO expression and macrophage polarisation in an enriched alveolar macrophage population from children with CF and controls. We demonstrated that 15-LO expression and activity in the CuFi-1 CF bronchial epithelial cell line is broadly similar to that found in the NuLi-1 non-CF bronchial epithelial cell line.

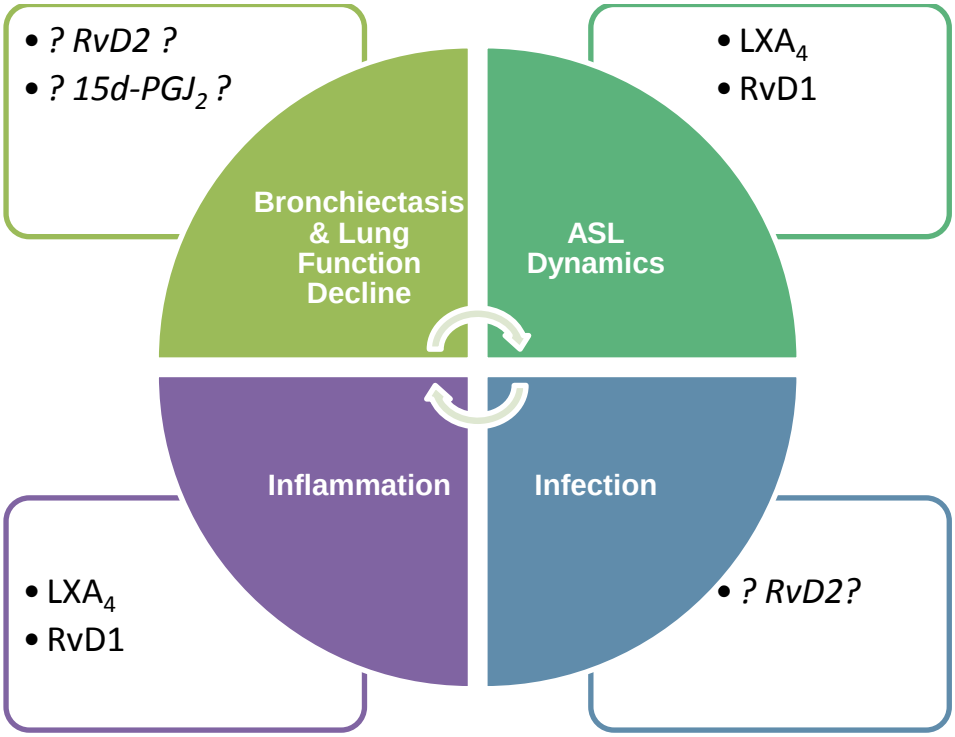

Figure 8-1 Evidence is accumulating that Pro-resolution mediators have beneficial effects on the key elements of airway disease pathogenesis in CF

We will consider the accumulated evidence (presented here and in the literature) that SPMôs have beneficial effects on each step in the pathogenesis of airway disease in CF, look at avenues to explore moving forwards, and consider how these concepts could be applied in therapy.

8.2 SPMôs on ASL Height in CF

CF is caused by mutation of the CFTR gene which leads to a loss of chloride secretion and sodium hyper-absorption, resulting in a lowering of the ASL height and impaired muco-ciliary clearance and impaired innate defence. The restoration of ASL height by LXA_4 was previously reported (85, 356, 393). In this thesis we demonstrate that RvD1 also restores ASL height in both the CuFi-1 differentiated, polarised bronchial epithelial cell model and in primary CF bronchial epithelium. In CuFi-1 cells this effect involves the FPR2 receptor. This physiological effect of SPMôs may have evolved to allow the ASL to respond to the requirement to enhance airway clearance as part of restoring tissue homeostasis in the context of active resolution after an inflammatory process. The restoration of ASL height

would be expected to lead to improved muco-ciliary clearance. This ñflushingò function, enhancing muco-ciliary clearance in the airways would allow irritants and inflammatory debris to be cleared from the lungs. Furthermore, increased ASL height might also affect the ionic composition of the ASL (including pH) and by dilution create a more favourable environment for the function of innate microbial peptides and potentially improve the bactericidal capacity of the ASL in CF. The downstream consequences of increased ASL height on innate defence in the CF airway remain to be demonstrated in a more complex model taking account of the roles played by ciliary beat frequency in muco-ciliary transport and the specific salt and pH range sensitivities of the characteristic CF pathogens.

8.3 SPMôs on Airways Infection in CF

Predisposition to airways infection is a key element of CF lung disease pathogenesis. Various factors interplay to allow the initial colonisation of the lung by characteristic pathogens; impaired muco-ciliary clearance , increased binding of pathogens to epithelial surfaces (135), reduced bacterial killing by innate antimicrobial peptides (128), reduced macrophage phagocytosis and impaired bacterial killing (225), and impaired phagolysosomal acidification (126). A context is generated whereby the microbiome is altered (177), characteristic pathogens have established inter-relationships, niches and entered into biofilms, the lung is structurally damaged and mucus plugging gives rise to hypoxic micro-environments which promote further infection of the lung.

A critical consideration for the application of SPMôs in CF therapy will always pertain to the safety and appropriateness of active resolution of inflammation in the context of the chronically infected CF airway with its intrinsic predisposition to infection (53). Recognising the difference between an anti-inflammatory or immune paralysis mechanism of action, versus a pro-resolution mechanism with antimicrobial properties becomes both interesting and important (253). The SPMôs are reported to improve bacterial killing in animal models of sepsis; LXA_4 administration resulted in a reduced bacterial load in a mouse model of *P. aeruginosa* infection (315), and a panel of SPMôs (Resolvin D1, Resolvin D5 and Protectin D1) improved the containment of a bacterial challenge in animal infection models acting in synergy with antibiotics (316). The function of anti-microbial peptides in the ASL are salt and pH-sensitive (128, 129). SPMôs described to

increase the ASL height (LXA_4 reported in the literature(355), and RvD1 reported in this thesis) might conceivably improve innate antimicrobial peptide function by changing the pH by bicarbonate secretion or ionic concentration of the ASL by hydration (128).

We have demonstrated that RvD1 increases the phagocytic capacity of primary CF alveolar macrophages, and improves intracellular killing of the key CF pathogen, *Pseudomonas aeruginosa*. Moreover, Resolvin D2, in particular, has potent anti-microbial properties (308) and we demonstrated that higher levels of Resolvin D2 detected during CFPE in sputum from children was associated with better recovery of lung function post CFPE. Since a higher concentration of RvD2 with its adjuvant anti-microbial properties was associated with a better outcome in our small cohort, we might be more optimistic that SPMôs could conceivably be applied safely under antibiotic cover. This approach is reasonably novel and in light of the lessons of prior anti-inflammatory strategies for CF(241), would need to be rigorously tested for safety and efficacy in appropriate CF animal models prior to taking this consideration any further.

We now recognise that the airway is not sterile (177) and a field of investigation is currently emerging to attempt to understand host-microbiome interactions. The possibility that SPMôs might play roles in microbiological house-keeping, or that microbes themselves could act synergistically to influence the active resolution of inflammation by the host adds a new layer of complexity to our understanding of the normal restoration of lung homeostasis following self-limited inflammation.

8.4 SPMôs on Airway Inflammation in CF

A normal host response to bacterial infection involves neutrophil recruitment that serves to contain and eliminate the bacterial insult, and is followed by an active resolution phase. The key features of tissue resolution (performed by SPMôs) have been described as; limiting neutrophil recruitment and effector functions, limiting the production of pro-inflammatory mediators and initiating macrophage phagocytosis and repair (253). CF is characterised by prolonged, ineffective neutrophil mediated inflammation, a hyper-inflammatory phenotype, failure of bacterial containment and failed resolution of inflammation ï perhaps CF is in fact the exemplifier disease for the failure of tissue resolution.

The underlying basis for the failure to actively resolve inflammation in the CF airway is incompletely understood, and controversy had surrounded prior reports of reduced LXA_4 concentration in CF airway samples (315, 352). Furthermore, the tendency for neutrophils to migrate into tissues is influenced by the relative concentrations of LXA_4 and LTB_4 (304, 360). LXA_4 is generated by a ñclass switchò in eicosanoid lipid mediator synthesis from Leukotrienes to Lipoxins via up-regulation of 15 LO (264). In this thesis (and the associated publication (370)) we reported that children with CF have a lower LXA_4 to LTB_4 ratio than control children measured from BAL fluid, even in the absence of recognised culturable pathogens, related to deficient 15-LO2 expression. This finding may in part explain the excessive neutrophil burden and the failure to actively resolve inflammation observed in CF airways.

The ability of LXA_4 to reduce bronchial epithelial IL8 secretion was previously reported (323). Here, we reported that RvD1 attenuates TNFŬ stimulated IL8 secretion via preservation of IKB in CuFi-1 bronchial epithelial cells. This physiological effect could serve to abate the hyper-inflammatory phenotype of the CF bronchial epithelial cell and antagonise IL8 mediated neutrophil chemotaxis.

The activity of the 15-LO enzyme plays a key role in the biosynthesis of the Lipoxins (257), D- Series Resolvins (262), Protectins (270) and the PPARɔ agonists 15-HETE, 9 & 13-HODE (271-274). We reported that the expression of 15-LO type 2 was depressed in the cellular fraction of BAL from children with CF. We observed positive relationships between 15-LO2 expression and the abundance of macrophages in the pellet, and with the LXA_4 / LTB_4 ratio among control children. These relationships were lost in children with CF. Moreover, to compound the reported SPM biosynthetic defect, essential fatty acid deficiencies are reported in CF, affecting the availability of parent compounds such as DHA and EPA (345, 346). The knock-on consequences of the failure to up-regulate 15-LO expression may be far reaching and include the failure to initiate the active resolution of inflammation in CF and arrest of the downstream resolution sequence. Furthermore, it could be involved in the unopposed recruitment of neutrophils, their failure to undergo apoptosis and be removed by efferocytosis.

8.5 SPMõs in Bronchiectasis & Lung Function Decline in CF?

Factors centrally associated with the development of bronchiectasis and lung function decline in CF include; Neutrophil Elastase burden (66); nutrition (155); early acquisition of *Pseudomonas aeruginosa* (167); and, the frequency of episodes of pulmonary exacerbation and the associated acute loss of lung function (155, 473). In our small observational study, we reported significant positive associations between the concentrations of Resolvin D2 and PPARɔ agonist 15-d-Prostaglandin-J2 measured in sputum during CFPE and the full recovery of FEV_1 post exacerbation. The mechanisms by which resolution mediators such as Resolvin D2 and 15d-Prostaglandin J2 could protect against loss of lung function during CFPE warrant further evaluation.

Whilst the study was small and there were technical issues with the detection of other SPMõs, many compounds that demonstrate beneficial effects in mouse models or controlled in vitro experiments do not bear out in the complex context of human disease. These findings linked pro-resolution mediators to an important outcome among children being treated for CFPE, and highlighted a context for the consideration of resolution mediators to be applied as candidate adjuvants to antibiotic therapy. Given that in our study, those children who did not recover their pre-exacerbation baseline FEV_1 fully lost a mean of 6.6% predicted FEV_1, clinically meaningful gains in lung health for children with Cystic Fibrosis by adjuvant administration during CFPE are credible goals. In subsequent clinical studies of this phenomenon, follow up spirometry post CFPE would be important to evaluate whether short term FEV_1 decline is attended by irreversible loss of lung function.

An overview of the projected beneficial effects of SPMõs in the context of CF airway disease is presented in Figure 8-2.

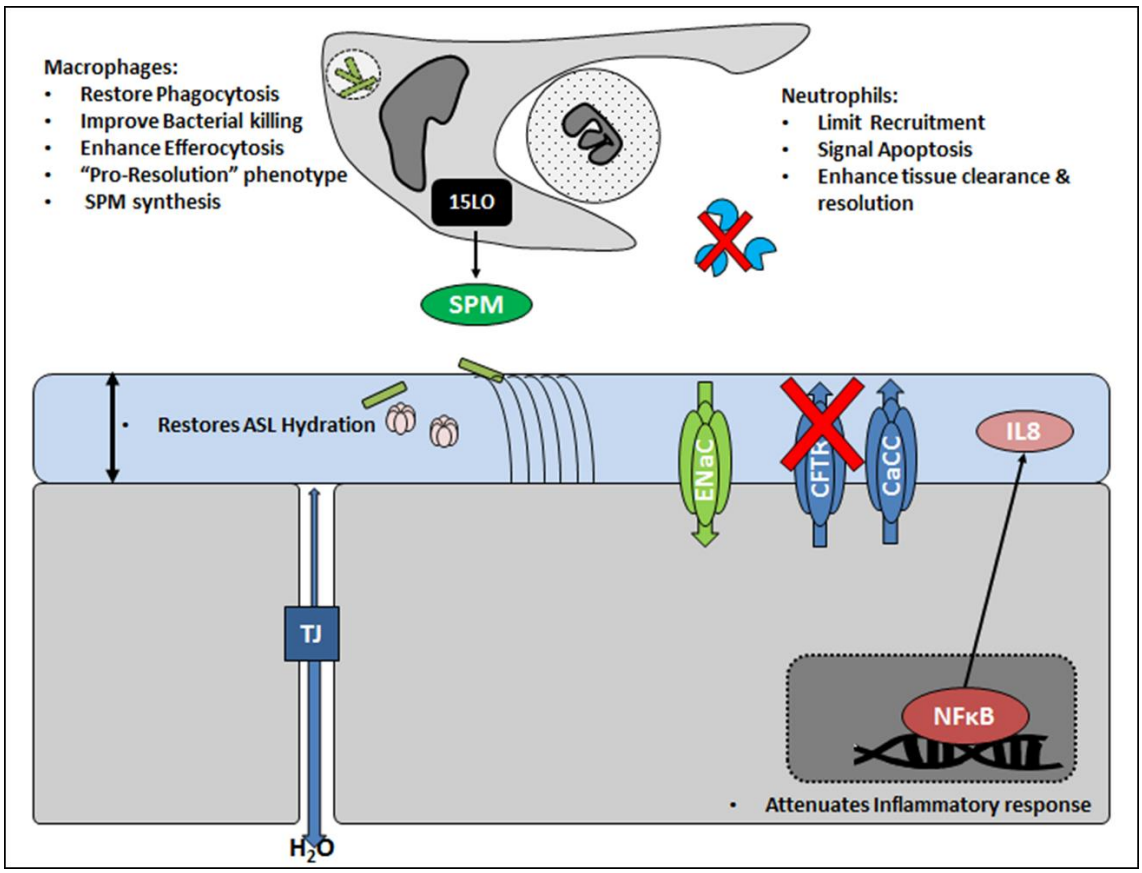

Figure 8-2 An illustration the physiological effects of SPMôs in CF lung disease

SPMôs can restore ASL height and barrier integrity. SPMôs can attenuate NFκB mediated pro-inflammatory signalling and reduce the secretion of neutrophil chemo-attractant IL8. SPMôs limit neutrophil recruitment and promote their tissue clearance and efferocytic uptake. SPMôs have the potential to reduce the release of free neutrophil Elastase into the airway by reducing the initial neutrophil burden and aiding in the removal of dead and dying neutrophils from the airway. SPMôs can promote CF macrophage mediated phagocytosis and intracellular killing of *Pseudomonas aeruginosa*. The synthesis of one these mediators, LXA_4, is defective in CF due to reduced expression of a key enzyme in its production pathway. SPMôs themselves, could potentially drive macrophages towards a resolution phenotype, attended by a cascade of de novo SPM synthesis.

8.6 Putting it all together ï could the resolution defect in the CF airway be attributed to alveolar macrophages?

Macrophages play key regulatory roles in the active resolution of inflammation. They receive, interpret and transmit signals to communicate tissue injury or invasion, and conversely, containment, resolution, homeostasis and repair. By phagocytosis they remove pathogens, inflammatory and cellular debris (433). The differentiation of a tissue macrophage reflects a complex interplay between intrinsic differentiation signalling pathways and environmental inputs received from neighbouring cells (434). Substantial cross-talk operates between; macrophage polarisation phenotype; macrophage function in efferocytosis; the local cytokine and lipid mediator milieu; PPARɔ expression and activation within macrophages; the capacity of a macrophage to synthesise pro-resolution mediators, and the lipid mediator profile secreted by macrophages. Many of the findings of this thesis share a common signature related to the regulation of the active resolution of inflammation by macrophages (roles played by LXA_4 /LTB_4 balance, 15-LO expression, RvD2, and PPARɔ agonist 15d-PGJ2 in resolution in CF). A consideration therefore of cross-talk in the regulation of macrophage function, involved in directing and enacting the active resolution is warranted. A deeper understanding of the regulation of active resolution regulation by airway macrophages might provide avenues to progress the study of failed resolution in the CF airway.

8.6.1 Cross-talk in M2 Macrophage polarisation - IL4, STAT6, 15-LO, PPARɔ, and efferocytosis

IL4 acts through a Jak-STAT6 (Signal Transducer and Activator of Transcription 6) signalling pathway (475). STAT6 is phosphorylated, dimerizes and translocates to the nucleus where it acts as a transcription factor, and controls genes associated with an Alternative Activation phenotype in Macrophages (M2 polarisation)(434). M2 macrophages are anti-inflammatory in their function, regulate wound healing, and are polarised by IL4 & IL13, via STAT6 signalling and PPARɔ activation (274, 307, 434). IL4 and STAT6 signalling also influence the expression of both 15-LO (274, 476, 477) and PPARɔ (274) (illustrated in Figure 8-3).

15-LO is up-regulated to initiate the ñswitchò towards resolution of inflammation (264), and its expression by macrophages is characteristic of M2 polarisation

(307). 15-LO is subject to transcriptional regulation by PGE2, IL4 and IL13 (264, 282, 477), and SPM biosynthesis (involving 15-LO) both marks, and effects the transition towards tissue resolution (264).

Kapoor et al reported that during normal wound repair, there is a shift in the metabolism of arachidonate from Prostaglandin E2 during the acute inflammatory phase to Prostaglandin D2 during the repair phase. PGD2 derivative 15-d-Prostaglandin J2 mediates its pro-resolution effects via PPARɔ, which is also up-regulated during the resolution phase (330).

A core function of SPMõs is to enhance the uptake of apoptotic neutrophils (259) whereas in CF, impaired neutrophil efferocytosis is reported (218, 219). Neutrophil Elastase was shown to cleave calreticulin, inhibiting phosphatidylserine mediated ingestion of apoptotic cells (220). Neutrophil efferocytosis, however, has important downstream consequences. Efferocytosis drives the polarisation of macrophages towards a pro-resolution phenotype stimulating production of 15-LO and 15-HETE via a PPARɔ/TGFƀ pathway (300, 312). During macrophage differentiation, natural PPARɔ ligands are formed which increase macrophage mediated efferocytosis via the induction of genes such as efferocytic surface receptors (478). Furthermore, efferocytic uptake was shown to enhance neutrophil efferocytosis by macrophages and induce de novo SPM synthesis (313) (illustrated in Figure 8-3).

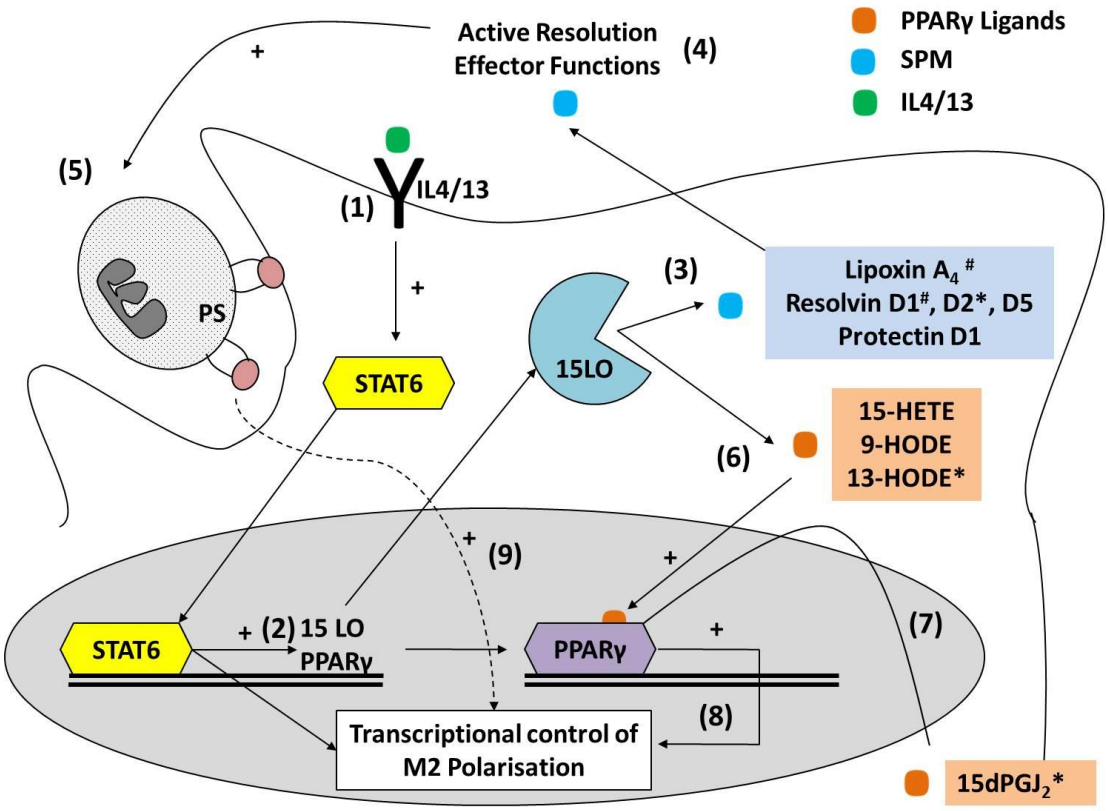

Figure 8-3 Schematic illustrating elements of the cross-talk in macrophage resolution regulation; IL4, STAT6, 15-LO, PPARɔ, M2 polarisation and apoptotic cell efferocytosis

(1) IL4 signals via a Jak-STAT6 pathway to up-regulate both (2) 15- LO and PPARɔ expression, and genes associated with an M2 polarisation phenotype. (3) 15-LO activity drives the biosynthesis of SPMs; LXA_4, Resolvins D1, D2 and D5 and Protectin D1, which in turn have pro-resolution effector functions (4) and stimulate neutrophil efferocytosis (5). PPARɔ activation by 15-LO products; 15-HETE, 9-HODE and 13-HODE (6), or by 15-d-PGJ2 (7) promotes M2 differentiation (8), and inhibits pro-inflammatory gene expression by trans-repression of NFκB. M2 macrophages have higher efferocytic capacity (9) and following exposure to PS (phosphatidylserine) on apoptotic cells accelerate resolution of inflammation. (*Indicates mediators associated with improved lung function recovery from CFPE in this thesis. # Indicates mediators with demonstrated beneficial physiological effects in CF models and includes both findings presented in this thesis and published by other investigators in the literature).

Taken together the observations that; 15-LO activity is important in LXA_4 /LTB_4 balance (disturbed in CF) and required for Resolvin D2 biosynthesis (associated with better recovery); and, that PPARɔ activation (associated with better recovery) and 15-LO expression play a key role in macrophage polarisation (332), a number of the findings of this thesis converge on the role played by macrophage polarisation in regulating the active resolution of inflammation.

Moreover, given that under certain conditions (both adaptive and maladaptive) inflammation does not ñself-resolveò and SPM biosynthesis does not arise, e.g. in animal models of peritoneal sepsis when a higher inoculum of bacteria is administered (316), or in CF, we reason that there must be an account taken of the context in the mechanistic operation of the ñswitchò towards resolution. The sensory component of this switch may be regulated through an integration of pro-inflammatory and pro-resolution signals, via the competing influences on STAT signalling pathways culminating in macrophage phenotype polarisation (434). Contextual factors which might have competing input into this regulation are illustrated in Figure 8-4. It is likely that, in CF, the combination of innate immune dysfunction, persistent pathogen colonisation and the intrinsic hyper-inflammatory response combine to make it more difficult to reach the tipping point towards resolution of inflammation

Improved understanding of the ñswitchò from propagation of inflammation to its active resolution might allow us to identify targets that could be modified to push the switch and tip the inflammatory profile into resolution. The role of alveolar macrophages in the active resolution of inflammation requires further study in CF.

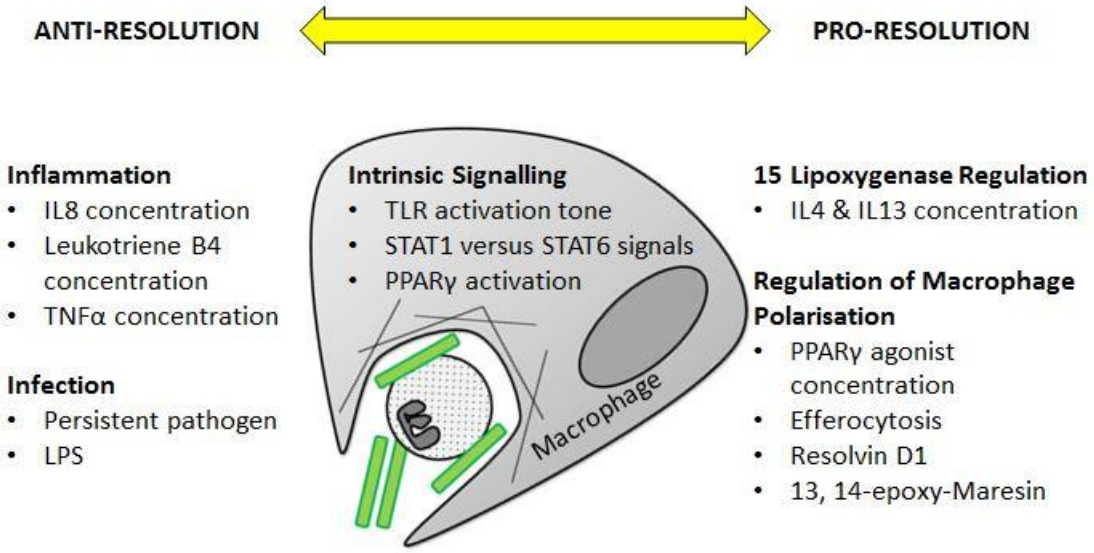

Figure 8-4 Schematic illustrating contextual factors effecting airway macrophages that could play roles in the sensory arm of the ñswitchò, regulating the transition from propagation of inflammation to its active resolution

8.7 Future Work - Key Questions to be addressed

¶ Given that the function of anti-microbial peptides in the ASL are pH-sensitive (128, 129) - do LXA_4 and RvD1, by elevating the ASL height, affect the ASL pH / restore the bactericidal activity of innate anti-microbial peptides?

Pezzulo et al. used elegant methods to evaluate the ASL pH, using a pH-sensitive planar optical probe, and the bacterial killing capacity of ASL using *S. aureus* linked to gold grids placed on the airway surface (128). Similar methods could be employed to evaluate the effects of LXA_4 and RvD1 upon ASL pH and bactericidal function using the primary CF bronchial epithelial culture model.

¶ What is the mechanism behind RvD1 mediated enhanced intracellular killing of *P. aeruginosa* by primary CF alveolar macrophages?

Candidate mechanisms by which RvD1 might have enhanced intracellular killing of *P. aeruginosa* by CFAM include; effects on the macrophage actin cytoskeletal

network (405) - accelerating internalisation and recruitment of vesicles leading to the maturation of the phagolysosome; effects on the acidification of the phagolysosome and the consequent activity of defensins ;and, effects on the activity of phagolysosome associated Nitric Oxide Synthase (479) in generating reactive oxygen species. The effect of RvD1 on the kinetics of CFAM cytoskeletal rearrangement and recruitment of vesicles leading to the maturation of the phagolysosome could be studied by treating CFAMôs with RvD1 during bacterial exposure and using fluorescence linked antibodies to identify early, intermediate, and late phagosomes and phagolysosomes. Effects of RvD1 on phagolysosomal acidification in CFAMôs could be explored by incubating macrophages with dextran linked to pH sensitive fluorescein and then measuring fluorescence emission (229). Effects of RvD1 upon ROS production within CFAM could be evaluated by measuring changes in the fluorescence of 29,79-dichlorodihydrofluorescein diacetate (DCF) (230) which fluoresces after cellular oxidation.

- Could RvD1 improve CFAM mediated killing of other CF pathogens such as *Staphylococcus aureus, Burkholderia cepacia*, or Non-tuberculous mycobacteria?
- What is the rank order of potency of SPMôs with respect to anti-microbial function / are there particular synergies with antibiotic classes?

In this piece of work we tested the efficacy of RvD1 in enhancing CFAM mediated killing of *P. aeruginosa*. However, CF pathogens possess divergent repertoires of evasion strategies, for example, *B. cepacia* in particular, has been described to invade and survive inside macrophages (480). Future experiments could test the effect of RvD1 upon the bactericidal capacity of CF alveolar macrophages against *B. cepacia* or *S. aureus*. Furthermore, other SPMôs either singly, in combination, and or, co-administered with antibiotics could be tested for bactericidal efficacy using the CFAM model we have developed, to gain a better understanding of their relative potency.

- Does the in vitro effect of RvD1 upon ASL height & CFAM mediated killing of *P. aeruginosa* translate into improved containment of a bacterial challenge in an animal model of CF e.g. CF mouse / pig model?

A critical step towards translation of this work for therapy involves testing the efficacy of the SPMõs in an animal model of CF lung disease. Particular compounds within the SPM class are expected to have cell type / tissue type specific roles and actions (253). A good candidate SPM, or panel of SPMõs for translation could be that which is most potent in containing a bacterial challenge. Candidates could be tested in the context of bacterial challenge in an animal model and compared against endpoints such as; clinical condition of the animal, resolution indices, reduction in bacterial burden, and airways physiological parameters.

- Could the restoration of macrophage mediated neutrophil efferocytosis reduce the neutrophil burden and trigger the active resolution of inflammation?
- Would this then cascade the biosynthesis of the whole orchestra of resolution mediators?

We demonstrated that RvD1 enhanced phagocytosis by CFAM. Dalli et al. reported a rank order of potency of SPMõs with respect to neutrophil phagocytosis; the most potent reported to be Protectin D1, followed by Maresin 1 and Resolvin D3, then Resolvin D2 and, finally, RvD1 (481).

The effect of SPMõs upon neutrophil efferocytosis by CFAM remains untested. It remains to be determined whether SPMõs could bypass neutrophil elastase mediated cleavage of calreticulin expressed upon apoptotic neutrophils, and independently improve efferocytic uptake. To study the effect of SPMõs upon CFAM mediated neutrophil efferocytosis, macrophages could be exposed to apoptotic neutrophils (either from BAL fluid or aged neutrophils isolated from blood) in the presence of Neutrophil Elastase (in BAL fluid or exogenously added) (to recapitulate the cleavage of the calreticulin). Changes in the rate of double positivity for macrophage marker CD68 and neutrophil marker Myeloperoxidase (MPO) by Flow cytometry would indicate changes in the rate of efferocytic uptake. The conditioned culture medium could be captured for the evaluation of the SPM profile (by LC-MS/MS) elaborated by the post efferocytosis.

Alternatively an SPM could be administered to an animal model of CF by inhalation or enterally by gavage (482). The lungs could then be lavaged to assess the neutrophil burden and the quantity of macrophages with evidence of neutrophil ingestion by flow cytometry. The lavage fluid could also be tested for the lipidomic footprint (by LC-MS/MS) post SPM administration.

- Is there a role for SPMõs as adjuvant therapeutics in CFPE to hasten the resolution of inflammation, shorten the required duration of antibiotic treatment or limit the extent of structural pulmonary damage incurred during exacerbation?

A significant body of work still lies ahead to achieve the stated aim to evaluate the role of SPMõs as adjuvant therapeutics in CFPE. This leads us on to a consideration of how to progress this work towards translation into therapy.

8.8 Pathways towards Application of Pro-resolution mediators in CF Therapy

The overarching aim of this work has been to gain a greater understanding of the role of SPMs in CF lung disease, with a view to translate new developments in the resolution field for therapeutic application in CF.

8.8.1 Proof of Principle

We have put forward a case õin principleòfor the consideration of SPMõs as candidate therapeutics for CF. Notably, we have performed *in vitro* work on primary human CF tissues and observational studies with children with CF. We have illustrated a context for application and demonstrated positive associations between the concentrations of pro-resolution mediators and recovery from CFPE. To bolster this õproof of principleòan important piece of work, moving forward, is to define the best candidate(s) to be tested in the most suitable context for application to give the most robust chance for these compounds to fulfil their potential as candidate therapeutics.

8.8.2 Which compound / combination?

The cases for the application of LXA_4, RvD1 and RvD2 are the most developed. LXA_4 enhances ASL height, restores epithelial barrier function, attenuates

inflammation, regulates neutrophil recruitment and enhances repair in CF models (465). RvD1 increases ASL height, attenuates IL8 secretion and restores phagocytic and bactericidal capacity to CF alveolar macrophages. RvD2 has potent antimicrobial properties and is associated with a favourable outcome from CFPE. The comparison between SPMõs as regards their anti-microbial potency has yet to be performed. If the strategy is to trigger the whole orchestra of SPM synthesis by driving macrophage mediated neutrophil efferocytosis, then the SPM which does this with the greatest potency is of the greatest interest (481). Maresin 1 has autacoid activity and drives feed-forward amplification of SPM synthetic capacity (298). Further work is required to understand functional redundancy in this class of compounds, their synergies and refine the answer to the question ï which compound / combination?

8.8.3 Stability

An important challenge in considering SPMõs for therapy regards the chemically unstable nature of the lipid compounds. They are also rapidly degraded by endogenous prostaglandin dehydrogenases (284). To this end, stable analogues have been synthesised (303, 483). A recent study of the benefit of 15(R/S)-methyl-LXA_4 in the topical treatment of infantile eczema demonstrated both safety and efficacy (365). The efficacy of stable analogues could be tested against their naturally occurring counterparts *in vitro*. Whether it is safe and desirable to have prolonged chemical stability / longer tissue exposure to these potent compounds needs to be evaluated.

8.8.4 Which route and dose?

Both oral and inhalation routes could be considered for administration. Oral administration of RvD1 was reported to result in rapid accumulation in plasma and systemic efficacy in a mouse model (482). Off target pro-resolution effects might in fact be considered desirable in CF, since it is a multi-organ disease (e.g. there are currently no effective drug therapies for CF associated liver disease). The airway effects of LXA_4 inhalation challenge have been evaluated in a pilot study of eight asthmatic and healthy adult subjects. The challenge was tolerated, had no adverse effect on pulse or blood pressure and demonstrated favourable effects on specific airway conductance (364). The physiological effects of SPMõs have been

described to be potent in the pico to nano-molar range. Again, further work is merited in *in-vitro* models / in CF animal models.

8.8.5 Contextual Factors

The most appropriate context for the application of SPMõs in CF therapy could be debated. Our finding that LXA_4 production was relatively depressed compared to pro-inflammatory mediators at clinical baseline in CF raises the case that a chronic administration strategy of a stable SPM analogue might yield clinical benefit. However, several issues discourage from this lead approach. Firstly, safety concerns associated with the use of immune modulators outside the context of antibiotic co-administration in CF (241). Secondly, the recognition that the evaluation of clinical outcomes for chronic administration of immune modulators in a childhood cohort is fraught with difficulties arising particularly from the long lead time to detect benefit, and the large number of children needed to treat to show a significant treatment effect over time. Thirdly, the natural context for the liberation of SPMõs is in the context of ñactive resolutionò of acute inflammation in concert with a host (+/- antibiotic augmented) response to eliminate bacterial pathogens.

Urbach et al. previously reported a significant increase in LXA_4 concentration in sputum after the completion of antibiotics for CFPE, with inverse correlation to IL8 concentration (354). Given reports that SPMõs have both pro-resolving action and act as antibiotic adjuvants, we directed our attention to a consideration of SPMõs as candidate adjuvants to antibiotic therapy in the context of CFPE. Developing this concept we demonstrated that RvD2 and 15d-PGJ2, in particular, were associated with improved FEV_1 recovery from CFPE. There are recognised clinical endpoints in CFPE which could be easily evaluated for benefit, including; length of stay in hospital, length of antibiotic administration, extent of clinical recovery post CFPE (change in FEV_1, weight gain). In the context of chronic lung infection, we could consider SPMõs (or their stable analogues) as adjuvant treatments in combination with intravenous antibiotic administration, used to achieve bacterial containment and creating a context appropriate for the active resolution of inflammation.

In considering any strategy to use an SPM to drive a cascade of endogenous SPM biosynthesis (by driving efferocytosis), account must be taken of the availability of

DHA and EPA as substrates. Efforts are on-going to evaluate the therapeutic benefit of DHA in CF (345). DHA supplementation alone, as it relates to SPM biosynthesis might be limited in its impact due to reduced 15-LO expression and thus a limited capacity to utilise DHA to synthesise the downstream bioactive SPMôs.

8.8.6 Timing

In our study of CFPE, RvD2 was detected in sputum even in the early days of antibiotic administration. It peaked in concentration at Day 6-8. In addition to validating these findings in a larger cohort, further work is needed to compare the effectiveness of SPM administration given in an animal model throughout the duration of antibiotic treatment for CFPE, as compared to initiating treatment after day 4-5.

8.8.7 Animal studies

Well conducted animal studies could serve to answer a number of the outstanding questions outlined in this chapter. Furthermore, conducting these studies would advance our progress towards translation by demonstrating safety and efficacy and defining the best SPM candidates and refining treatment protocols. Furthermore such studies would be essential to satisfy regulatory requirements for clinical drug development.

8.8.8 Drug Trials

One of the biggest hurdles facing the development of any drug for clinical application surrounds funding Phase III clinical trials. The cost of conducting a Phase III clinical trial has been estimated at 200-300 million USD, or 8-9 million USD for an ñorphan diseaseò (484). Thus, there must be the potential to recoup the costs of developing the drug by making profits from the sale of the drug. This brings several factors to the fore; the size of the population of sufferers of the condition, their capacity to pay for the drug, the cost-benefit analysis of treatment, patents and intellectual property rights over the development of the drug.

Two pathways to consider in navigating a path through the funding and regulatory obstacles to test SPMôs for CF therapy include; (a) CF as the ñtrail blazerò, and; (b)

SPM development for another mass market application and adoption of the compounds into the field of CF therapy following licencing for the other application. CF therapy has advantages in terms of drug development such as the key role played by the CF Association in advocating for and actively participating in drug development, including funding early phase clinical drug development (the ñCF drug development pipelineò).

In CFPE, there is a cost associated with weeks of hospitalisation and antibiotic administration. If there were to be proven benefits in terms of recovery of lung function following CFPE, this might potentially translate into survival gains and then the cost-benefit analysis includes an examination of the increase in ñquality adjusted life yearsò.

In regulatory terms, CF has been categorised as a ñrare/orphanò disease and Kalydeco was registered as an ñOrphan Medicineò (485). The regulations for ñorphan diseasesò allow for smaller Phase III trials and therefore reduced costs of drug development.

Notably, these compounds are not unique to CF application. Resolution of inflammation poses a challenge across the entire spectrum of disease pathogenesis. Significant research effort is underway to investigate the role of SPMôs in atherosclerosis, cardiovascular disease, Alzheimerôs disease, sepsis, and autoimmune disease to name a few. There are on-going clinical phase III trials of Resolvin analogues being tested for application in the context of dry eye disease and periodontal disease. Should these trials prove that these compounds are beneficial in those contexts, it may be possible (and significantly less costly) to test their application for ñoff labelò applications in CF therapy.

However, the specific cell targets and tissue type and disease specific roles of this class of compounds, has not fully been explored. To illustrate this point it is pertinent to consider the example of the prostaglandins; a non-redundant class of eicosanoid compounds that find application in diverse aspects of therapy, e.g. Prostaglandin E2 (Dinoprostone) is used clinically to soften the cervix and induce uterine contraction and has vasodilator activity, whereas Prostaglandin E1 (Alprostadil) is used to prevent the closure of the ductus arteriosus. Particular

compounds within the SPM class might not be functionally redundant, and as such the particular SPM analogues currently in trial might not be those best suited to a CF application. Therefore, further work is required to understand the differences between the various resolvins, protectins and maresins and distinguishing between functional redundancy, and tissue / cell type specific actions of each mediator.

8.8.9 Biomarker applications

A potential application of our findings might be to validate a combination of pro-resolution mediators (including Resolvin D2, 15-d-PGJ2 and 13-HODE) measured in sputum at Day 6-8 of CFPE as a biomarker panel to predict the likelihood of successful recovery from CFPE. This could be used to aid treatment decisions e.g. for intensification or completion of therapy.

8.8.10 Alternatives to direct SPM supplementation

We have observed positive associations between PPARɔ agonist concentrations measured in sputum during CFPE and full recovery of pre-morbid FEV_1. To that end our work lends weight to the body of opinion recommending the development of PPARɔ agonists for CF application (362, 363). Furthermore, the wider resolution field have proposed a range of alternative strategies to rectify resolution deficits and enhance SPM biosynthesis including; the use of aspirin to generate aspirin triggered isoforms of the SPMs; statins; glucocorticoids; supplementation of EPA and DHA; and the inhibition of Prostaglandin biosynthesis (486).

8.8.11 Will there be a need for CF therapeutics in the post-corrector/modifier era?

Whilst the field continues to celebrate the success of achieving therapeutic benefit via CFTR modulation strategies, for the majority of people with CF, these gains are not expected to be sufficient to completely prevent lung function decline, rather to slow the decline. Restoration of airway hydration, muco-ciliary clearance, and strategies to reduce lung inflammation and heighten anti-microbial responses are likely to remain core goals of CF therapy for the majority.

8.9 Conclusion

We have shown that in CF *in vitro* bronchial epithelial cell models, RvD1 restored ASL height, attenuated NFκB mediated pro-inflammatory signalling and reduced the secretion of the neutrophil chemo-attractant IL8. In primary CF alveolar macrophages, RvD1 promoted phagocytosis and intracellular killing of *Pseudomonas aeruginosa* (illustrated in Figure 8-5).

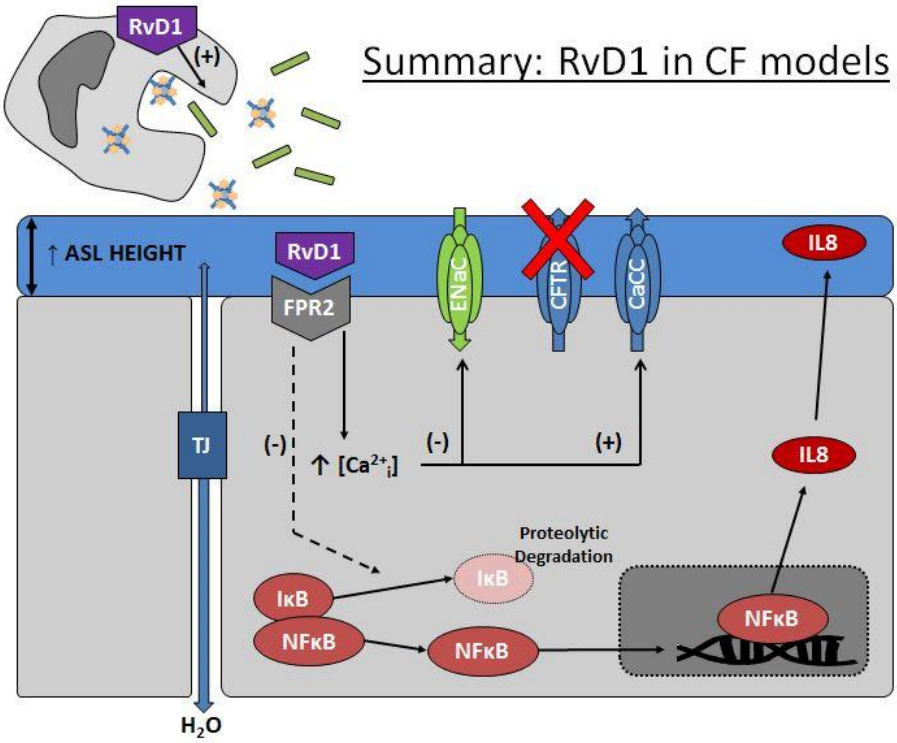

Figure 8-5 Schematic illustrating the beneficial physiological effects of RvD1 in CF models demonstrated in this thesis.

In CF *in vitro* bronchial epithelial cell models RvD1 restored ASL height via ALX/FPR2 receptor stimulation, reduced the secretion of the neutrophil chemo-attractant IL8 via preservation of IəB. In primary CF alveolar macrophages, RvD1 promoted phagocytosis and intracellular killing of *Pseudomonas aeruginosa*.

We demonstrated that the synthesis of LXA_4 is defective in CF. *In vivo* evidence was presented of impoverished 15 Lipoxygenase-2 gene expression associated with a depressed LXA_4 / LTB_4 ratio in the lower airways of children with CF (illustrated in Figure 8-6).

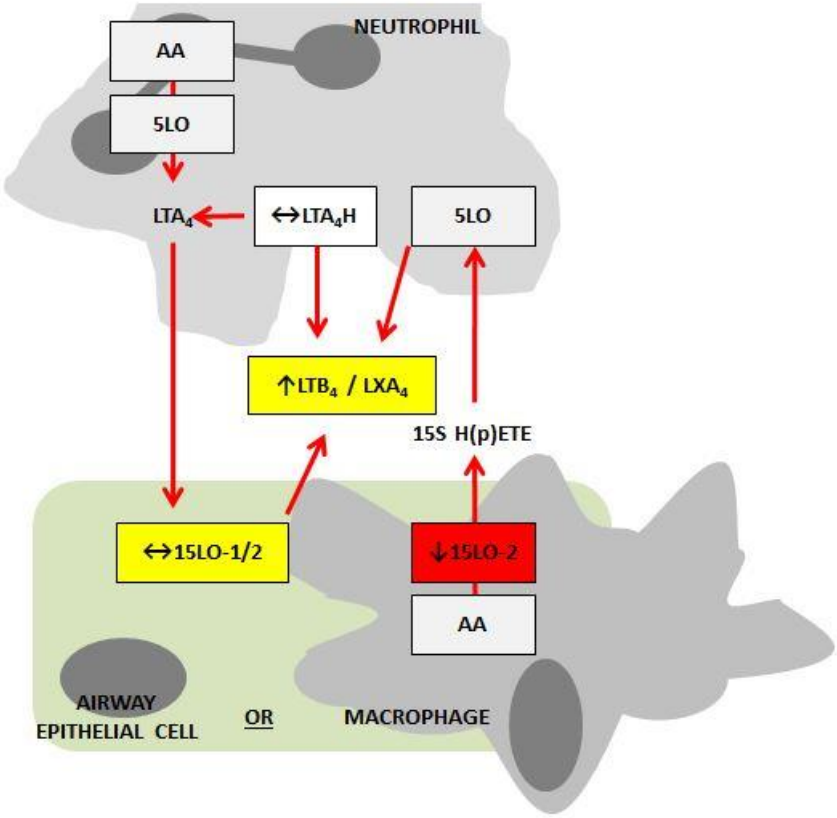

Figure 8-6 Schematic illustrating the finding of impoverished 15 Lipoxygenase-2 expression associated with a depressed LXA_4 / LTB_4 ratio in the lower airways of children with CF

Airway epithelial cell expression of 15 LO-1&2 were not significantly different in CF cells.

Moreover, favourable relationships between pro-resolution mediators RvD2 and 15-d-PGJ_2 concentrations in sputum and the full recovery of pre-exacerbation FEV_1 following CFPE have been demonstrated (Figure 8-7).

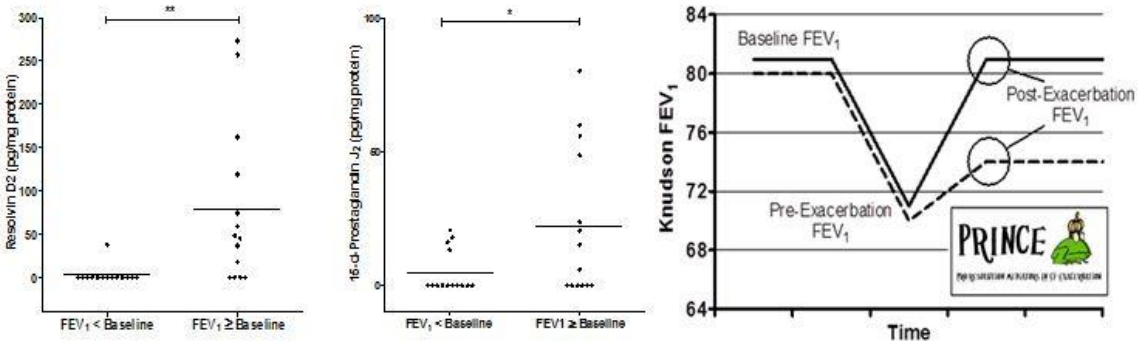

Figure 8-7 A summary of the key findings of the PRINCE study relating pro-resolution mediators in sputum to recovery of Baseline FEV_1 post CFPE
Higher concentrations of RvD2 and 15-d-PGJ_2 measured in sputum during CFPE were found among children who fully recovered their pre-exacerbation baseline FEV_1. *Graph of FEV_1 change over the course of time during CFPE adapted from Collaco et al (368) .

SPMâs have the potential to counteract the release of free Neutrophil Elastase into the airway by reducing the neutrophil burden and removing dead and dying neutrophils from the airway. We optimized methods to develop a better understanding of 15-LO regulation in CF alveolar macrophages and macrophage polarisation. These studies advance novel strategies to tip the balance in favour of active resolution of prolonged frustrated neutrophilic inflammation. Restoration of physiological concentrations of SPMâs, could potentially drive macrophages towards a resolution phenotype, and be attended by a cascade of de novo SPM biosynthesis and drive resolution of inflammation in the CF airway. The most critical step in advancing this work will be in testing SPMâs in an animal model of CF faced by a bacterial challenge to mimic CF pulmonary exacerbations.

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