Abstract

Inflammatory lung disease is the major life-limiting factor of cystic fibrosis (CF) and occurs through a self-sustaining cycle of airway obstruction, infection and inflammation. Although there is no consensus regarding the pathways responsible for the excessive inflammation, reducing lung-damaging pro-inflammatory responses are likely to be beneficial for CF patients.

Using CF (IB3-1) and non-CF control (C38) respiratory cells, the host-pathogen interaction between the airway epithelium and the common CF pathogens P. aeruginosa and B. cepacia was investigated. Using purified Toll-like receptor (TLR) ligands and different knock-out strains of P. aeruginosa, TLR5 was identified as the receptor mediating much of the increased inflammatory response to CF pathogens.

To validate TLR5 as an anti-inflammatory target, the disease modifying effects of the functionally relevant TLR5 c.1174C>T single nucleotide polymorphism (rs5744168) was analysed in approximately 80% of Canada’s CF population. rs5744168 encodes a premature stop codon and the T allele is associated with 45.5 – 76.3% reduction in flagellin responsiveness. CF patients carrying rs5744168 (CT or TT) had a significantly higher body mass index than CF patients homozygous for the common allele (CC) (p=0.044); however, similar improvements in lung function associated with the T allele were not statistically significant.

Since TLR5 mediates much of the excessive inflammation to P. aeruginosa, it is of interest to understand the mechanisms underlying this dysregulated immune response. By combining gene expression arrays with network analyses and biochemical assays, ER stress was identified as a potential mechanism dysregulating p38 MAP kinase activity and leading to potentiated immune responses.

Together, this thesis provides data underscoring the importance of TLR5-mediated excessive pro-inflammatory immune response by CF airway cells to P. aeruginosa.
The association of the \textit{TLR5\textsuperscript{392STOP}} SNP with higher BMI in adult CF patients indicates an important role for TLR5 in CF disease severity. Finally, ER stress may potentiate the immune response to flagellin by signalling through p38 MAP kinase, supporting an emerging paradigm in which the imbalance of protein homeostasis can lead to altered signalling events. Strategies to inhibit either TLR5 signalling, ER stress signalling or to improve the cellular protein homeostasis may prove useful in treating life limiting inflammation in CF.
Preface

Ethics approval was obtained for collection of blood samples from UBC C&W Research Ethics Board (ethics certificate #H09-01192).

Permission for reproduction of Chapter 2 and 3 in whole has been obtained from the The American Association of Immunologists, Inc.

The study presented in Chapter 2 of this work was published in the Journal of Immunology on June 1 2008 (accepted for publication April 3, 2008; Copyright 2008. The American Association of Immunologists, Inc.). The study was designed by CJ Blohmke with assistance from SE Turvey. Bacterial strains depicted in Table 2.1 were a generous gift from DP Speert, REW Hancock and KD Smith. Bacterial strain PAKΔfliC (pUCP22::fliC) was generated by J Overhage. Figure 2.2 was produced by DG Hancock. Figure 2.4 was produced by IM Elias. Data in Figure 2.8 was produced by CR Lane. Data shown in Figure 2.5 and 2.6 were generated by CJ Blohmke with the help of RE Victor and AF Hirschfeld. Patient samples were provided by CF patients through AGF Davidson and PG Wilcox. CJ Blohmke performed all remaining experiments, analysed the data and wrote the manuscript with assistance from SE Turvey.

The study presented in Chapter 3 of this work was published in the *Journal of Immunology* on December 15 2010 (accepted for publication October 11, 2010; Copyright 2010. The American Association of Immunologists, Inc.). The study was designed by CJ Blohmke with assistance from SE Turvey. Genotyping of CF patient samples was performed by D Stefanowicz and AJ Sandford. Patient data was derived from the Canadian Consortium for CF Modifier Studies at the Hospital for Sick Children with help from M Corey, R Dorfman, PR Durie and J Zielenski. Statistical data analysis was performed by J Park and D Daley. Figure 3.1, panel D was produced by J Schneiderman. CJ Blohmke designed and performed all remaining experiments and wrote the manuscript.


The study in described in Chapter 4 was designed by CJ Blohmke with assistance from SE Turvey. Gene expression array experiments were conducted in collaboration with REW Hancock, ML Mayer, R Falsafi and the Jack Bell Micro Array facility. Gene expression data analysis was performed by ML Mayer, CD Fjell and CJ Blohmke. Figure 4.1 was generated by M Mayer. CJ Blohmke generated the data for Figures 4.2-4.5, wrote the manuscript and analysed the data.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AEC</td>
<td>Airway epithelial cell</td>
</tr>
<tr>
<td>aGM1</td>
<td>Asialo GM1</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ASL</td>
<td>Airway surface liquid</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar liquid</td>
</tr>
<tr>
<td>BCC</td>
<td><em>Burkholderia cepacia</em> complex</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculovirus inhibitor of apoptosis repeat</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BP</td>
<td>Biological process</td>
</tr>
<tr>
<td>C38</td>
<td>CF corrected control cell line</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase activation recruitment domain</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFRD</td>
<td>CF-related diabetes</td>
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<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>CIITA</td>
<td>Class II transactivator</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DE</td>
<td>Differentially expressed</td>
</tr>
<tr>
<td>DZ</td>
<td>Dizygous</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic initiation factor 2α</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated protein degradation</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FEV1</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>GI</td>
<td>Gastro-intestinal</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>Grp78</td>
<td>Glucose-regulated protein, 78kDa</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td><em>Hemophilus influenzae</em></td>
</tr>
<tr>
<td>IB3-1</td>
<td>CF cell line</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IEC</td>
<td>Intestinal epithelial cell</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor IκB kinase</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>Ipaf</td>
<td>ICE-protease activating factor</td>
</tr>
</tbody>
</table>
IRAK1  IL1R associated kinase 1
IRAK4  IL1R associated kinase 4
IRE-1α  Inositol requiring enzyme 1
IRF  Interferon regulatory factor
IκBα  inhibitor κ B-α
JNK  Janus kinase
kb  Kilo base
KC  Keratinocyte chemoattractant
kDa  Kilo dalton
KO  Knock-out
LDH  Lactat dehydrogenase
LPS  Lipopolysaccharide
LRR  Leucin-rich repeat
MALP-2  Macrophage-activating lipopeptide 2
MAP kinase  Mitogen activated protein kinase
MBL  Mannose binding lectin
MOI  Multiplicity of infection
mRNA  Messenger ribonucleic acid
MyD88  Myeloid differentiation factor 88
MZ  Monozygous
NBD1  Nucleotide binding domain 1
NE  Neutrophil elastase
NEMO  NF-κB essential modulator
NF-κB  Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR  NOD-Like Receptor
NOD  Nucleotide binding oligomerization domain
ORA  Over-representation analysis
P. aeruginosa  Pseudomonas aeruginosa
PAb-hTLR5  Polyclonal antibody against human TLR5
PAMP  Pathogen associated molecular pattern
PBMC  Peripheral blood mononuclear cell
PCL  Periciliary layer
PERK  PKR-like eukaryotic initiation factor 2α kinase
PI  Pancreas insufficiency
PPIA  Cyclophilin A
PKA  Protein kinase A
PRRs  Pattern recognition receptor
PS  Pancreas sufficiency
PYD  Pyridding domain
qPCR  Quantitative polymerase chain reaction
ROS  Reactive oxygen species
S. aureus  Staphylococcus aureus
SNP  Single nucleotide polymorphism
T3SS  Type three secretion system
TAB  TAK1-binding protein
TAK1  Transforming growth factor-activated kinase 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/Interleukin-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Tm</td>
<td>Tunicamycin</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
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<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TRAF6</td>
<td>Tumor necrosis factor receptor associated factor 6</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
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<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor protein-inducing IFN-γ</td>
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<tr>
<td>Ubc13</td>
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<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
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<tr>
<td>XBP-1</td>
<td>X-box binding protein 1</td>
</tr>
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Acknowledgement

The past years have been an incredible journey through different cities and countries leaving me with so many invaluable experiences. This endeavor would not have been possible without the tremendous help from many different people.

I want to express my deepest gratitude to Stuart Turvey, my supervisor and mentor. I am indebted to you for taking this chance and showing the flexibility of dealing with 'some German from Amsterdam'. Your supervision is tremendous and your patience and support incredible. I decided to commit to this work in your lab because I knew that I can learn so much from you. As your first PhD student, I hope I could ‘cater’ to your expectations and made this a memorable experience for you. On that note, many thanks to David Speert for being so tremendously supportive at the beginning of this work.

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Finally, Patrizia – I cannot put in words how grateful I am for your understanding and support, your belief in me and your love. Muito obrigado!
Dedication

Für meine Familie - Susanne, Robert, Andreas und Julian Blohmke.

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1. Introduction

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder caused by a loss-of-function mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene [1]. CF is a life limiting disease and affects approximately 1 in every 3500 live births, making it the most common monogenic cause of death in the Caucasian population [2]. Although our understanding of the disease has increased significantly and better treatment options have been developed over the past three decades, the median age of survival is still only 47 years today [3]. New therapeutic approaches are critically needed to increase the life expectancy, and importantly, also the quality of life for people living with CF.

1.1.1 Genetics

The CFTR gene located on chromosome 7, is 230kb (kilo bases) and encodes a 1480 amino acid cyclic adenosine monophosphate- (cAMP) controlled transmembrane chloride channel of the ABC-binding cassette transporter superfamily [4, 5]. A single mutation in the gene can result in the loss of function of the chloride channel. Over 1,500 mutations in the CFTR gene that give rise to the CF phenotype have been described. They are categorized into five classes reflecting their different molecular consequences [6]. Class 1 mutations are nonsense mutations or premature stop codons that result in a lack of CFTR messenger RNA (mRNA) synthesis. Class II mutations are missense mutations that translate successfully, however the protein is misfolded and consequently retained in the ER and degraded. In contrast, class III and IV mutations do not lead to folding defects. The protein reaches its final destination in the cell membrane but is dysfunctional due to either defective regulation of the channel function (class III) or decreased chloride ion conductance (class IV). The fifth class of mutations results in
a decreased rate of synthesis of functional CFTR protein (class V) [6-8]. The majority of the disease causing mutations discovered is represented only by a small number of patients. The most prevalent mutation among the Caucasian population is characterized by the loss of three base pairs, resulting in the deletion of a phenylalanine residue at the position 508 (ΔF508) in the amino acid sequence of CFTR [9]. This mutation belongs to the Class II category and leads to retention of the protein in the ER [10]. Approximately 1 in 25 healthy people are heterozygous carriers of this mutation and it is found in about 70% of CF patients [8].

### 1.1.2 Modifier Genes

Since the discovery of the CFTR gene in 1989 [1], tremendous advances in improving life quality and life expectancy have been made. However, disease severity is poorly correlated with CFTR genotype, making it difficult to predict disease outcomes based on the mutation alone [11, 12]. Twin and sibling studies have shown a higher level of concordance of clinical outcome measures (forced expiratory volume in one second, FEV₁) in monozygous (MZ) twins when compared to dizygous (DZ) twins [13, 14], indicating that genetic determinants other than CFTR mutations can modify the outcome of the disease (modifier genes). Modifier genes are loci containing gene variants (such as single nucleotide polymorphisms, SNPs) that affect the clinical manifestation of a disease. Unfortunately, it is difficult to determine genetic risk factors and the identification of modifier genes in CF remains largely elusive. To date three modifier genes have been discovered and replicated in CF – TGF-β, MBL-2 and IFRD1 [15-17]. All of these have been associated with either decline of lung function or time of acquisition of Pseudomonas aeruginosa.
1.1.3 Pathophysiology

The CFTR protein is expressed in a variety of organs. However, its best known function as a chloride channel is restricted to epithelial cells of the gastrointestinal (GI) tract, the pancreatic duct, the reproductive tract, secretory glands and the airway epithelium [18-20], reflecting a dominant role in epithelial tissues. The channel is activated by cAMP-dependent phosphorylation of the nucleotide binding domain 1 (NBD1) by protein kinase A (PKA), which opens the channel to allow chloride ions to flow across the plasma membrane [4]. Besides gas exchange, the lung tissue has important homeostatic functions. The tissues lining the airways are composed of mucus-secreting goblet cells and ciliated epithelial cells. Goblet cells produce large amounts of mucus which has important functions in trapping inhaled foreign particulate materials and pathogens. A periciliary liquid (PCL) overlaying the ciliated cells ensures the permanent beating motion of the cilia to move the mucus towards the throat (mucociliary escalator). At the pharynx, the mucus is either swallowed or expectorated, thus removing anything foreign that is contained within the mucus. This important homeostatic function is highly dependent on the viscosity of the overall airway surface layer (ASL; PCL and mucus), which determines the velocity and efficiency of the mucociliary escalator. The ASL volume is regulated by active transepithelial absorption of sodium via the epithelial sodium channel (ENaC). When sodium enters the cells by ENaC-dependent transport, water and chloride follows. In turn, if the volume of the ASL needs to be increased, CFTR-activated chloride transport inhibits ENaC-dependent sodium absorption and transports chloride into the extracellular space followed by sodium and water [21-23]. The loss-of-function mutation in CFTR results in chloride impermeability. The retention of chloride ions inside the cell and the resulting lack of ENaC inhibition leads to the hyper-absorption of sodium ions and water, leaving the ASL dehydrated [24]. These dry secretions result in the collapse of the PCL, paralyzing the mucociliary escalator. This prevents the removal of mucus, particulates and bacteria, thereby facilitating colonization of the CF airways with pathogens such as P. aeruginosa.
The accumulation of dehydrated mucus on the surface of epithelial cells results in disease manifestation in several organs. While symptoms include gut obstruction (meconium ileus), CF-related diabetes (CFRD), infertility and liver disease, the pulmonary manifestation represents the most significant cause of morbidity and mortality in CF [6, 8]. Dysregulated ion homeostasis in CF patients results in thick and viscous mucus accumulation in the distal airways, leading to lung obstruction, the decline of lung function and inhibition of airway defence mechanisms (such as the mucociliary escalator) [25-27].

1.1.4 Lung Disease and Inflammation

Along with mucus accumulation, chronic pulmonary inflammation and infection are the major hallmarks of CF lung disease. Inflammation is evident by the increased production of pro-inflammatory markers including interleukin-8 (IL-8), elastase, interleukin-1 (IL-1) and interleukin-6 (IL-6) [28-32]. Neutrophil infiltration of the lung tissue is characteristic of inflammatory lung disease and is likely mediated by increased IL-8 production and its interaction with the neutrophil chemokine receptors CXCR1 and CXCR2 [33, 34]. Due to unopposed proteolytic activity in the CF lung, neutrophils are essentially inactivated by the cleavage of CXCR1. While CXCR2 is still active resulting in further attraction of neutrophils into the lungs, inactive CXCR1 prevents neutrophils from executing their anti-microbial functions [35]. Furthermore, the cleaved fragment of CXCR1 can stimulate further IL-8 production [35]. Inactivated neutrophils become necrotic and release their antibacterial effector molecules, such as reactive oxygen species (ROS), in an uncontrolled fashion increasing the adverse nature of the environment. Additionally, neutrophil-derived DNA further thickens the mucus [36] and the release of neutrophil elastase (NE) contributes to destruction of lung tissue, obstruction and further susceptibility to infection [37-39].
The inefficiency of mucus removal and anti-bacterial defence mechanisms of neutrophils results in the infection of the CF lung with bacteria, viruses and fungi. Intriguingly, only a small repertoire of bacterial species including *P. aeruginosa*, *Haemophilus influenza*, *Staphylococcus aureus* and species of the *Burkholderia cepacia* complex (BCC) infect and colonize the airways of CF patients [8]. In the early stage of disease, predominantly viruses, fungi and some bacteria (*H. influenzae*, *S. aureus*) infect CF patients. *P. aeruginosa* follows and colonizes approximately 80% of CF patients by early childhood [40]. Infection with species of the BCC are often associated with severe decline of lung function and sometimes, if invasive, with end-stage disease [41]. While these are the most prominent pathogens linked to clinical presentation, evidence is emerging that suggests that other microorganisms may also contribute significantly to disease progression and severity [42, 43].

Mucopurulent plugging of the airways followed by infection and uncontrolled inflammation represents a self-perpetuating vicious cycle leading to bronchiectasis, dramatic loss of lung function and ultimately to death. To date, there is no consensus over whether infection precedes inflammation or if inflammation is caused intrinsically. Data generated from fetal, immortalized human cell lines showed enhanced activation of pro-inflammatory pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Activator Protein-1 (AP-1) signalling in the absence of infection [44]. Furthermore, several studies showed increased pro-inflammatory markers in bronchoalveolar lavage (BAL) fluids of CF patients in the absence of culturable infection [31, 45]. These studies suggest intrinsic pro-inflammatory events, however, it is disputed what molecular mechanism underlies this phenotype. While data by Perez et al. have suggested the lack of CFTR channel function as potential cause [46], other have proposed endoplasmic reticulum (ER) stress-induced inflammatory responses due to the
retention of misfolded CFTR in the ER [47]. In contrast, a study by Armstrong et al. in 2005 compared pro-inflammatory markers in the BAL fluids of infected, uninfected CF infants and control subjects, and concluded that infection initiates and sustains the inflammation seen in the CF lung [48]. Data from the recently developed CFTR knock-out (KO) pig model showed that newborn CF pigs have airway development abnormalities, but no inflammation could be detected at birth. In contrast, hours after birth the lungs of the CFTR KO piglets developed signs of CF lung disease including airway inflammation, mucus accumulation and infection with several bacterial species. These findings indicate that the lungs of CF pigs have a significant defect in host-defence mechanisms [49, 50]. The fact that antibodies against P. aeruginosa can be detected 6 to 12 months prior to isolation of the organism from respiratory secretions such as sputum or BAL fluids [51], raises concerns about the sensitivity of currently available detection methods. Regardless of whether it is infection or inflammation that occurs first, it is generally accepted that chronic inflammatory responses in the CF airways are the major cause of progressive lung disease, and ultimately, death. Consequently, insights into the molecular mechanisms underlying CF pulmonary inflammation are critical for the development of novel anti-inflammatory therapeutic strategies.

1.1.5 Therapeutic Approaches

In a medical context, CF has been an incredible story of success. While life expectancy did not exceed early infancy when CF was first described by Dorothy H. Andersen in 1938 [52], the average age of survival today is in the mid-40s [3]. Especially in the past 30 years, life expectancy has increased dramatically due to the discovery of new treatment strategies. Different physiotherapeutic approaches using inhaled mucolytics, such as Dornase alpha (a deoxynuclease), and hypertonic saline have been proven to alleviate the burden of pulmonary obstruction [36, 53, 54]. The use of β-lactam, macrolide and aminoglycoside antibiotics [6] and improved clinical practices have made significant differences in infection control,
resulting in a significant rise in the median age of survival. Finally, more attention is being paid to the inflammatory component of CF lung disease. Several clinical trials of anti-inflammatory drugs in CF patients have shown improvement in clinical outcome measures such as FEV$_1$ and body mass index (BMI) [55-57]. Unfortunately, adverse side effects render these treatment options unacceptable.

Table 1.1: Selection of Currently On-Going or Completed Clinical Trials for CF Therapeutics.

<table>
<thead>
<tr>
<th>Effect/Target</th>
<th>Effect/Target</th>
<th>Progress</th>
<th>ClinicalTrials.gov ID$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linezolid</td>
<td>Antibiotic against gram-positive bacteria resistant to other antibiotics.</td>
<td>Phase II</td>
<td>NCT00625703</td>
</tr>
<tr>
<td>SB656933</td>
<td>CXCR2 antagonist to prevent neutrophil infiltration.</td>
<td>Phase II</td>
<td>NCT00903201</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>Treatment of infections with fungi.</td>
<td>Phase IV</td>
<td>NCT00528190</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>Anti-inflammatory by reducing ROS.</td>
<td>Phase I</td>
<td>NCT00308243</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>Macrolide antibiotic to target pulmonary infection.</td>
<td>Treatment effectiveness</td>
<td>NCT00205634</td>
</tr>
<tr>
<td>AZD1236</td>
<td>Anti-inflammatory by inhibiting matrix metalloproteases.</td>
<td>Phase II</td>
<td>NCT00812045</td>
</tr>
<tr>
<td>VX-770</td>
<td>Chloride channel potentiator for certain mutations.</td>
<td>Phase III</td>
<td>NCT00909532</td>
</tr>
<tr>
<td>Ataluren</td>
<td>Over-come effects of non-sense mutations such as stop codons.</td>
<td>Phase III</td>
<td>NCT00803205</td>
</tr>
<tr>
<td>QAU145</td>
<td>Inhibits prostasin regulated ENaC activity.</td>
<td>Phase II</td>
<td>NCT00506792</td>
</tr>
</tbody>
</table>

$^a$ [http://www.clinicaltrials.gov](http://www.clinicaltrials.gov)

In order to develop new anti-inflammatory therapies without undesirable side effects, research into the molecular mechanisms mediating CF pulmonary inflammation has increased significantly in the past decade. Several approaches targeting the trafficking or functional defects of CFTR, as well as understanding the molecular pathways underlying increased immune responses to pathogens, have generated promising results with several potential drugs entering clinical trials (Table 1) [58].
1.2 Innate Immunity

The two major defence mechanisms of the human body, the innate and the adaptive immune system, orchestrate a tremendously efficient response to infections with viruses, bacteria, fungi and parasites. Although both arms of the immune system are extremely powerful, often they rely on each other to provide adequate protection from infection.

The adaptive immune system consists of T- and B-lymphocytes, which are produced by a unique mechanism termed clonal selection. The principles of this mechanism define the specificity of the adaptive immune system: each lymphocyte produces a single type of receptor with unique antigen specificity, interaction between the antigen and the receptor activates the lymphocyte, the activated lymphocyte rapidly divides and produces clones expressing the same specific receptor (clonal expansion), and finally lymphocytes recognizing self-antigens are inactivated through a variety of sophisticated tolerance mechanisms. The variability of the T-cell receptor enables the host to respond to virtually any antigen. T-cell activation relies on the presentation of specific antigens by antigen presenting cells (APCs) such as macrophages and dendritic cells (but also B-cells), which leads to the production of different messenger molecules such as cytokines to further shape a specific immune response, or to destroy infected cells. Activation of B-cells results in antibody secretion into the circulation to opsonize a specific pathogen, neutralize and label it for destruction by phagocytic cells and the complement system (see below). A hallmark of the adaptive immune system is the immunological memory that it develops to a specific pathogen, enabling a specific lymphocyte clone to quickly respond to re-occurring infection, facilitating clearance of the pathogen before symptoms can develop. In contrast to the adaptive immune system, which requires up to five days to mount an effective immune response after initial antigen encounter, the innate immune system is activated within minutes of infection.
1.2.1 The Innate Immune System

While the importance of the adaptive immune system is undisputed in the defence against harmful pathogens, the innate immune system is likely to be crucial in activating and shaping the adaptive immune response, thus establishing it as the first line of defence against microorganisms. It consists of physical barriers and mechanisms (epithelia, mucociliary escalator), cellular components (neutrophils, macrophages, dendritic cells), humoral components (cytokines, chemokines, antimicrobials and host defence peptides) and a limited number of receptors [59].

Cells of the innate immune system are of hematopoietic and non-hematopoietic origin. Non-hematopoietic cells such as epithelial cells (lining the GI, the genitourinary and the respiratory tract) and skin cells fulfil an important barrier function, and are also characterized by their immune responsiveness (e.g. cytokine production). In contrast, hematopoietic cells such as neutrophils, macrophages, dendritic cells, eosinophils and mast cells cover several important effector functions, such as clearance of pathogens by phagocytosis and release of effector molecules such as cytokines, chemokines and other bactericidal molecules (humoral components). Furthermore, phagocytic cells can act as a bridging point between the innate and the adaptive immune system by uptake and presentation of antigens (APCs) from engulfed pathogens to activate lymphocytes.

Augmenting these important defence functions, the humoral components of the innate immune system consist of complement proteins, LPS-binding protein, C-reactive protein (CRP), antimicrobial peptides, cytokines and chemokines. These circulating immune proteins are involved in sensing and effector mechanisms to facilitate the clearance of infections. The complement system for example, consists
of three sub pathways which all result in the activation of the C3 convertase, responsible for the production of the opsonin C3b. The three pathways differ solely in their induction and all lead to the activation of C3. The classical pathway is characterized by binding of the initiating protein C1q to the surface of the pathogen, to CRP or antibodies bound to the pathogen. The Mannose-binding lecting (MBL) pathway utilizes MBL to recognize pathogenic surface molecules and induces a sequence of reactions resulting in the activation of C3. The alternative pathway is characterized by the spontaneous hydrolysis (‘tick-over’) of C3 on the surface of any microbe. Although the production of the opsonin C3b is the main outcome of the complement pathway, it can also result in a protein complex (‘membrane attack complex’) that directly destroys the pathogen. The complement pathway presents a few key examples of the interrelatedness between the adaptive and the innate immune system. Thus, C1q can bind to B-cell derived antibodies on the surface of pathogens. Furthermore, opsonisation of pathogens by C3d (a breakdown product of C3b) greatly enhances B-cell effector and memory function [60]. The complement system is a sophisticated biochemical mechanism providing a quick and efficient response mechanism to microbes invading the host, as well as a cross-link combining the functions of the adaptive and the innate immune system.

Mucosal infection is primarily cleared by phagocytes such as neutrophils and macrophages that are recruited by chemokines produced by epithelial cells [61]. Chemokine production is initiated upon recognition of pathogens by ligation of germ-line encoded PRRs. Because microbes can rapidly evolve, PRRs recognize specific pathogen associated molecular patterns (PAMPs) that are essential for the survival of the pathogen and are therefore highly conserved molecules, including nucleic acid structures [59]. PRRs consist of different receptor families – cytosolic proteins such as nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and membrane bound Toll-like receptors (TLRs). NLRs consist of a C-terminal leucine-rich repeat (LRR) domain responsible for recognition of the ligand, and a central NOD or NACHT (NAIP, CIITA, HET-E and TP-1 protein) domain
important for oligomerization of the proteins. At the N-terminus, these receptors have different domains (pyrin (PYD), baculovirus inhibitor of apoptosis repeat (BIR), TOLL/interleukin-1 receptor homology (TIR) or caspase activation recruitment domains (CARDs)) reflecting the different downstream pathways activated upon ligation of the LRR domain [62]. Ligation of these receptors leads to the activation of a large multi-protein complex consisting of oligomerized NLR components and scaffolding proteins, termed inflammasome. The inflammasome recruits the inactive form of caspase-1 (pro-caspase-1) and cleaves it into active caspase-1. While caspase-1 plays an important role in apoptosis, it also processes pro-IL-1β and pro-IL-18 into active cytokines [63]. Furthermore, stimulation of NLRs can lead to the activation of transcription factors such as NF-κB, AP-1 and IRFs (interferon regulatory factor). These transcription factors are responsible for regulating inflammatory and apoptotic pathways driving inflammatory responses or cell death [64]. While NLRs play an important role in innate immune recognition, TLRs sense extracellular and phagocytosed microbes to activate an immune response. TLRs present yet another clear example of the interrelatedness of the innate and adaptive immune response. Thus, that they are expressed not only by innate immune cells but also by T- and B-lymphocytes, further distorts the simplistic view of innate and adaptive immunity as two separate host-defence mechanisms.

1.2.2 Toll-like Receptors

TLRs are expressed both in the membrane of epithelial and immune cells and in intracellular compartments such as the lysosome [65], hence they often present the first contact with microbes. TLRs were first described as the receptors mediating the protective immune response to fungal infections in Drosophila [66]. To date, 10 TLRs have been described in humans. They can be divided into subfamilies according to the PAMPs recognized, e.g. TLR7-9 being specific for nucleic acids [67]. Aside from PAMPs, TLRs are also capable of recognizing host proteins such as fibrinogen, cleaved CXCR1 and heat-shock proteins Hsp60 and Hsp70 [35, 68].
Prominent TLR ligands are lipopolysaccharide (LPS) (TLR4), glycoproteins (TLR1/2) and flagellin (TLR5) (Table 1.2).

**Table 1.2: Toll-like Receptors and Their Ligands.**

<table>
<thead>
<tr>
<th>Toll-like receptor</th>
<th>Example ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Triacyl lipopeptides</td>
</tr>
<tr>
<td>TLR2</td>
<td>Zymosan, Lipotechoic acid, Lipoproteins, Peptidoglycan, Diacyl lipopeptides</td>
</tr>
<tr>
<td>TLR3</td>
<td>Poly (I:C) double-stranded RNA, Single-stranded RNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS, Fusion protein, Hsp60? Hsp70?</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
</tr>
<tr>
<td>TLR6</td>
<td>Diacyl lipopeptides, Zymosan, MALP-2</td>
</tr>
<tr>
<td>TLR7</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>TLR8</td>
<td>Single-stranded RNA, R-848</td>
</tr>
<tr>
<td>TLR9</td>
<td>Unmethylated CpG DNA, CpG ODNs, Herpes virus</td>
</tr>
<tr>
<td>TLR10</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

\(^{a)}^{)\text{Compiled from [67, 69-71].}\)

The type I integral membrane proteins consist of a LRR domain on the extracellular side, that is responsible for the recognition of PAMPs, whereas the intracellular side is characterized by a TIR domain [68]. Upon ligation of the LRR domain, TLRs activate two major signalling pathways resulting in the transcription of host response programs to microorganisms. With the exception of TLR3, all TLRs signal through the myeloid differentiation factor 88 (MyD88)-dependent pathway leading to the activation of NF-κB (Figure 1.1). While TLR4 activates the MyD88-dependent
pathway, it is also known to activate the signalling cascade downstream of TLR3 through the adaptor molecules TIR-domain-containing adaptor protein-inducing IFN-γ (TRIF) and TRIF-related adaptor molecule (TRAM) (MyD88-independent pathway) resulting in the activation of IRF3 and/or IRF7 [65].

1.2.3 TLR5 Signalling Cascade

To date, the only identified ligand to stimulate TLR5 is flagellin [72]. Flagellin is the protein subunit of the molecular tail flagellum (pl. flagella), which provides some bacterial species with motility to move towards nutrient sources [73]. By means of the crystal structure of Salmonella typhimurium, four distinct protein domains of flagellin were characterized: N- and C-terminal α-helix domains (D0), central α-helices (D1), and hypervariable central regions consisting of β-sheets (D2 and D3) [74, 75]. The TLR5 recognition site for flagellin has been mapped to amino acid residues 89-96 in the N-terminal D1 domain, and mutation of these residues abolishes the TLR5 stimulating capacity as well as motility [76, 77]. Another study has identified the C-terminal end of the D1 domain as indispensable for IL-8 secretion in Caco-2 cells, indicating this region as essential for immune recognition as well [78]. Upon TLR5 activation, the TIR domain of the receptor associates with the TIR domain of MyD88. Together they recruit IL1R associated kinase (IRAK) 4 via their death domains [79]. Activated IRAK4 phosphorylates IRAK1, which joins the complex formation [80-82]. This is necessary for the recruitment of tumor necrosis factor receptor (TNFR) associated factor (TRAF) 6. IRAK1, which acts as an adaptor between the receptor signalling complex and TRAF6, undergoes autophosphorylation resulting in the dissociation from the TLR5-MyD88-IRAK4 complex without affecting the association of IRAK1 with TRAF6 [83, 84]. IRAK1-TRAF6 then interacts with the ubiquitin-conjugating enzyme E2 variant 1 (Uev1A) and the ubiquitin-conjugating enzyme 13 (Ubc13), leading to the poly-ubiquitination
Figure 1.1: Canonical TLR5-Signalling Cascade.
TLR5 activates MyD88 which leads to phosphorylation of IκBα through the IKK complex. Degradation of IκBα allows NF-κB to translocate into the nucleus to start the transcription of target genes.
and activation of TRAF6 to associate with transforming growth factor (TGF)-β-activated kinase 1 (TAK1) and the TAK1-binding proteins, TAB1-3 [85]. Somewhere within this sequence of protein interaction events, IRAK1 dissociates and is degraded. In this new multi-protein complex, it is TAK1 that phosphorylates the inhibitor IκB kinase (IKK) complex, consisting of IKK-α, -β (catalytic subunits) and IKK-γ (the regulatory subunit a.k.a. NF-κB essential modulator (NEMO)). The activated IKK complex phosphorylates inhibitor κ B-α (IκBα), leading to its degradation. Consequently, NF-κB is released and can translocate into the nucleus where it starts its transcriptional program [84].

It is widely accepted that TLR5 activates the canonical MyD88-dependent TLR signalling pathway resulting in the nuclear translocation of NF-κB. Along with this common pathway, it is also known that TLR signalling can activate alternative cascades. Wang et al. provided compelling evidence that TAK1 is one of the kinases at which the signalling pathway bifurcates. While poly-ubiquitinated TRAF6 activates TAK1 dependent stimulation of the JNK-p38 kinase pathways [85], the kinase Tpl1 can also be directly phosphorylated by TAK1, which leads to the activation of the extracellular signal regulated kinase (ERK) 1 and 2 pathways [86, 87]. Stimulation of these alternative pathways results in the activation of AP-1 and CREB (cAMP Response Element Binding) transcription factors [87]. Although this has been shown to be true for stimulation of some TLRs, p38 Mitogen Activated Protein (MAP) kinase, ERK1/2 and Janus kinase (JNK) are known to be activated by TLR5 signalling. This shows that TLR5 activates both the canonical and alternative branches to mount an appropriate immune response to pathogenic infection [88] (Figure 1.2).
Figure 1.2: Alternative Signalling Pathways Downstream of TLR5.

Ligation of TLR5 can result in the activation of the MAP kinase pathway (p38 MAP kinase; JNK, Janus Kinase; ERK, extracellular signal regulated kinase).
1.3 Epithelial Host Response to *P. aeruginosa*

1.3.1 *P. aeruginosa*

*P. aeruginosa* is a gram-negative, rod-shaped, opportunistic bacterial pathogen that primarily infects immune-compromised individuals such as burn victims, the immunosuppressed and CF patients [89]. It is an important nosocomial pathogen and a major cause of ventilator-associated pneumonia which results in a high rate of mortality [90]. A study investigating BAL fluids from children with CF concluded that 97% of the subjects investigated were colonized with *P. aeruginosa* by age 3 [91]. The pathogen expresses a wide array of virulence factors ranging from pili, flagellin, LPS and glycoproteins on the surface to effector molecules such as ExoS, ExoT, ExoU and ExoY which are secreted by a type III secretion system (T3SS). Due to its genetic flexibility, it is highly adaptable to its environment. Furthermore, it expresses a repertoire of effective defence molecules such as elastase, alkaline protease, lecithinase and its pigment pyocyanine [89]. The interactions with the host are variable, but the expression of TLR ligands such as LPS, flagellin and glycoproteins suggest the involvement of TLRs in the recognition of *P. aeruginosa*.

1.3.2 Recognition of *P. aeruginosa* by Airway Epithelial Cells

While macrophages and neutrophils play an important role in clearing infections from the lungs, the respiratory epithelium is also a significant contributor to the host-response to *P. aeruginosa*. Not only is the mucociliary escalator provided by these cells, but the interactions between the bacterium and epithelial cells are also crucial to subsequent immune events. The recognition of *P. aeruginosa* by immune cells is likely mediated by several PAMPs such as pili, LPS, flagellin or glycoproteins abundantly expressed on the surface of the microorganism. Upon interaction with the epithelial cells, signalling components are rapidly expressed on the apical surface of the cells leading to further stimulation and the production of pro-inflammatory cytokines [92]. In fact, the respiratory epithelium is an important
source of the neutrophil attractant chemokine IL-8 that recruits neutrophils to the site of infection [61].

*In vivo* mouse and *in vitro* human models have produced conflicting results with regard to identifying the specific TLRs recognizing *P. aeruginosa*. Studies have suggested that the glycolipid asialoGM1 (aGM1) may act as a receptor for flagellin or pili and collaborates with TLR2 or TLR5 in recognizing the pathogen [93-95]. While one study showed that TLR4/5−/− double KO mice succumb faster to *P. aeruginosa* infection compared to single KO mice [96], others have shown that TLR2/4−/− double KO mice are not hypersusceptible, and are able to clear and survive *P. aeruginosa* challenge despite lower levels of cytokine expression (KC and TNF-α) [97]. In stark contrast, 100% of the TLR2/4−/− KO mice challenged with a flagellin-deficient strain of *P. aeruginosa* showed significantly decreased neutrophil infiltration and succumbed quickly to the infection, while specific cytokine production was either unaffected (TNF-α) or decreased (KC and IL-6). Interestingly, pre-challenge of these mice with purified flagellin saved 100% of the litter mates [98]. In both studies, MyD88 KO mice did not produce any cytokines upon bacterial challenge and did not survive infection. Although these studies highlight the importance of airway expressed TLR5, it also demonstrates the importance of differentiating between inflammation, survival and pulmonary clearance of bacteria. It appears that the orchestrated activation of these TLR pathways is important to mount a full and protective immune response. Furthermore, it needs to be carefully distinguished between the cell types investigated when asking questions about the recognition of bacterial pathogens. Janot *et al.* provided convincing evidence that in the absence of bone marrow-derived cells, TLR5 is responsible for recognition of flagellin by the airway epithelium [99], whereas murine macrophages rely on TLR2, 4 and 5 to recognize *P. aeruginosa* [100]. Although these findings contribute greatly to our understanding of host-pathogen interactions, caution is required when extrapolating this knowledge into the human setting due to the inherent physiological differences between human and murine airways. Conflicting results
from *in vitro* studies using human immortalized airway epithelial cell (AECs) and primary cell cultures showed that AECs are responsive to both flagellin and LPS [101, 102]. However, *P. aeruginosa* driven NF-κB activation and cytokine production is predominantly mediated by TLR5 [101, 103-105]. Although many studies have investigated the mechanism using sophisticated models, it seems that no consensus exists over the TLRs that drive the immune response to *P. aeruginosa* in the lung.

### 1.3.3 Flagellin and TLR5 Signalling in CF

The lungs of CF patients typically produce an increased inflammatory response to pathogens such as *P. aeruginosa*. Different hypotheses as to what drives this heightened inflammation have been formulated and tested over the past 20 years. TLRs are likely to play a role in CF pulmonary disease since TLRs are expressed by the airway epithelium [106, 107]. Furthermore, pathogens frequently infecting and colonizing the CF airways express common TLR ligands [92, 108, 109]. No agreement exists over the role of TLRs in the airways of CF patients. Since CF patients are readily colonized with *P. aeruginosa*, it is of great interest to understand how this pathogen is recognized and whether TLR ligation triggers the increased immune response seen in CF lungs. The few reports from the literature indicate that CF AECs are responsive to LPS and that TLR4 can contribute to IL-8 expression [103, 107]. However, it remains controversial as to whether TLR4 is involved in the immune response to *P. aeruginosa* in CF [110]. Despite these conflicting results, the involvement of TLR4 in the recognition of *P. aeruginosa* cannot be ruled out because human TLR4 might only recognize certain LPS structures. Changes in the acetylation pattern of LPS contribute significantly to the human TLR4-LPS interaction. It is known that the LPS of *P. aeruginosa* derived from CF lungs has variable molecular structures, resulting in either gain or loss of TLR4 stimulatory capacity [111, 112]. Despite the numerous mouse studies performed, only one report showed that pro-inflammatory signalling is mediated by surface glycolipid
asialoGM1 in conjunction with TLR5, indicating the potential role of TLR5 in the *P. aeruginosa* induced lung inflammation [95].

### 1.4 Thesis Objectives

The goal of the present thesis was to investigate the host-pathogen interactions between CF airway epithelial cells and *P. aeruginosa*, and to understand the mechanisms mediating the increased pro-inflammatory immune response seen in the CF lung. The first part of this thesis investigates the role of TLRs in the immune response of CF airway epithelial cells to *P. aeruginosa* and identifies TLR5 as the receptor mediating much of the pro-inflammatory cytokine production seen in immortalized CF AECs. In the second part, the aim was to validate the findings of the initial observation by evaluating the effect of a functionally relevant SNP in the *TLR5* gene on important clinical outcome parameters in the Canadian CF population. Finally, while exploring the mechanisms underlying the increased cytokine production in response to TLR5 stimulation, the data presented in the third part suggest a novel pathway integrating different stress responses triggered in the CF cell, leading to the hyper-inflammatory phenotype. Ultimately, these studies contribute important knowledge about host-pathogen interactions in the CF lung. Identifying the mechanisms leading to the increased inflammatory responses in CF, will likely contribute to the design of specific therapeutic approaches that may increase the quality and length of life of CF patients.
2. **Innate Immunity Mediated by TLR5 as a Novel Anti-Inflammatory Target for Cystic Fibrosis Lung Disease**

2.1 **Introduction**

CF is an autosomal recessive disorder resulting from a mutation in the *CFTR* gene. Although understanding and treatment of the disease have developed significantly over the past two decades, new treatment options for patients with CF are critically needed since the median age for survival is still only in the mid-40s [3].

Lung disease, the major cause of death in CF, is caused by a self-sustaining cycle of airway obstruction, infection, and inflammation [113]. CF lung disease is characterised by neutrophilic airway inflammation, increased expression of pro-inflammatory cytokines, and infection by a narrow repertoire of bacterial pathogens, with *P. aeruginosa* and *B. cepacia* complex being the most clinically significant pathogens. Current therapy for CF lung disease relies upon antibiotics to treat bacterial infections, combined with airway clearance strategies to mobilize viscid secretions [20].

Anti-inflammatory therapy has been shown to be beneficial for patients with CF, especially for younger patients with mild disease. Clinical trials using non-specific global immunosuppressants — corticosteroids and ibuprofen — established that targeting excess inflammation is helpful in improving the lung disease of CF patients [56, 114]. Unfortunately, safety concerns render these currently available anti-inflammatory medications unacceptable for routine use. While it remains unclear whether the *CFTR* mutation itself causes a pro-inflammatory milieu in the CF airways, or whether inflammation is always secondary to infection, this controversy is less relevant for the treatment of CF patients [115]. Airway infection and
inflammation are virtually universal in CF patients (regardless of the order in which they develop), and reducing inflammation is likely to augment current treatments and improve the clinical outcome in CF. To date, studies focused exclusively on identifying the mechanisms responsible for inflammation in CF have yielded conflicting results (as recently reviewed in [45]); therefore, rather than specifically investigating the mechanism of inflammation, this study aims to identify an immunological pathway that can be blocked to reduce CF airway inflammation.

Targeting the signalling cascade responsible for over-production of inflammatory cytokines is a particularly attractive treatment approach for reducing inflammation in the CF lung. There is currently no consensus concerning molecular pathway(s) critical for the increased inflammation seen in CF. This study explores whether the TLR signalling pathway is responsible for the hyper-inflammatory response seen in CF. TLRs allow for the recognition of diverse microbial epitopes, inducing a cascade of effector responses within minutes [116]. Individual TLRs recognize a distinct, but limited, repertoire of conserved microbial products; for example, well characterized receptor-ligand pairs include TLR4 and lipopolysaccharide (LPS), TLR5 and flagellin, TLRs1/2/6 and lipoproteins. TLR signalling is a likely contributor to the excess inflammation seen in the CF lung since: (a) functional TLRs are expressed by CF airway epithelial cells and by hemopoietic cells within the lung [106, 107]; (b) TLR signalling generates the cytokines known to be upregulated in the CF lung, particularly IL-8, IL-6, IL-1β and TNF-α [31, 32]; (c) TLRs are able to respond to ligands expressed by the major CF pathogens, including *P. aeruginosa*, *B. cepacia* complex, *S. aureus* and *H. influenzae* [92, 108, 109].

To address the gap in our understanding of inflammation in CF, the present study examined whether TLR signalling is responsible for the excessive inflammation seen in CF and whether inhibiting TLR signalling can reduce this damaging inflammation. Rather than relying exclusively on cellular or animal models of
disease, the data show that the TLR pathway is dysregulated in fresh blood cells of humans living with CF — making these findings a clinically relevant starting point for characterization of the TLR pathway that may be targeted for treatment of CF lung disease.
2.2 Materials and Methods

2.2.1 CF and CF-Corrected Respiratory Epithelial Cells

To study the inflammatory response of CF airway cells, experiments were performed on the well-characterized immortalized CF and CF-corrected lung epithelial cells: IB3-1 — compound heterozygote for the ΔF508 and W1282X CFTR mutations; and C38 — a "corrected" CF cell line derived from IB3-1 after stable transfection with CFTR (American Type Culture Collection) [117]. Cells were grown in pre-coated flasks (100μg/mL bovine serum albumin, 30μg/mL bovine collagen I, 10μg/mL human fibronectin) in LHC-8 Basal Medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 1% penicillin-streptomycin-amphotericin B solution at 37˚C and 5% CO₂. The C38 and IB3-1 cell lines were seeded into pre-coated 96 well plates at a density of 5x10⁴ cells per well 16 hours prior to use in stimulation assays. To assess cell viability after the 48 hour stimulation with bacteria or TLR-ligands, release of a cytoplasmic enzyme (lactate dehydrogenase, LDH) into the supernatant by damaged cells was measured using a LDH cell cytotoxicity kit (Roche).

2.2.2 CF and Healthy Control Peripheral Blood Mononuclear Cells

Blood samples from CF patients were obtained at BC Children’s Hospital and St Paul’s Hospital, Vancouver, Canada. The diagnosis of CF was established by typical clinical features, increased sweat chloride concentrations (> 60 mmol/l) and detection of CF-inducing mutations. All patients with CF were clinically stable at the time of blood donation, and we excluded any subjects who were receiving systemic immunosuppressive medications, such as oral corticosteroids and azithromycin (due to potential immunomodulatory activity). All samples were obtained with informed consent. Control samples were obtained from children undergoing elective surgery for non-infectious, non-inflammatory conditions and from healthy adult
volunteers. The University of British Columbia Clinical Research Ethics Board approved all studies. The technique for isolating PBMCs has been described [118].

Table 2.1: Bacterial Strains Utilized in This Study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Clinical isolate from the CF lung, motile, classic morphology</td>
<td>D. Speert</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Clinical isolate from the CF lung, motile, mucoid</td>
<td>D. Speert</td>
</tr>
<tr>
<td><em>Burkholderia multivorans</em></td>
<td>Clinical isolate from the CF lung, motile, non-mucoid</td>
<td>D. Speert</td>
</tr>
<tr>
<td><em>Burkholderia cenocepacia</em></td>
<td>Clinical isolate, from the CF lung motile, non-mucoid</td>
<td>D. Speert</td>
</tr>
<tr>
<td>PAK wt</td>
<td><em>P. aeruginosa</em> laboratory strain, non-mucoid, motile</td>
<td>[119]</td>
</tr>
<tr>
<td>PAKΔfliC</td>
<td><em>P. aeruginosa</em> non-motile derivative of PAK wt; <em>fliC</em> interrupted by gentamycin cassette</td>
<td>[119]</td>
</tr>
<tr>
<td>PAKΔfliC (pUCP22::fliC)</td>
<td><em>P. aeruginosa</em> motile derivative of PAKΔfliC; complemented with a plasmid containing the coding region of the <em>fliC</em> gene (pUCP22::fliC).</td>
<td>Present study, REW Hancock</td>
</tr>
<tr>
<td>PAO1 wt</td>
<td><em>P. aeruginosa</em> laboratory strain, motile, smooth LPS structure</td>
<td>[120]</td>
</tr>
<tr>
<td>PAO1ΔfliC</td>
<td><em>P. aeruginosa</em>, non-motile derivative of PAO1 wt, <em>fliC</em> interrupted by tetracycline cassette</td>
<td>[121]</td>
</tr>
<tr>
<td>PAO1ΔfliM</td>
<td><em>P. aeruginosa</em>, non-motile derivative of PAO1 wt, <em>fliM</em> interrupted by tetracycline cassette</td>
<td>[121]</td>
</tr>
<tr>
<td>PAO1ΔflgE</td>
<td><em>P. aeruginosa</em>, non-motile derivative of PAO1 wt, <em>flgE</em> interrupted by tetracycline cassette</td>
<td>[121]</td>
</tr>
</tbody>
</table>

2.2.3 Bacterial Strains, Culture Conditions and Swimming Motility Assays

Bacterial strains used in this study are outlined in Table 2.1. For complementation of the knockout mutant *P. aeruginosa* PAKΔfliC, the *fliC* gene was amplified by a tailed PCR using the primers fliCUE (AAAAAGAATTCGCGGCGAGATCGCAAGCTCAGG) and fliCDB.
(AAAAAGGATCCCCTCCACGTACCGCGTGAGTGACCG) together with genomic DNA from *P. aeruginosa* PAO1 as a template. The PCR product was isolated, hydrolyzed with *Eco*RI and *Bam*HI, and cloned into the broad-host-range vector pUCP22 [127]. In the resulting plasmid, pUCP22::fliC, the *fliC* gene was arranged colinear to and downstream of the *lacZ* promoter. The plasmid was transferred to *P. aeruginosa* PAKΔ*fliC* by electroporation resulting in strain *P. aeruginosa* PAKΔ*fliC* (pUCP22::fliC). For the stimulation assays bacteria were harvested at late-log or early stationary phase of growth. To control for motility differences between wild-type and flagellin-mutant strains, bacteria were heat killed before use. Swimming motility was assessed by inoculation into soft LB-agar (0.3%) and after 24 hours colony diameter was measured with ≤5mm considered immotile. To assess motility and TLR5 stimulatory capacity of clinical isolates of *P. aeruginosa* during chronic infection, isolates from 17 patients (10 female, 7 male) were chosen from a library of clinical isolates collected from the CF clinics at BC Children’s Hospital and St. Paul's Hospital in Vancouver. Four to twelve isolates were chosen per patient and in total 151 isolates were tested.

### 2.2.4 Cell Stimulation Assay With Whole Bacteria or Pure TLR Ligands

The C38/IB3-1 cell lines and fresh PBMCs were challenged with pure TLR ligands or heat-killed whole bacteria at a variety of multiplicities-of-infection (MOIs). Optimal concentrations of TLR ligands were determined in pilot experiments (Table 2.2). After 24 hours (PBMCs) or 48 hours (C38/IB3-1) culture supernatants were harvested and cytokine concentration in the supernatant was measured. In some experiments TLR5 activation was inhibited using a neutralizing polyclonal antibody raised against the extracellular region of human TLR5 (PAb-hTLR5, 6µg/ml, InvivoGen) with a purified rat IgG1 (eBioscience) serving as a control.
2.2.5 Flow Cytometry

Cellular composition of CF and control PBMCs was assessed by flow cytometry. Cells were stained with anti-CD3-PE, anti-CD19-FITC, anti-CD16-FITC and anti-CD14-FITC antibodies, as well as isotype controls, after blocking with 10% human serum (BD Biosciences). Cells were acquired on a FACSCalibur (BD Biosciences) and were analysed using FlowJo software (Tree Star, Inc.).

2.2.6 Human TLR5-NF-κB Luciferase Reporter Assays

The TLR5 reporter cell line has been described [77]. CHO K1 cells were stably transfected with human TLR5 cDNA (cloned into the pEF6 V5/His TOPO vector (Invitrogen)) and ELAM-LUC (Promega) plasmids. Cells were stimulated for 3-4 hours with heat-killed whole bacteria, TLR ligands and controls before assaying for luciferase activity (Promega). For studies assessing the TLR5 stimulatory capacity of clinical isolates, a fold change above 4.5 was considered positive based on control studies using wild-type and flagella-deficient strains of *P. aeruginosa*.

2.2.7 Gene Expression Quantification

Total RNA isolated from airway epithelial cells (RNeasy Plus Mini Kit, Qiagen) was transcribed to cDNA with oligo-dT primers (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Quantitative PCR (qPCR) primers were designed for SYBR Green chemistry to amplify β-actin (5′-GTTGCGTTACACCCCTTTCTT-3′, 5′-ACCTTCACCGTTCCAGTTT-3′, 148bp), TLR5 (5′-TGTTGCAACTTGCTGGAAAC-3′, 5′-AACTGTCAGCCTGTTGGAGTG-3′, 170bp) and *lpaf* (5′-TGCCCAGAAATCGAAGCCCTGATA-3′, 5′-TTGGAGCAACAAGCCCTCAGCAAG-3′, 201bp). Relative gene expression in the
CF (IB3-1) compared to CF-corrected (C38) airway cells was analysed by the $2^{-\Delta\Delta C_{t}}$ method using $\beta$-actin as a reference housekeeping gene.

**Table 2.2: Optimized TLR Ligand Concentrations.**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Mode of action</th>
<th>Concentration (µg/ml)</th>
<th>Airway cell stimulation</th>
<th>PBMC Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Unstimulated control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PAM3CSK4</td>
<td>TLR1 &amp; TLR2</td>
<td>10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Peptidoglycan</td>
<td>TLR2</td>
<td>20</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>TLR4</td>
<td>1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Flagellin</td>
<td>TLR5</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Zymosan</td>
<td>TLR2 &amp; TLR6</td>
<td>100</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>ssRNA</td>
<td>TLR7 &amp; TLR8</td>
<td>10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CpG</td>
<td>TLR9</td>
<td>100</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>IL-1R</td>
<td>1</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2 depicts the TLR ligands used for cell activation, the relevant TLR engaged and the optimal concentration of each ligand for both airway epithelial cell and PBMC stimulation. The optimal concentration for each ligand was determined in pilot experiments by titration on airway epithelial cells or PBMCs from healthy volunteer donors.

### 2.2.8 Western Blotting

Bacterial whole cell lysates were assessed for flagellin expression by Western blot using a previously described techniques [123].

### 2.2.9 Statistical Analysis

Groups were compared using the unpaired Student’s t-test, two-way ANOVA with Bonferroni post-test for multiple comparisons, or by regression analysis, as appropriate using GraphPad Prism (Version 4.0). $P$-value less than 0.05 was considered significant.
2.3 Results

2.3.1 CF Respiratory Epithelial Cells Mount an Exaggerated Pro-Inflammatory Response to a Range of Relevant Bacterial Pathogens

Initial studies on the inflammatory response in the CF airway were performed on the well-characterized CF and CF-corrected lung epithelial cells — IB3-1 and C38. The cell lines were stimulated with: (a) two clinical isolates of *P. aeruginosa* strain (classic and mucoid); and (b) two clinical isolates of the Bcc — *B. multivorans* (genomovar II) and *B. cenocepacia* (genomovar III). CF respiratory epithelial cells produced 2 to 8-fold more of the pro-inflammatory cytokine IL-6 than the CF-corrected cell line when stimulated with whole bacteria (*p*<0.001 for all pathogens tested determined by 2-way ANOVA with Bonferroni post-test) (Figure 2.1). In addition, the CF cell line produced more pro-inflammatory IL-6 than the CF-corrected cells following exposure to *Streptococcus pneumoniae* (serotypes 4 and 14) — a pathogen not typically associated with CF lung disease (data not shown).

Cell cytotoxicity assays demonstrated that the viability of the respiratory epithelial cells following bacterial stimulation was equivalent between CF and CF-corrected cells (data not shown). Data from pilot experiments demonstrated that production of a variety of inflammatory cytokines (specifically IL-1β, TNF-α, and IL-8) was well-correlated with IL-6 secretion following innate immune activation of airway epithelial cells, hence, for most experiments only IL-6 production is shown.
Figure 2.1: Vigorous Pro-Inflammatory Response Generated by CF Respiratory Epithelial Cells Following Exposure to CF Pathogens. CF-corrected (C38) and CF (IB3-1) airway epithelial cells were stimulated with a range of heat-killed whole bacteria at varying multiplicities-of-infection (MOI). IL-6 concentration in cell supernatants after 48 hours stimulation was determined by ELISA. In all cases, the CF (IB3-1) cell line produced significantly more IL-6 than the CF-corrected (C38) airway cells for all CF pathogens tested (p<0.001 for all pathogens tested). Values represent mean ± SEM of 3 experiments performed in triplicate.
2.3.2 Pro-Inflammatory Response Generated by CF Peripheral Blood Mononuclear Cells Following Exposure to *Pseudomonas aeruginosa* is Increased Compared to Healthy Controls

Subtle technical artefacts, possibly related to immortalization and long-term culture, may alter the pro-inflammatory phenotype of any cell line [45]. To address this concern and to validate any findings in CF respiratory epithelial cells, similar experiments were performed using blood cells donated by patients with CF. Freshly isolated peripheral blood mononuclear cells (PBMCs) were selected to investigate the immune response in CF. Fresh PBMC’s have several advantages over other possible human CF samples: (i) PBMCs are not subject to alterations that may emerge from long-term cell culture, cloning and immortalization; (ii) cells within the PBMC population, particularly lymphocytes and some monocytes/macrophages, express functional CFTR protein [124, 125]; and (iii) the need to study enough subjects makes it impractical to collect cells through other means, such as bronchoalveolar lavage or biopsy.

Showing a phenotype similar to CF airway epithelial cells, CF PBMCs produced significantly more pro-inflammatory IL-6 after stimulation with *P. aeruginosa* (PAK strain) than did cells from healthy controls at every multiplicity of infection tested (Figure 2.2a; \( p=0.0003 \)). Control experiments showed that differences in cytokine production were not simply related to differences in proportions of monocytes, T-, B- & NK-cells in the PBMCs isolated from CF patients and healthy controls (Figure 2.2b). Hence both CF airway epithelial cells and blood mononuclear cells generate an exaggerated pro-inflammatory response following exposure to bacterial pathogens.
Figure 2.2: Elevated Pro-Inflammatory Cytokine Production by CF PBMCs Following Exposure to P. aeruginosa.
(a) PBMCs from patients with CF (n=5) and healthy age-matched controls (n=4) were exposed, in triplicate, to heat-killed P. aeruginosa (PAK strain) at varying multiplicities-of-infection (MOI) and controls. After 24 hours IL-6 concentration in the supernatant was measured. CF patients produced higher amounts of pro-inflammatory IL-6. Values represent mean ± SEM. Statistical testing was performed by regression analysis demonstrating that slopes of the lines were identical, but the intercepts were significantly different (p=0.0003) indicating that CF PBMCs produce more IL-6 than control PBMCs at baseline and following stimulation with PAK strain bacteria.
(b) Composition of the PBMC population of CF (n=6) and healthy control (n=6) subjects was determined by flow cytometry after staining for monocytes, T-, B- & NK-cells using monoclonal antibodies against CD14, CD3, CD19 and CD16, respectively. Values represent mean ± SEM.
2.3.3 TLR Ligands, Particularly Flagellin, Induce a Marked Inflammatory Response in CF Respiratory Epithelial Cells

A range of receptor families are involved in innate immune recognition of microbes. These receptor systems include TLRs, the NOD-like proteins and TNF-α receptor 1 (TNFR1) [126]. To assess specifically the contribution of TLR signalling to the enhanced inflammatory response generated by CF airway cells following exposure to whole bacteria, respiratory epithelial cells were stimulated with purified TLR ligands and IL-6 production was measured (Figure 2.3).

Concentrations of TLR ligands resulting in optimal IL-6 production were selected in pilot experiments (Table 2.2). IL-6 production following stimulation with both flagellin and IL-1β was significantly higher in CF respiratory epithelial cells compared to the CF-corrected cell line, while signalling through other TLRs (including the LPS-specific TLR4) was minimal in airway cells. Together these data suggest that TLR signalling is dysregulated in the CF airway cells, and that flagellin is a particularly potent stimulator of the airway innate immune response.
Figure 2.3: CF Respiratory Epithelial Cells Respond More Vigorously to Flagellin Stimulation.
(a & b) CF-corrected (C38) and CF (IB3-1) cell lines were stimulated with a range of pure TLR ligands at concentrations selected by titration to optimize IL-6 production (see Table 2.2 for details). CF respiratory epithelial cells responded most vigorously to flagellin stimulation. Values represent mean ± SEM of 2-3 experiments performed in triplicate. Statistical analysis was performed by two-way ANOVA using the Bonferroni correction for multiple comparisons and *** signifies p<0.001.
2.3.4 Analysis of TLR Responses in CF Peripheral Blood Mononuclear Cells

To further explore the hypothesis that TLR signalling contributes to dysregulated cytokine production occurring in CF and to verify our cell line data, TLR function was studied in primary PBMCs donated by 23 children with CF and 20 healthy age-matched controls. As cells within the PBMC population are known to recognize a broader repertoire of TLR ligands than airway epithelial cells [106, 127], the PBMCs were predicted to produce pro-inflammatory cytokine in response to a wider variety of TLR ligands than the airway epithelial cells. Hence, the aim of these experiments was to assess if global TLR responsiveness was dysregulated in primary cells from CF patients. Freshly isolated PBMCs were exposed to an optimized panel of TLR ligands and controls for 24 hours, and cytokine production was measured (Figure 2.4). The general observation from this data series was that for every condition studied, children with CF produced more pro-inflammatory cytokines (i.e. IL-6, TNF-α & IL-1β) following TLR stimulation than healthy age-matched controls, and these differences reached statistical significance for a number of conditions tested. In contrast, production of the anti-inflammatory cytokine IL-10 was indistinguishable between cases and controls. Together these data confirm our findings from immortalized respiratory epithelial cells and suggest that TLR signalling contributes to dysregulated cytokine production seen in CF.
Figure 2.4: Elevated TLR-Mediated Pro-inflammatory Cytokine Production by Peripheral Blood Cells Donated by CF Patients Compared to Healthy Age-Matched Controls. Freshly isolated PBMCs from patients with CF (n=23) and healthy age-matched controls (n=20) were stimulated with a range of TLR ligands (outlined in Table 2.2) and cytokine production was measured by cytometric bead array. CF PBMCs produced higher amounts of pro-inflammatory cytokines (i.e. IL-6, TNF-α and IL-1β) but equivalent levels of the anti-inflammatory cytokine, IL-10. Values represent mean ± SEM. Statistical analysis was performed by two-way ANOVA using the Bonferroni correction for multiple comparisons. *, ** & *** signify p<0.05, 0.01 & 0.001, respectively.
2.3.5 Flagellin is The Ligand That Mediates The Excessive Inflammatory Response Generated by CF Respiratory Epithelial Cells in Response to P. aeruginosa

Since the CF airway epithelial cells responded most vigorously to flagellin (Figure 2.3), it was important to determine the contribution of flagellin to the enhanced inflammatory response generated by CF airway epithelial cells following exposure to P. aeruginosa. The experimental strategy was to stimulate respiratory epithelial cells with bacteria expressing a full complement of pathogen-associated molecular patterns, but lacking flagella. Besides the wild-type (PAK & PAO1) and isogenic flagella-deficient strains of P. aeruginosa (PAKΔfliC, PAO1ΔfliC, PAO1ΔflgE & PAO1ΔfliM), a complemented strain in which the coding region of the fliC gene was replaced (PAKΔfliC (pUCP22::fliC)) was characterized. The flagella-deficient strains lacked both swimming motility and the capacity to activate TLR5 (Figure 2.5 a, b, d, e). To control for differences in motility between wild-type and flagella-deficient strains, all bacteria were heat-killed before use in stimulation assays. Strikingly, the absence of flagellin significantly reduced pro-inflammatory IL-6 production by the CF respiratory epithelial cells following exposure to P. aeruginosa (Figure 2.5 c, f). Complementation of the flagellin-knockout mutant of P. aeruginosa (PAKΔfliC) with the coding region of the fliC gene (PAKΔfliC (pUCP22::fliC)) resulted in the normalization of motility, TLR activating capacity, and pro-inflammatory impact of the flagellin-deficient strain (Figure 2.5a-c), verifying the role of flagellin in the inflammatory response generated by the CF airway cells. Hence, strategies to block flagellin recognition may have the potential to reduce the excessive and damaging pro-inflammatory response occurring in the CF lung, ultimately improving the outcome for patients with CF.
Figure 2.5: Stimulation with Flagella-Deficient Strains of *P. aeruginosa* Abolishes Pro-Inflammatory IL-6 Production by CF Respiratory Epithelial Cells. Compared with wild-type bacteria (PAK & PAO1), isogenic flagella-deficient strains of *P. aeruginosa* (PAKΔfliC, PAO1ΔfliC, PAO1ΔflgE and PAO1ΔflhM) lack swimming motility (a & d) and fail to activate a TLR5 reporter cell line — CHO-K1 cells stably expressing human TLR5 and an NF-κB luciferase reporter (b & e). Complementation of the flagellin-knockout mutant of *P. aeruginosa* (PAKΔfliC) with the coding region of the fliC gene (PAKΔfliC pUCP22::fliC) normalized both the motility defect and TLR5 activating capacity. CF (IB3-1) airway epithelial cells were stimulated with these characterized bacteria. To control for differences in motility between wild-type and flagella-deficient strains, all bacteria were heat-killed before use. (c & f) IL-6 concentration in cell supernatants after 48 hours stimulation was determined by ELISA. Values represent mean ± SEM of 3-6 separate experiments all performed in triplicate. Statistical analysis was performed by two-way ANOVA using the Bonferroni correction for multiple comparisons. *, ** & *** signify p<0.05, 0.01 & 0.001, respectively.

2.3.6 Inhibition of TLR5 Activation Normalizes The Inflammatory Response Generated by CF Airway Epithelial Cells Following Exposure to *P. aeruginosa*

In order to identify a novel anti-inflammatory target for CF lung disease, it was necessary to determine which receptor was mediating flagellin recognition by the airway epithelial cells. TLR5 is the best-studied physiological receptor for flagellin in
vertebrates; however, recent evidence also implicates IL-1-converting enzyme (ICE) protease-activating factor (Ipaf) in the recognition of intracellular flagellin [128]. Expression of the genes encoding these two flagellin receptors was quantified in the airway epithelial cells. TLR5 mRNA expression was 5.5-fold higher in the CF (IB3-1) compared to control (C38) airway epithelial cells (p=0.0002, unpaired Student’s t test). In contrast, the mRNA expression of Ipaf was indistinguishable between CF and control cells (p>0.05, unpaired Student’s t test) (Figure 2.6).

Based on the observation that TLR5 mRNA was increased in CF airway epithelial cells, combined with the established physiological role for TLR5 in airway cell biology, it was essential to examine if TLR5 inhibition would reduce the excessive inflammatory response generated by CF airway cells following exposure to CF pathogens. To achieve efficient and specific TLR5 inhibition a neutralizing polyclonal antibody raised against the extracellular region of human TLR5 was utilized. The anti-TLR5 antibody reduced the flagellin response to baseline in both the TLR5 reporter cell line and the CF airway epithelial cells. Importantly, the anti-TLR5 antibody did not alter IL-1β responsiveness; confirming that the inhibition of flagellin recognition was occurring specifically at the level of TLR5 rather than through non-specific inhibition of a downstream signalling molecule, as the TLR5 and IL-1β signalling pathways differ only in the ligand binding domain of the receptor (Figure 2.7 a & b). Furthermore, the anti-TLR5 antibody significantly reduced pro-inflammatory IL-6 production by CF airway epithelial cells following exposure to the PAK strain (p<0.01) and a classic clinical isolate (p<0.01) of P. aeruginosa (Figure 2.7 c & d). Importantly, TLR5 inhibition also significantly reduced IL-8 secretion in response to these strains of P. aeruginosa (PAK p<0.01; classic clinical isolate p<0.01).
Figure 2.6: TLR5 mRNA and Protein Expression is Increased in CF Airway Epithelial Cells. TLR5 and Ipaf gene expression in the CF (IB3-1) and CF-corrected (C38) airway cells was determined by quantitative PCR. Relative gene expression was analysed by the $2^{-\Delta\Delta CT}$ method using $\beta$-actin as a reference gene (a). Protein expression was determined by flow cytometry (b). Values represent mean ± SEM of 2 separate experiments all performed in triplicate. Statistical analysis was performed by unpaired Student’s t-test and *** signifies $p<0.001$. 
2.3.7 Clinical Isolates of *P. aeruginosa* Maintain TLR5 Stimulatory Capacity During The Evolution of Chronic CF Lung Infection

A potential limitation of therapy to target the TLR5-flagellin interaction in CF is the observation that most environmental *P. aeruginosa* strains and those isolated early in CF infections are highly motile, whereas some of strains isolated from older chronically infected CF patients are immotile. To assess formally both motility and TLR5 stimulatory capacity of *P. aeruginosa* during chronic infection, 151 clinical isolates of *P. aeruginosa* derived from 17 patients representing 304 patient years of disease were analysed. Motility of isolates decreased significantly with increased patient age (*p*=0.006 by linear regression). Specifically, 81.2% of isolates from patients aged less than 6 years were motile compared with only 16.7% motility of isolates from CF patients aged 30-36 years (Figure 2.8a). In contrast the decline in TLR5 activating capacity of *P. aeruginosa* was less pronounced and did not reach statistical significance, with 87.5% of isolates from patients aged less than 6 years activating TLR5 compared with 60.0% of isolates from CF patients aged 30-36 years (*p*=0.06 by linear regression). Overall, 113 of 151 (75%) clinical isolates of *P. aeruginosa* activated TLR5. In these experiments 55 isolates (36.4% of total) were identified that were non-motile but TLR5-activating. To confirm that these non-motile but TLR5-activating isolates of *P. aeruginosa* did express immunogenic flagellin, Western blots on whole protein extracts of the bacteria were performed using a polyclonal antibody against the type a (45-kDa) flagellin of *P. aeruginosa* P1. Western blot analysis confirmed that 51 of 55 (93%) non-motile but TLR5-activating isolates of *P. aeruginosa* stained strongly for immunoreactive flagellin (Figure 2.8b).
Figure 2.7: Specific Inhibition of TLR5 Abolishes the Excessive Inflammatory Response Generated by CF Airway Epithelial Cells Following Exposure to *P. aeruginosa*.

(a & b) A neutralizing polyclonal antibody raised against the extracellular region of human TLR5 (α-TLR5) specifically blocked flagellin responsiveness in both the TLR5 reporter cell line and CF airway epithelial cells (IB3-1). (c & d) The anti-TLR5 antibody significantly reduced pro-inflammatory IL-6 production by CF (IB3-1) airway epithelial cells following exposure to the PAK strain and a classic clinical isolate of *P. aeruginosa*. Values represent mean ± SEM of 3 experiments performed in duplicate. * & ** signify \( p < 0.05 \) & 0.01, respectively.
Figure 2.8: Clinical Isolates of *P. aeruginosa* Maintain TLR5 Stimulatory Capacity During the Evolution of Chronic CF Lung Infection.

(a) The motility and TLR5 stimulatory capacity of 151 clinical isolates of *P. aeruginosa* were analysed. Motility of isolates decreased significantly with increasing patient age (*p*=0.006 by linear regression) while the decline in TLR5 activating capacity was not significant (*p*=0.06 by linear regression). (b) Western blot of whole bacteria protein extracts using a polyclonal antibody against the type a (45-kDa) flagellin of *P. aeruginosa* P1. Figure shows representative clinical isolates of *P. aeruginosa* characterized for their motility and TLR5 activating capacity with PAK wt and PAKΔfliC serving as the positive and negative controls, respectively.
2.4 Discussion

Lung damage is the major life-limiting complication of CF. Chronic infection and inflammation are the hallmark of CF lung disease. Novel therapies to target lung inflammation, when administered in combination with airway clearance and antimicrobials, are predicted to improve the lives of people with CF. The challenge has been to identify the molecular pathway(s) mediating the excessive inflammation occurring in the CF airway. The data in this study show that TLR-mediated innate immune responsiveness is increased in both CF respiratory epithelial cells and fresh blood cells from subjects living with CF. Moreover, inhibition of the TLR5-flagellin interaction markedly reduced the pro-inflammatory response of immortalized CF respiratory epithelial cells following exposure to the predominant CF pathogen *P. aeruginosa*. These data suggest that TLR5 activation may represent a novel anti-inflammatory target for improving CF lung disease. A potential criticism of many published studies investigating airway inflammation in CF is the reliance on immortalized airway epithelial cells. Aware of this limitation, experiments were performed utilizing an extensively studied pair of CF and control airway epithelial cells (IB3-1/C38; for examples see [129-131]) and the fundamental observations were validated in primary blood cells donated by CF and control subjects.

Bacteria infecting the CF lung must interact with the airway epithelium. Respiratory epithelial cells are an important component of the innate immune system forming a physical and immunologic barrier to inhaled bacteria. Surprisingly, although *P. aeruginosa* expresses ligands to activate a variety of TLRs (specifically TLRs 1, 2, 4, 5, 6 and 9 [92, 108, 109, 132]), the present data demonstrate that airway epithelial cells almost exclusively rely upon TLR5 to sense *P. aeruginosa* through its flagellin protein (Figures 2.5 & 2.7). In the absence of TLR5 activation, the interaction of *P. aeruginosa* with respiratory epithelial cells does not initiate a significant host inflammatory reaction. LPS was initially predicted to be the major
immunostimulatory component expressed by *P. aeruginosa*; however, evidence suggests that the TLR4-mediated response to LPS is minimal in airway cells due to a lack of the necessary co-receptors, including MD2 and CD14 [133-135]. Moreover, the observation that TLR4 knockout mice show no enhanced susceptibility to *P. aeruginosa* lung infection provides further evidence that the TLR4-LPS interaction alone is unlikely to account for the florid airway inflammation observed in CF [96, 97]. In summary, these data suggest that a TLR5 inhibitor is likely to minimize the inflammatory response generated by airway epithelial cells following infection with clinically-relevant flagellated bacteria such as *P. aeruginosa* and members of the *B. cepacia* complex. In contrast, TLR5 is unlikely to be involved in the recognition of non-motile pathogens such as *S. aureus* and *H. influenzae*, and their contribution to chronic inflammation is debatable as these pathogens tend to be found in the lungs of young CF patients, are generally less virulent than other CF pathogens and are quickly over-grown by *P. aeruginosa* [136].

An important recent discovery is that mammalian cells rely on (at least) two molecular systems to sense flagellated bacterial pathogens [128]. Extracellular flagellin is recognized by TLR5, activating NF-κB and leading to the secretion of a variety of cytokines, including IL-6 and TNF-α. Intracellular flagellin is detected by Ipaf, initiating IL-1β processing and the secretion of mature IL-1β. This is the first study to investigate the relative roles of TLR5 and Ipaf in CF lung infection, demonstrating that TLR5 plays a critical role in recognizing flagellated bacteria, including *P. aeruginosa*. The present observation that CF airway epithelial cells rely heavily upon TLR5 to sense bacterial pathogens is consistent with the established clinical observation that in the CF lung extracellular bacterial infection predominates, and the respiratory epithelium remains intact despite accumulation of neutrophils, mucin, and bacteria in the airway [89]. Previous studies have shown that the presence of flagellin expressed by *P. aeruginosa* [133, 137, 138] triggers an inflammatory response by airway epithelial cells, but none of these studies have established definitively the identity of the receptor mediating this inflammatory response.
response. The present experiments using a neutralizing anti-TLR5 antibody (Figure 2.7) and others using dominant negative TLR5 constructs [92, 105] establish that TLR5 mediates airway cell recognition of *P. aeruginosa* and *B. cepacia* complex; moreover, the data demonstrate that a specific TLR5 inhibitor is able to significantly reduce the hyper-inflammatory response generated by CF airway epithelial cells following exposure *P. aeruginosa* — the most clinically-relevant bacterial pathogen infecting the CF lung.

The challenge in developing an anti-inflammatory medication for CF lies in reducing harmful inflammation without unacceptably increasing infections or other untoward effects. A number of observations indicate that TLR5 is a particularly exciting therapeutic target for blocking airway inflammation in CF. First, TLR5 inhibition only blocks the response to flagellin, leaving all other innate immune response mechanisms intact. Second, humans with defective TLR5 signalling have been identified, providing unique insights into the biological effects of interfering with TLR5 function. Approximately 10% of Caucasians carry a polymorphism in the ligand-binding domain of TLR5 (*TLR5*<sup>392STOP</sup>) that acts in a dominant fashion to abolish flagellin signalling [139]. Individuals carrying the *TLR5*<sup>392STOP</sup> polymorphism appear to be entirely healthy, although they are at slightly increased risk of developing Legionnaires’ disease following exposure to *Legionella pneumophila* [139]. Importantly, this *TLR5* polymorphism does not render human carriers universally susceptible to infection with flagellated bacteria, as it had no measurable impact on susceptibility to typhoid fever caused by *Salmonella enterica* serovar Typhi [140]. Finally, mouse studies provide further support for the potential safety of TLR5 inhibition as *TLR5<sup>−/−</sup>* and WT mice show similar rates of survival following *P. aeruginosa* lung infection [96] and infection with flagellin-deficient strains of *P. aeruginosa* delays the time to death [141]. Together, these data suggest that a specific TLR5 inhibitor may be useful for reducing the excess inflammation in CF, while having an acceptable side-effect profile. Despite these reassuring theoretical arguments, caution and humility must be used when interfering with the finely tuned
immune system. All consequences of our manipulations are impossible to predict, and will be appreciated only through rigorous preclinical experimentation and well-conducted clinical trials.

The potential therapeutic value of a TLR5 inhibitor is further supported by the demonstration that 75% (113 of 151) of clinical isolates of *P. aeruginosa* obtained from the lungs of CF patients retained the capacity to activate TLR5 (Figure 2.8). The library of *P. aeruginosa* isolates tested in this study is likely to be representative of CF patients treated in a large North American centre as 151 clinical isolates of *P. aeruginosa* derived from 17 patients representing over 300 patient years of disease were analysed. The significant loss of motility of *P. aeruginosa* during the evolution of chronic CF lung infection has been observed by others [123, 142]; however, the present study is the first demonstration that TLR5 activating capacity of *P. aeruginosa* is relatively retained in the CF lung across the lifespan. The observation that 34% of the clinical isolates of *P. aeruginosa* appear to have ‘paralyzed’ flagellum (i.e. TLR5 activating but non-motile) is not without precedent. This paralyzed flagellum phenotype has been noted in clinical isolates of *P. aeruginosa* and is seen in PAK-*motABC* which has deletions in both sets of *mot* genes encoding the flagellar motor proteins, resulting in loss of motility but preservation of flagellin synthesis [123, 143].

Other evidence suggests a central role of flagellin and TLR5 in the cyclical exacerbation of inflammatory lung disease in CF patients. For example, sputum neutrophil elastase is now recognized to be a useful clinical biomarker that negatively correlates with lung function in CF [144]. Since the neutrophil elastase content of CF sputum is known to transiently repress expression of a number of *P. aeruginosa* genes including *fliC*, which encodes flagellin; a model of CF lung inflammation linking TLR5 activation, flagellin expression and neutrophil elastase concentration is emerging [142, 145, 146]. When neutrophil numbers and neutrophil
elastase concentrations in the CF lung are low, *P. aeruginosa* proliferates, assembles a flagellum, and stimulates a robust inflammatory response via TLR5. Neutrophils are then recruited to the airway and when neutrophil elastase concentrations reach a threshold, flagellin synthesis is suppressed and TLR5-flagellin mediated inflammation abates. As neutrophil numbers wane, flagellin synthesis resumes, and there is a resurgence of inflammation. Hence, TLR5 inhibition may be useful in breaking this repetitive cycle of airway infection and inflammation that ultimately causes respiratory failure in CF patients.

In conclusion, through the use of both respiratory epithelial cells and fresh blood cells donated by patients with CF, I have shown that TLR signalling results in excessive pro-inflammatory cytokine production by cells with dysfunctional CFTR protein. Moreover, the data presented in this chapter identifies a single molecule — TLR5 — as a novel anti-inflammatory target since inhibition of the TLR5-flagellin interaction abolishes the damaging inflammatory response generated by immortalized CF airway cells *in vitro*. Although more studies utilizing both primary CF airway cells and animal models are required, the potential utility of TLR5 inhibition is further supported by the demonstration that clinical isolates of *P. aeruginosa* generally maintain the ability to activate TLR5 even during chronic lung infection when motility is lost. Ultimately, it is anticipated that these preclinical studies may be the first step in developing a new therapy for safely reducing airway inflammation and improving CF lung disease.
3. Toll-like Receptor 5 as an Anti-Inflammatory Target and Modifier Gene in Cystic Fibrosis

3.1 Introduction

Lung disease, the major cause of death in CF, is caused by chronic infection and inflammation. Current therapies for CF address airway infection with antibiotics and airway obstruction using physiotherapy clearance techniques combined with mucolytics. Safe and clinically-acceptable therapies to target airway inflammation are likely to augment current treatments and improve the clinical outcome in CF [113]. Clinical trials have demonstrated that anti-inflammatory therapy is beneficial for patients with CF, improving important clinical outcomes such as lung function and body weight. Oral corticosteroids helped maintain pulmonary function, but unfortunately adverse side-effects render this approach unacceptable [57, 114, 147]. The non-steroidal anti-inflammatory, ibuprofen, taken at high doses for a long duration, slowed the progression of lung dysfunction and body weight was better maintained in the ibuprofen-treated group [56, 148, 149]. In addition to corticosteroids and ibuprofen, increasing evidence shows that azithromycin, a macrolide antibiotic with both anti-microbial and anti-inflammatory activity, benefits people with CF [150-153].

Clinical experience with these relatively non-specific anti-inflammatory medications provides ‘proof of concept’ evidence that targeting inflammation can be beneficial in CF. The challenge is now to identify new, specific anti-inflammatory targets. Modulating the function of the innate immune system is a particularly attractive treatment approach, since activation of the innate immune system is central to the inflammatory response occurring in the CF lung [154, 155]. Innate immunity relies upon a series of germline-encoded receptors, including Toll-like receptors (TLRs), to sense infectious organisms and to trigger an acute inflammatory response. Work by our group and others suggests that modulation of TLR function, and particularly
TLR5 inhibition, has therapeutic potential to improve the inflammatory manifestations of CF [156, 157]. TLR5 is the receptor for flagellin, the protein component of bacterial flagella expressed by classic CF bacterial pathogens, particularly P. aeruginosa [72]. Emerging evidence indicates that the TLR5-flagellin interaction plays a central role in driving the inflammatory response triggered by P. aeruginosa [92, 96, 100, 105, 157, 158] and inhibition of TLR5 normalizes the inflammatory response generated by CF airway epithelial cells following exposure to P. aeruginosa [157]. In this study cellular model systems were used to further explore the central role of TLR5 in driving the inflammatory response of airway cells against P. aeruginosa. The experiments in this chapter were deliberately focused on the response of CF airway epithelial cells, because evidence suggests that radioresistant lung epithelial cells are essential for triggering the innate immune responses in the lung [99].

While data from our group and others, in a variety of animal and cellular model systems, indicate that inhibition of TLR5 may decrease inflammation in CF, it would be much more compelling if the benefits of TLR5 modulation could be confirmed using an independent experimental design based on the outcome of people living with CF. The identification of CF modifier genes represents a powerful, clinically-relevant strategy to discover and validate new therapeutic targets. Modifier genes are loci containing variants that affect the clinical manifestation of a disease. Modifier genes have received considerable attention in CF: patients with an identical CFTR genotype (e.g. ΔF508 homozygous) show great variability in lung function [11, 12, 159], and twin studies have confirmed a significant non-CFTR genetic contribution to this variation in CF lung disease [13]. To further validate TLR5 as an anti-inflammatory target, the disease modifying effects of the TLR5 c.1174C>T single nucleotide polymorphism (SNP) (rs5744168) were analysed in a large representative cohort of Canadian CF patients [15, 160]. TLR5 c.1174C>T encodes a premature stop codon (i.e. TLR5<sup>392STOP</sup>) in the ligand binding domain of TLR5. Allele T is found in 6.1% of Canadian CF patients and acts in a dominant
fashion with respect to allele C, to significantly reduce flagellin responsiveness [139, 161, 162]. Consequently, through determining the disease modifying effects of the TLR5 c.1174C>T SNP, this study examines the impact of reduced TLR5 responsiveness on the health of a large cohort of Canadian CF patients.

The goal of this study was to empower the development of new therapies to treat the inflammatory component of CF lung disease by exploring the potential of TLR5 modulation to improve the outcome in CF. I hypothesized that reducing damaging inflammation, through the modulation of TLR5 responsiveness, would improve the clinical outcome in CF. By combining in vitro cellular immunology approaches with a large in vivo population-based assessment of TLR5 c.1174C>T SNP as a genetic modifier in Canadian CF patients, these results provide further evidence that TLR5 may represent an effective therapeutic target for improving the outcome in CF.
3.2 Materials and Methods

3.2.1 Airway Epithelial Cell Lines and Peripheral Blood Mononuclear Cells

To study the inflammatory response of CF airway cells, experiments were performed on two well-characterized sets of immortalized CF and non-CF airway epithelial cells: (i) IB3-1 (compound heterozygote for the ΔF508 and W1282X) and C38 (IB3-1 transfected with CFTR using an adeno-associated viral vector) [117]; and (ii) CuFi-1 (ΔF508 homozygous) and NuLi (wild-type CFTR genotype) [163]. It is essential to validate any findings in at least two pairs of CF and control airway epithelial cells, because subtle technical artefacts, possibly related to immortalization and long-term culture, may alter the pro-inflammatory phenotype of any cell line (reviewed in [45]). Importantly, airway epithelial cells grown at air-liquid interface or as submerged cultures have been shown to have similar patterns of cytokine production [164, 165]. IB3-1/C38 cells were grown in pre-coated flasks (100 µg/ml BSA, 30 µg/ml bovine collagen I, 10 µg/ml human fibronectin) in LHC-8 basal medium (Invitrogen) supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% penicillin-streptomycin-amphotericin B solution at 37°C and 5% CO2. NuLi and CuFi cells were grown in flasks (pre-coated with 60ug/ml Human Placental Collagen Type VI (Sigma)) in serum-free Bronchial Epithelial Growth Medium (BEGM) (Lonza) supplemented with SingleQuot additives in BEGM Bullet Kit (Lonza) and 50ug/ml G-418 (Invitrogen) at 37°C and 5% CO2. Sixteen hours prior to use in stimulation assays, the NuLi/CuFi and C38/IB3-1 cell lines were seeded into pre-coated 96 well plates at a density of 3-5×10⁴ cells per well. To assess cell viability after stimulation with bacteria or TLR ligands, release of a cytoplasmic enzyme (lactate dehydrogenase, LDH) by damaged cells was measured using a LDH cell cytotoxicity kit (Roche).
With approval of the University of British Columbia Clinical Research Ethics Board and subject consent, DNA samples from healthy volunteer donors were obtained and genotyped for the TLR5 c.1174C>T SNP. Individuals with relevant TLR5 genotypes subsequently donated blood, peripheral blood mononuclear cells (PBMCs) were isolated and the cells were seeded into 96-well plates at a density of 1x10^5 cells per well, according to protocols described previously [118].

3.2.2 Stimulation Assay with Pure TLR Ligands and Whole Bacteria

PBMCs were challenged with pure TLR ligands (ultrapure LPS from *E. coli* 0111:B4 1ng/ml; recombinant endotoxin-free flagellin from *S. typhimurium* 40ng/ml, Invivogen). Culture supernatants were harvested after 24h and cytokine concentrations (IL-6 and IL-8) were quantified by ELISA. Airway epithelial cells were exposed to heat-killed whole bacteria (*P. aeruginosa* PAK wild-type or PAKΔfliC, a non-motile derivative of PAK wild-type in which *fliC* is interrupted by a gentamycin cassette) or live whole bacteria (*P. aeruginosa* PAO1 wild type) at a variety of multiplicities-of-infection (MOIs). Stimulation time was 24 hours for heat-killed bacteria but was reduced to 4 hours for live bacteria to minimize pathogen-driven cytotoxicity. In some experiments TLR5 activation was inhibited using a neutralizing polyclonal antibody raised against the extracellular region of human TLR5 (PAb-hTLR5, 6-25µg/ml, InvivoGen) with a purified rat IgG1 (eBioscience) serving as a control.

Table 3.1: Primer Sequences Used for Quantitative PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>IL6</em></td>
<td>TGTGAAAGCAGCAAAGAGGCACTG</td>
<td>ACCAGGCAAGTCTCCTCATTGAAT</td>
</tr>
<tr>
<td><em>IL8</em></td>
<td>GTGCAGAGGGTTGGGAAGATTT</td>
<td>TGCATCTGGCAACCCTACAACAGA</td>
</tr>
<tr>
<td><em>PPIA</em></td>
<td>TAAAGCATAGGGTCCTGGCATCT</td>
<td>ATCCAAACCACCTCAGTCTTGGCAGT</td>
</tr>
</tbody>
</table>
3.2.3 Gene Expression Quantification

Total RNA isolated from airway epithelial cells (RNeasy Plus Mini Kit, Qiagen) was transcribed to cDNA with oligo-dT primers (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Quantitative PCR (QPCR) primers were designed for SYBR Green chemistry to amplify peptidylprolyl isomerase A (i.e. cyclophillin A, PPIA), IL-6 and IL-8 (Table 3.1) under standard cycling protocols using a 7300 Real-Time PCR System (Applied Biosystems). Relative gene expression in the CF compared to non-CF airway cells was analysed by the $2^{\Delta\Delta Ct}$ method using cyclophillin A as a reference gene.

3.2.4 Description of The Canadian CF Modifier Study Patient Cohort

The Canadian CF Modifier Study has been described in detail elsewhere [15, 160]. Briefly, at the time of this study the cohort consisted of 2,219 CF patients with samples available for genotyping who were recruited from 37 specialized CF clinics across Canada. The protocols for this study were approved by ethical review boards at the University of British Columbia, the Hospital for Sick Children and all other participating institutions. Informed consent was obtained from each individual or his/her guardian. Clinical parameters analysed were: (a) mean FEV$_1$ % predicted=mean forced expiratory volume in 1 second was calculated for each subject from all available FEV$_1$ measurements in the 3 years before recruitment. At each visit, FEV$_1$% predicted was calculated using paediatric [166] and adult [167] prediction equations based on age, height and sex; (b) zBMI=body mass index (BMI) expressed as standard deviations above or below median for age and sex based on the most recent height and weight measurement.
**Table 3.2: TLR5 C.1174C>T Genotype Data.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paediatric</td>
<td>531</td>
<td>64</td>
<td>2</td>
<td>597</td>
</tr>
<tr>
<td>Adult</td>
<td>407</td>
<td>56</td>
<td>3</td>
<td>466</td>
</tr>
</tbody>
</table>

### 3.2.5 Genotyping

The *TLR5* c.1174C>T SNP (*TLR5*<sup>392STOP</sup>, rs5744168) genotyping was performed in a 384-well format using a commercially available TaqMan assay (C_25608804_10, Applied Biosystems). Twelve positive and twelve negative controls were included in each plate. End-point analysis (allelic discrimination) was performed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems).

### 3.2.6 Statistical Analyses for The CF Modifier Study

To minimize the overlap of records in the transition from paediatric to adult CF clinics, patients were stratified into paediatric (<18.5 years of age) and adult groups by the current age, which was defined as the age at the last visit to the clinic. Genotypes for the *TLR5* c.1174C>T SNP in both paediatric and adult groups were in Hardy–Weinberg equilibrium, and after combining CT and TT genotypes a Fisher's exact test showed no evidence of genotype difference by gender. Considering the small number of individuals with the TT genotype, it was most appropriate to fit dominant models instead of additive or genotypic models (Table 3.2). Linear models were fitted to ascertain the modifying effect of *TLR5* c.1174C>T. Confounders were identified by univariate analyses and sensitivity analyses were performed to ensure that univariate analyses results were not driven by extreme values. Confounders included mean age (i.e. mean of ages at which FEV<sub>1</sub>% predicted was measured), number of clinic visits (i.e. the number of visits with FEV<sub>1</sub>% predicted values) and zBMI. Considering the number of sib-pairs in the paediatric and adult group (46 and 15, respectively), both linear and mixed effects
models were fitted to assess the effect of the within family correlation in variance estimation. Since the outputs of the two models did not differ significantly, we only reported the results based on the linear regression. The data were analysed using SAS (SAS Institute Inc, Version 9.1.2) procedure GLM, mixed and genmod. The non-parametric Kruskal-Wallis test was considered to see if the medians of the zBMI in the TLR5 c.1174C>T genotype groups differed significantly and in order to deal with the within family correlation, the test was repeated after excluding the younger siblings.
3.3 Results

3.3.1 TLR5-Flagellin Interaction is a Major Mediator of The Inflammatory Response Generated by CF Airway Epithelial Cells Following Exposure to \textit{P. aeruginosa}

To assess the relative contribution of the TLR5-flagellin interaction to the inflammatory response generated by CF airway epithelial cells, the experimental strategy was to alternatively block both sides of the TLR5-flagellin host-pathogen interaction. To remove the pathogen contribution, airway epithelial cells were stimulated with PAKΔ\textit{fliC}, a laboratory strain of \textit{P. aeruginosa} that expresses a full complement of pathogen-associated molecular patterns but lacks flagella. Strikingly, the absence of flagellin significantly reduced pro-inflammatory IL-6 and IL-8 production at the mRNA (\(p<0.05\) & \(p<0.01\)) and protein level (\(p<0.0001\)) by the CF airway epithelial cells following exposure to \textit{P. aeruginosa} (Figure 3.1 a-c). A neutralizing polyclonal antibody raised against the extracellular region of human TLR5, which has been shown to efficiently and specifically inhibit TLR5, was used to remove the host contribution of the TLR5-flagellin interaction [157]. The anti-TLR5 antibody significantly reduced pro-inflammatory IL-6 production by CF airway epithelial cells following exposure to \textit{P. aeruginosa} (Figure 3.1 d & e). Importantly, the anti-TLR5 antibody was equally effective in reducing inflammation regardless of whether the bacteria were heat-killed (PAK wt strain, \(p=0.004\)) or live (PAO1 wt strain, \(p<0.0001\)). Experiments using live bacteria were performed using PAO1 rather than PAK, as PAK was profoundly cytotoxic to these cell lines. Lactate dehydrogenase (LDH) release assays confirmed that there was no significant cytotoxicity to CF or control cells under our experimental conditions following stimulation with live PAO1 (data not shown).
Figure 3.1: Inhibition of The TLR5-Flagellin Interaction Significantly Reduces The Pro-Inflammatory Response of CF Airway Epithelial Cells Following Exposure to *P. aeruginosa*.

(a-d) CF (CuFi-1) and control (NuLi) airway epithelial cells were stimulated with a heat-killed PAK or PAKΔfliC at varying multiplicities-of-infection (MOI). mRNA expression was analysed after 2 hours, while supernatants were harvested after 24h for cytokine quantification. mRNA expression is shown for the 50:1 MOI. In some experiments an anti-TLR5 antibody or isotype control was added. (e) CF-corrected (C38) and CF (IB3-1) airway epithelial cells were stimulated for 4 hours with live PAO1 and IL-6 secretion into the supernatant was quantified by ELISA. Values represent mean ± SEM of 3-4 separate experiments all performed in triplicate. Statistical analysis was performed by two-way ANOVA using the Bonferroni correction for multiple comparisons (a & b) and semi-logarithmic nonlinear regression (c-e). * & ** signify *p*<0.05 & 0.01, respectively.
3.3.2 TLR5 c.1174C>T SNP Significantly Reduces Flagellin Responsiveness

To facilitate the genetic modifier study, the aim was to identify a functional variant in TLR5 that is common in the population. Hawn et al. described the functional impact of the TLR5 c.1174C>T SNP which encodes a premature stop codon [139]. The functional impact of TLR5 c.1174C>T was assessed using PBMCs from individuals who were heterozygous for the premature stop codon (CT genotype) and control subjects homozygous for the common allele (CC genotype). Subjects homozygous for the premature stop codon (TT genotype) make up less than 1% of the population and were not included in this analysis. PBMCs were stimulated with the TLR5 ligand, flagellin (endotoxin-free, 40ng/ml), and the TLR4 ligand, LPS (ultrapure, 1ng/ml). Heterozygous carriers of the premature stop codon (CT genotype) demonstrated significantly decreased IL-6 and IL-8 production (76.3% and 45.5% reduction, respectively; \( p < 0.0001 \)) after stimulation with flagellin compared to controls (CC genotype), whereas following LPS stimulation the inflammatory response was slightly lower in the controls (Figure 3.2). These data confirm the functional impact of the TLR5 c.1174C>T SNP on flagellin responsiveness.

3.3.3 Relationship Between The TLR5 c.1174C>T SNP and Clinical Outcome Variables in CF

After the TLR5 c.1174C>T SNP was successfully genotyped in 99.4% of the 2219 CF patient samples, the subjects were stratified as outlined in the flow chart in Figure 3.3. Specifically, to reduce genetic variability at the CFTR locus, only CF patients who had insufficiency of the exocrine pancreas (PI) and carried severe CFTR mutations on both alleles were included. For the purposes of this study, exocrine pancreatic function status was based on clinical status and CFTR mutations [15, 168, 169]. PI status was assigned if both CFTR mutations were known to be associated with PI. Pancreatic sufficient (PS) patients carried at least
one known pancreatic sufficient mutation. Otherwise, those with \textit{CFTR} mutations which were unidentified or not clearly associated with PS or PI were classified as PS or PI based on the reported clinical use of pancreatic enzymes. Furthermore, given that a modifying effect of \textit{TLR5} would only be expected to occur in patients infected with flagellated bacteria, patients not infected with \textit{P. aeruginosa} were excluded. Finally, patients infected with organisms of the \textit{B. cepacia} complex (BCC) were excluded from analysis, because infections with BCC are associated with a worse prognosis at all levels of pulmonary function, and BCC may cause 'cepacia syndrome', characterized by rapid pulmonary deterioration, septicemia and

![Figure 3.2: TLR5 C.1174C>T SNP is Functionally Relevant Significantly Reducing Flagellin Responsiveness.](image)

Pro-inflammatory cytokine production by PBMCs upon stimulation with pure recombinant flagellin (40ng/mL) is markedly reduced in individuals carrying the TLR5 premature stop codon (CT genotype, n=5) compared to control individuals homozygous for the common allele (CC genotype, n=5). Response to lipopolysaccharide (LPS, 1ng/mL) was examined as a TLR5-independent control. Statistical comparisons made by two-way ANOVA using the Bonferroni correction for multiple comparisons. *, ** & *** signify \(p<0.05, 0.01 \& 0.001\), respectively.
death in about 20% of infected CF patients [170, 171]. Table 3.3 summarizes the basic demographic data of the subjects after stratification.

The goal was to examine the relationship between the TLR5 c.1174C>T SNP and two clinical characteristics that have been associated with mortality among patients with cystic fibrosis: FEV₁ and body weight [172]. These were relevant end-points for examining the modifying impact of a TLR5 variant known to reduce TLR5 function, as clinical trials evaluating the anti-inflammatory benefits of ibuprofen and azithromycin often demonstrated improvements in both FEV₁ and body weight [56, 148-153].

**Table 3.3: Demographic and Clinical Characteristics of The CF Population.**

<table>
<thead>
<tr>
<th></th>
<th>Paediatric (n=597)</th>
<th>Adult (n=466)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (yr)</td>
<td>0.36 (-0.57 to 12.72)</td>
<td>0.75 (-0.003 to 59.4)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>11.54 (6.02 to 18.50)</td>
<td>25.04 (16.33 to 61.31)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>17.83 (11.89 to 29.33)</td>
<td>21.41 (14.54 to 35.98)</td>
</tr>
<tr>
<td>zBMI</td>
<td>-0.24 (-5.16 to 2.13)</td>
<td>-0.31 (-4.99 to 2.26)</td>
</tr>
<tr>
<td>Mean FEV₁ % predicted</td>
<td>88.82 (19.18 to 132.40)</td>
<td>61.11 (15.34 to 127.50)</td>
</tr>
</tbody>
</table>

Values represent the median (and range) for the cohort

In both the paediatric and adult CF patients, zBMI was not normally distributed. Non-parametric tests were considered to see if the medians of the zBMI in the two genotype groups differed significantly (Table 3.4). Based on the non-parametric Kruskal-Wallis test, adults with CF carrying the TLR5 premature stop codon (CT or TT genotype) had higher zBMI than CF patients homozygous for the common fully functional allele (CC genotype) ($p=0.044$). This difference between the CT/TT and CC genotypes was equivalent to CF patients carrying the premature stop codon (CT or TT genotype) having a BMI 1.18kg/m² higher, which equates to a 3.3 kilogram weight increase for a person 167cm (5 feet 6 inches) tall. To test the sensitivity of
this association and assess the impact of within family correlation, the analysis was repeated after excluding the younger sibling of each sib-pair (n=15) and the association between the TLR5 c.1174C>T genotype and zBMI was lost (p=0.119). In an attempt to account for the duration of infection with P. aeruginosa, two relationships were examined: (a) the Spearman correlation between the duration of infection and BMI, stratified by TLR5 genotype; and (b) the relationship between the duration of infection and TLR5 genotype by the Kruskal-Wallis test. These analyses suggested that the direction and strength of association between zBMI and TLR5 c.1174C>T genotype were not altered significantly when we accounted for the duration of infection with P. aeruginosa.

While the TLR5 c.1174C>T SNP was associated with differences in BMI in adults with CF, no significant modifying effect of the TLR5 c.1174C>T SNP on lung function was observed in either the paediatric or adult CF patients regardless of adjustment for the confounders (Table 3.4).

Table 3.4: Relationship Between TLR5 C.1174C>T Genotype and Clinical Outcomes.

<table>
<thead>
<tr>
<th></th>
<th>Paediatric (n= 597)</th>
<th>Adult (n= 466)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT/TT</td>
</tr>
<tr>
<td>zBMI</td>
<td>-0.239</td>
<td>-0.241</td>
</tr>
<tr>
<td>Mean FEV₁, % predicted</td>
<td>88.720</td>
<td>90.765</td>
</tr>
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<td></td>
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</tbody>
</table>

¹P-values based on the Kruskal-Wallis test. ²P-values obtained from fitting a linear model unadjusted for confounders. ³P-values obtained from fitting a linear model adjusted for confounders: mean age, number of visits and zBMI.
Figure 3.3: Flow Chart Outlining CF Patients Included in The Analysis of TLR5 as A Modifier Gene in CF.
Flow chart provides an overview of subjects included in the analysis. The following abbreviations are used: DoB-date of birth; PS/PI-pancreatic sufficient/pancreatic insufficient; PA-infected with *P. aeruginosa*; BCC-infected with members of the B. cepacia complex. Subjects were considered to be infected with *P. aeruginosa* if they had one or more positive cultures, and subjects who subsequently eradicated *P. aeruginosa* were not excluded from analysis.
3.4 Discussion

The aim of this study was to explore the potential of TLR5 as an anti-inflammatory target to improve the clinical outcome in CF. As controlling inflammation has been shown to be beneficial for CF patients; most attention has focused upon the therapeutic potential of corticosteroids and ibuprofen. Unfortunately, clinical uptake of these treatment options is low due to significant adverse side effects. Indeed, recently published guidelines concerning the use of chronic medications for maintenance of CF lung health recommend against the use of oral corticosteroids in children [173]. Similarly, while guidelines now recommend the chronic use of oral ibuprofen to slow the loss of lung function, uptake of this therapy at CF centres has been very limited due to concerns about side effects, particularly gastrointestinal ulceration and renal damage [174]. To expand the repertoire of anti-inflammatory medications for CF, recent attention has turned to the immunomodulatory properties of macrolide antibiotics, especially azithromycin; with trials demonstrating improved lung function and body weight in those receiving azithromycin [150-153]. However, there are also potential challenges in using azithromycin, including the risk of selection of resistant bacteria [153, 175] and our lack of mechanistic understanding of the beneficial anti-inflammatory actions of macrolides [176]. Consequently, alternative anti-inflammatory agents with different modes of action and safety profiles are required to improve the outcome in CF.

Controlled inflammation is important for fighting infection, but in CF the inflammatory response is dysregulated and prolonged (reviewed in [25]). Inflammation in the CF lung is characterized by an intense neutrophilic infiltrate, high levels of pro-inflammatory cytokines (IL-8, IL-6, TNF-α, IL-1β) and evidence of activation of NF-κB and other pro-inflammatory signalling cascades. Specifically targeting the molecular pathways driving these aspects of CF airway inflammation is a particularly attractive therapeutic approach. The data presented in Chapter 2 show that the TLR5 – flagellin interaction mediates much of the exaggerated pro-
inflammatory cytokine production seen in immortalized CF airway cells following exposure to *P. aeruginosa* in vitro [157]. Two limitations of these previous experiments were the reliance on a single pair of immortalized CF and CF-corrected airway epithelial cells, and the fact that the bacteria used for the stimulation assays were heat-killed to control for differences in motility between wild-type and flagella-deficient strains of *P. aeruginosa*. To assess more thoroughly the potential of TLR5 as an anti-inflammatory target in CF, it was critical to address these weaknesses and expand the previous observations. In this study two pairs of CF and control airway epithelial cells were used to confirm that the interaction between TLR5 and flagellin plays a central role in triggering pro-inflammatory cytokine production by CF airway epithelial cells following exposure to the major CF pathogen, *P. aeruginosa* (Figure 3.1). The observation that blockade of TLR5 abrogated pro-inflammatory cytokine production by CF airway epithelia following exposure to live *P. aeruginosa* was of particular relevance to host-pathogen interactions occurring in the CF lung.

While live bacteria have the capacity to activate a wide variety of innate immune detector systems, including NOD-like receptors (NLRs) which can respond to both flagellin and elements of the bacterial type III secretion system [177-180], the present data indicate that following exposure to *P. aeruginosa* the production of IL-6 and IL-8 — key pro-inflammatory cytokines known to be present at high levels in the CF lung — is dependent upon signalling through TLR5.

When considering TLR5 as a novel anti-inflammatory target in CF, several potential limitations must be addressed. The first is the observation that most environmental *P. aeruginosa* strains and those isolated early in CF infections are highly motile, whereas some strains isolated from older, chronically infected CF patients are immotile [123, 142]. However, the potential therapeutic value of a TLR5 inhibitor is supported by the demonstration that 75% of clinical isolates of *P. aeruginosa* obtained from the lungs of CF patients aged from 1 year to 36 years retained the capacity to activate TLR5 [157]. Despite the significant loss of motility of *P. aeruginosa* during the evolution of chronic CF lung infection, the TLR5 activating
capacity of *P. aeruginosa* is relatively retained in the CF lung across the patient’s lifespan. A second factor to consider is that in the CF lung, *P. aeruginosa* appears to exist as biofilms [181]. These structured communities of *P. aeruginosa* encased in a self-produced polymeric matrix may fail to interact with the TLR5-expressing airway epithelium; nevertheless, biofilms are dynamic and flagellated planktonic organisms that break free from the bacterial community are likely to shed flagella within the airway lumen to activate TLR5. Finally, lipopolysaccharide (the ligand for TLR4) is often considered to be the most potent immunostimulatory molecule expressed by *P. aeruginosa*. While unique lipid A modifications occur in *P. aeruginosa* during chronic infection of the CF lung [112], accumulating evidence indicates that the TLR4-mediated response to LPS is minimal in airway cells because of a lack of the necessary co-receptors, including MD2 and CD14 [134, 135, 182].

With multiple lines of evidence in a variety of model systems all implicating TLR5 as a major driver of inflammation following pulmonary infection with *P. aeruginosa* [92, 96, 100, 105, 157, 158, 183], the second focus of this study was to explore the contribution of TLR5 to the health outcomes of patients living with CF. A powerful translational research approach for identifying and validating new therapeutic strategies is through the identification of genetic variants that modify CF clinical phenotypes. The data presented in this chapter establish *TLR5* as a biologically-plausible candidate gene that may modify clinical outcomes in CF. The *TLR5* gene harbours a polymorphism — *TLR5* c.1174C>T (rs5744168) — that encodes a premature stop codon at amino acid position 392 in the ligand binding domain of TLR5. Since allele T has been shown by our group (Figure 3.2) and others [139, 161, 162] to be associated with significantly impaired flagellin responsiveness, it is predicted that CF patients carrying the T allele would have improved clinical outcomes due to the endogenous anti-inflammatory effects of this *TLR5* variant.
We elected to investigate the disease-modifying impact of TLR5 c.1174C>T on two important prognostic indicators in CF — pulmonary function and nutritional status [170, 172, 184, 185]. The loss of lung function is characterized by approximately 2% decline of FEV\textsubscript{1} per year [186]. For body weight, low BMI values are usually associated with more severe disease and nutritional status is inversely correlated with mortality rate [172]. In the large representative CF cohort used in this study, adults with CF carrying the TLR5 premature stop codon (CT or TT genotype) had improved nutritional status (higher BMI), as measured by zBMI, compared with CF patients homozygous for the common fully-functional allele (CC genotype) (Table 4). In contrast to adults, the TLR5 c.1174C>T SNP was not associated with differences in the age and sex adjusted zBMI of children with CF. When examining pulmonary function, the analysis did not produce any evidence that the TLR5 premature stop codon modified lung function in the present CF cohort. Specifically, although the CT/TT genotype was associated with a higher mean FEV\textsubscript{1} % predicted (2.0% and 2.5% in paediatric and adult CF patients, respectively); these differences were not statistically significant (Table 4). Nevertheless, these results must be interpreted with some caution given that the association between the TLR5 c.1174C>T SNP and zBMI in adult CF patients was modest (p=0.044) and this association was lost during sensitivity analysis that excluded the younger sibling of each sib-pair, perhaps because of the loss of power caused by excluding 15 subjects. While the in vitro data establish TLR5 as a plausible candidate modifier gene in CF, the modifying impact of TLR5 will only be firmly established through replication studies in other CF cohorts. Moreover, formal establishment of a link between any observed clinical benefits and modulation of TLR5 function will require the use of animal models, such as mice and pigs with engineered defects in both CFTR and TLR5.

The present study design does not allow a direct investigation of the mechanism by which the TLR5 premature stop codon (CT or TT genotype) may mediate an improvement in the nutritional status of adults with CF. Nevertheless, inflammation
has been linked to energy expenditure in CF. Patients with CF have been demonstrated to have elevated energy expenditure leading to energy imbalance and the development of under-nutrition (reviewed in [187]). Of the many factors contributing to increased energy expenditure in CF patients, inflammation appears to be responsible for increases in resting metabolic rate. Indeed, Bell and colleagues demonstrated that intravenous antibiotic therapy for CF pulmonary exacerbations resulted in parallel reductions of host systemic inflammatory markers and resting energy expenditure together with an improvement in lung function and an increase in body weight [188]. A variety of other studies have revealed that acute respiratory exacerbations caused increased resting energy expenditure in many patients, which reverts to lower values after antibiotic therapy or administration of aerosolized dornase alpha (reviewed in [189]). Together these data suggest that inflammation and metabolic rate are co-regulated, consequently the down-regulation of the inflammatory response mediated by the TLR5 premature stop codon (CT or TT genotype) would be predicted to be accompanied by a reduction in the metabolic state and higher zBMI in CF patients. Clearly, due to the complex relationship between immunity, nutrition and metabolic events, additional studies are needed to explore the relationship between TLR5 variants and nutritional status in CF patients.

A strength of this study is the translational research design. Starting with in vitro models of the host-pathogen interaction occurring in the CF lung, TLR5 was confirmed as a major mediator of the inflammation occurring after exposure to P. aeruginosa (Figure 3.1). Furthermore, this study explored the potential in vivo consequences of TLR5 inhibition by determining if the functional TLR5 c.1174C>T SNP influences clinical outcomes in people living with CF. The data demonstrate that partial loss of TLR5 function appears to improve nutritional status in adults with CF is likely to be applicable generally, because the patient cohort was recruited from a population-based sample representing nearly 75% of the current Canadian CF population. When compared with the Canadian CF Patient Registry, the
demographic and clinical data in the recruited patient cohort were found to be nationally representative [15, 160]. A slightly surprising outcome of this study was the finding that, while the TLR5 c.1174C>T SNP appeared to modify BMI in adults with CF this TLR5 variant was not associated with differences in lung function. In the cohort there was a significant correlation between zBMI and mean FEV\textsubscript{1}% predicted ($p=0.0001$) which has been previously reported (for example see [190]). However, the Pearson product-moment correlation coefficient between the two variables was 0.35 and 0.38 for the paediatric and adult CF patients, respectively; indicating that the correlation between zBMI and mean FEV\textsubscript{1}% predicted was not perfect. It has been reported recently that mice genetically deficient in TLR5 develop a metabolic syndrome associated with increased weight that appears to be related to changes in the composition of the gut microbiota. These mouse data indicate that TLR5 dysfunction may modulate metabolic activity independently of lung function [191]. Intriguingly, the disease modifying impact of the TLR5 c.1174C>T SNP is reminiscent of the results of a recent trial testing azithromycin in CF patients who were not infected with *P. aeruginosa* in which this anti-inflammatory agent improved BMI but not lung function [153].

In summary, using complementary *in vitro* and population-based *in vivo* approaches, the data presented in this chapter demonstrate that: (a) inhibition of TLR5 responsiveness is associated with a reduced inflammatory response of CF airway epithelial cells to *P. aeruginosa*; and (b) the functional TLR5 c.1174C>T SNP, which significantly decreases TLR5 responsiveness, is associated with improved nutritional status in adults living with CF. Although follow-up studies are needed to specifically examine the impact of TLR5 on nutritional status, this study provides further preclinical evidence that strategies to inhibit TLR5 may improve the health outcome for patients with CF.
4. The Role of ER Stress in The Immune Response of CF Airway Epithelial Cells

4.1 Introduction

In CF lung disease, chronic airway infection and inflammation is the primary cause of morbidity and mortality leading to progressive pulmonary obstruction [113]. Although the role of inflammation in CF is well accepted, there is a lack of consensus over whether inflammation is intrinsically triggered as a result of the CFTR gene defect, or if it is caused by bacterial infection. Regardless of what causes the increased immune response, clinical interventions targeting inflammation have proven to be beneficial for CF patients, improving important clinical parameters such as BMI and FEV1, supporting the concept that lung inflammation is an attractive therapeutic target for treatment of CF [56, 114, 149]. The only currently available approaches to reduce inflammation are global anti-inflammatories such as corticosteroids or ibuprofen, which have demonstrated unacceptable side effects [55, 57]. Therefore, to treat lung inflammation successfully it is crucial to understand the specific biochemical mechanisms underlying the propagation of the inflammatory response to CF pathogens such as P. aeruginosa.

Several attempts have been undertaken to understand the biochemical events mediating inflammation and leading to an increased pro-inflammatory immune response in CF. The lung epithelium relies on innate immune receptors such as Toll-like receptors (TLRs) to recognize pathogens. Chapter 2 and 3 of the present thesis and others have shown that TLR5 plays a critical role in mediating much of the immune response to one of the clinically most important pathogens in CF, P. aeruginosa [157]. Not only is the pro-inflammatory response of CF cells normalized by blocking TLR5 but a SNP rendering TLR5 dysfunctional is associated with higher BMI in adult CF patients [192]. In 2006 McNamara et al. showed that asialoGM1
and TLR5 cooperate to mount a potent inflammatory response to flagellin mediated by ATP, Ca$^{2+}$ release and the activation of the ERK pathway [95] and p38 MAP kinase signalling is known to mediate flagellin-induced pro-inflammatory signalling in airway epithelial cells [193]. Other compelling evidence unravels the role of p38 MAP kinase in the TLR5 – *P. aeruginosa* mediated NF-κB activation and pro-inflammatory cytokine production in reporter cells and immortalized CF airway cells [194-196].

Although these reports implicate important signalling cascades potentially involved in mediating inflammatory responses to CF pathogens, it remains unclear what perturbs the signalling of these pathways leaving the CF lung epithelium hyper-responsive to bacterial stimuli. An attractive hypothesis suggests the endoplasmic reticulum (ER) is an important contributor to the hyper-inflammatory phenotype of CF epithelial cells. The most common *CFTR* mutation, ΔF508, which is carried by approximately 70% of CF patients [8], results in the misfolding of CFTR and the retention of the protein in the ER lumen [10]. Misfolded proteins form non-functional protein aggregates that do not pass the quality control system in the ER and are directed into the protein degradation pathway [197]. This system ensures that only correctly folded and functional proteins reach their target destination within or outside the cell. Three general mechanisms have evolved to cope with accumulation of unfolded proteins – transcriptional induction of folding chaperones, translational attenuation (regulation of protein influx) and up-regulation of the degradation machinery of misfolded proteins (regulation of protein efflux) [198].

Literature reports have suggested that ΔF508 protein expression causes ER stress [47, 199-201], likely due to accumulation of aggregated protein in the ER lumen. The ER is a sensitive organelle and perturbation of the protein homeostasis (proteostasis) due to deficiencies in folding, trafficking and degradation potentially contributes to a broad range of human diseases [202]. Thus, a decrease in proteasome activity could be dangerous for the cell as it is associated with chronic
obstructive pulmonary disease (COPD), various autoimmune diseases, and with the normal aging process [203-205].

The influx and efflux of proteins into and from the ER needs to be tightly regulated to prevent overloading of this compartment. In the case of ER stress, this is typically regulated by the unfolded protein response (UPR). The classical UPR consists of three major pathways initiated by the sensor proteins IRE-1α (inositol requiring enzyme 1) and PERK (PKR-like eukaryotic initiation factor 2α kinase), and the transcription factor ATF6 (activating transcription factor 6) [206]. IRE-1α activates the transcription factor X-box binding protein 1 (XBP-1) by alternative splicing through its endonuclease activity. The active form XBP-1s initiates a transcriptional program leading to the expression of chaperones resulting in an increase in the folding capacity of the ER [207]. The activation of ATF6 initiates a transcriptional program aimed to facilitate the clearance of the ER by activating the ER-associated protein degradation (ERAD) [206, 208]. Activation of PERK induces the phosphorylation of eukaryotic initiation factor 2α (eIF2α), which leads to a halt of mRNA translation and therefore attenuation of protein biosynthesis [209]. Furthermore, this pathway can lead to the activation of pro-apoptotic and pro-inflammatory pathways via ATF4 (activating transcription factor 4) and CHOP (C/EBP homologous protein) [210, 211]. Together these three arms mitigate ER stress by attenuating protein synthesis, facilitating protein folding and increase protein degradation to collectively alleviate ER stress.

While ER stress is evident in chronic lung disease (such as CF and COPD) [212, 213] several studies have implicated cross-talk mechanisms between the UPR and classical pro-inflammatory pathways including NF-κB signalling [206, 211, 214]. The goal of the present study is to investigate the mechanism underlying the hyper-inflammatory phenotype in CF epithelial cells (IB3-1) and the non-CF control cell
line (C38). By combining computational with biochemical approaches, the differentially activated pathways and transcription factors were determined at baseline and in response to flagellin. The results confirm previous hypotheses and literature reports indicating a role of ER homeostasis in inflammatory responses. Furthermore, the data identify a pathway that potentially integrates signals from both ER stress responses and infection, to result in one combined inflammatory output in CF. Taken together these results indicate that pre-existing ER stress can prime inflammatory pathways to over-react, resulting in increased pro-inflammatory cytokine production and, identifying ER stress as potential therapeutic targets to alleviate inflammation in CF.
4.2 Material and Methods

4.2.1 Airway Epithelial Cell Lines

To study the inflammatory response of CF airway cells, experiments were performed on the well-characterized immortalized CF and non-CF airway epithelial cells: (i) IB3-1 (compound heterozygote for the ΔF508 and W1282X) and C38 (IB3-1 transfected with CFTR using an adeno-associated viral vector) [117]. IB3-1/C38 cells were grown in pre-coated flasks (100 µg/ml BSA (Sigma), 30 µg/ml bovine collagen I (BD Bioscience), 10 µg/ml human fibronectin (BD Biosciences)) in LHC-8 basal medium (Invitrogen) supplemented with 10% (v/v) FCS (HyClone), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), and 1% penicillin-streptomycin-amphotericin B solution at 37˚C and 5% CO₂. Sixteen hours prior to use in stimulation assays C38 and IB3-1 cells were seeded into pre-coated 96- or 24-well plates at a density of 5x10⁴ or 2.5x10⁵ cells per well, respectively. To assess cell viability after stimulation with bacteria or TLR ligands, release of a cytoplasmic enzyme (lactate dehydrogenase, LDH) by damaged cells was measured using a LDH cell cytotoxicity kit (Roche).

4.2.2 Reagents and Inhibitors Used in This Study

Cells were stimulated with either tunicamycin (20µg/ml) (Calbiochem) or flagellin (100ng/ml) (Invivogen) and treated with p38 MAP kinase inhibitor SB203580 (Calbiochem).

4.2.3 Western Blotting

Cells were seeded in 6-well plates at a concentration of 1x10⁶ cells per well. Cells were stimulated with flagellin and subject to lysis with 200µl 1x Laemmli’s digestion
mix. The same volumes of protein were separated on a 10% SDS-PAGE and transferred to PVDF membranes (BioRad). Membranes were probed for p38, phospho-p38 and β-actin (Cell Signaling). Protein bands were quantified using ImageQuant (GE Healthcare).

4.2.4 Cell Stimulation Assays

Cells were seeded into 96-well plates (functional studies) or 24-well plates (gene expression studies) at a concentration of 5x10^4 or 2.5x10^5 per well, respectively, 16hs prior to stimulation. Cells were stimulated with optimal concentrations of tunicamycin and/or flagellin. For inhibitor studies cells were incubated with SB203580 (p38 MAP kinase inhibitor) 1h prior to stimulation with tunicamycin or flagellin. IL-6 concentrations were determined using a standard ELISA kit (BD Bioscience). For gene expression experiments cells were challenged with tunicamycin for 2, 4, 6, 8, 24, and 30hs before mRNA was extracted.

4.2.5 Gene Expression Quantification

Total RNA was isolated from airway epithelial cells (RNaseasy Plus Mini Kit, Qiagen) at 2, 4, 6, 8, 24 and 30hs of stimulation and transcribed to cDNA with oligo-dT primers (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Quantitative PCR (qPCR) primers were designed for SYBR Green chemistry to amplify peptidylprolyl isomerase A (i.e. cyclophilin A, PPIA), interleukin-6 (IL-6), XBP-1u, XBP-1s and, Grp78 (Table 1, Real-time PCR primers) under standard cycling protocols using a 7300 Real-Time PCR System (Applied Biosystems). Relative gene expression in the CF compared to non-CF airway cells was analyzed by the 2^−ΔΔCt method using cyclophilin A as a reference gene.
4.2.6 Gene Expression Array

Cells were stimulated in 6-well plates with 1ug/ml flagellin for 2hs. Cells were lysed and total RNA was harvested as described above. RNA quality was determined using Bioanalyzer 2100 (Agilent Technologies). mRNA was amplified using the RNA ampULSe kit (Kreatech) according to manufacturer's instructions. Amplified cRNA was hybridized to human HT-12 expression bead chips (Illumina) and raw data were first processed with Illumina BeadStudio version 3.4.0 to produce summary gene expression values. Further processing was performed with Bioconductor software (beadarray package) for quantile normalization. Differential expression of gene probes were calculated using the limma package [215]. Adjusted p-values were calculated using the Benjamini-Hochberg method [216]. Gene ontology (GO), transcription factor (TF) and pathway over-representation analysis (ORA) was performed using the MetaGEX platform (http://marray.cmdr.ubc.ca/metagex), implemented by CD Fjell, manuscript submitted).

Table 4.1: Quantitative PCR Primers Used in This Study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5' → 3')</th>
<th>Reverse (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6</td>
<td>TGTGAAAGCAGCAAAGAGGCCTCTG</td>
<td>ACCAGGCAAGTCTCTCATTGAAT</td>
</tr>
<tr>
<td>PPIA</td>
<td>TAAAGCATACGGGTCCGGCTCTG</td>
<td>ATCCAACCACCTGCTTCGTTGAA</td>
</tr>
<tr>
<td>Grp78</td>
<td>TGCCGTTCAAGGTGGTTGAAA</td>
<td>ACTGCATGGGTAACCTTCTTTCCC</td>
</tr>
<tr>
<td>XBP-1u</td>
<td>AGCAGTCAGACTACGTGCACCTCT</td>
<td>CCAGAATGCCCACAGGATATCAG</td>
</tr>
<tr>
<td>XBP-1s</td>
<td>ATGGATGCCCTGGTGCTGAAG</td>
<td>TGCACCTGCTGGCAGTC</td>
</tr>
</tbody>
</table>

4.2.7 Network Analysis

Network analysis was carried out as previously described [217, 218]. Briefly, a list of protein level interactions between differentially expressed genes (FC > +/- 1.4; p<0.05) was obtained using InnateDB [219]. Network analysis was carried out by visualizing these interactions into a single integrated network using Cytoscape (2.6.3 for Windows) and the software plugin Cerebral to appreciate cellular
directionality [220]. Further enrichment of the network was performed through the manual addition of interactions based on primary literature [221], focusing on genes curated in the KEGG pathway Protein processing in endoplasmic reticulum [KEGG:hsa04141] [222].
Figure 4.1: Interaction Network of Differentially Expressed Genes Between IB3-1 and C38 Cells Before and After Flagellin Stimulation.

The network visualizes the stress response and inflammatory signature in CF (IB3-1) cells compared to non-CF (C38) cells at baseline (a) and after flagellin stimulation (b). Colour of the nodes is proportional to its fold expression (green down-regulated, red up-regulated) in CF versus non-CF control cells.
4.3 Results

4.3.1 CF Cells Display Evidence of an ER/General Stress Response Combined with an Inflammatory Response Compared to Non-CF Cells

Multiple lines of evidence have shown that CF cells produce an increased pro-inflammatory immune response to flagellated pathogens such as *P. aeruginosa* via the flagellin-TLR5 interaction [157, 192, 195]. Currently it is not clear which events lead to the excessive immune response. To investigate the mechanism underlying the increased immune response to flagellin in an unbiased fashion, gene expression arrays were performed (Illumina platform) on IB3-1 and C38 cells at unstimulated conditions and 2hs after flagellin stimulation. The 2h time-point was chosen based on preliminary time-course experiments monitoring pro-inflammatory cytokine expression. The data was subject to extensive network analysis using complementary approaches of gene ontology (GO) and transcription factor (TF) over-representation analysis (ORA) methods (Figure 4.1, Table 4.2-4.4). A comprehensive network of interactions between differentially expressed genes was constructed, indicating the differential expression of genes representing pro-inflammatory signalling, ER associated protein degradation (ERAD, proteasome) and ER stress response mechanisms (UPR) (Figure 4.1). The TF ORA returned a signature of transcription factors activated downstream of the p38 MAPK signalling pathway (NF-κB, CREB1, AP-1) as well as transcription factors commonly activated by the UPR (XBP-1, ATF6, DDIT3) (Table 4.4).
Figure 4.2: Flagellin Induces Prolonged and Enhanced Activation of p38 MAP Kinase in CF Cells.

Cells were treated with 100ng/ml flagellin and cell lysates were subject to western blotting for p-p38 and total p38 after 5, 15, 30 and 45 minutes of stimulation (a & b). Densities of p-p38 protein bands were normalized to β-actin and fold expression compared to unstimulated is shown (b). CF cells (IB3-1) were incubated with 4μM SB203580 for 1h prior to 24hs of flagellin stimulation (100ng/ml) and IL-6 was measured by ELISA (c). Statistical analysis was performed by one-way ANOVA using Bonferroni correction for multiple comparisons (c). *p<0.05; ** p<0.01; ***p<0.001.

The findings of the TF ORA were mirrored in the GO ORA. When looking at the GO term ‘Biological Process’ (BP) (Table 4.2 & 4.3) a distinct MAP kinase stress response pattern was apparent (GO:0032874, p=0.02; GO:0000165, p=0.03;
GO:0000187, \( p=0.04 \)) as well as ER stress response mechanisms such as the retrograde protein transport (GO:0030970, \( p=0.0003 \)), ER overload response (GO:0.0006983, \( p=0.02 \)), calcium homeostasis (GO:0032469, \( p=0.03 \)), and innate immune pathways (GO: 0045089, \( p=0.006 \); GO:0043123, \( p=0.03 \)) which are regulated by ER stress response pathways [208, 210, 211] (Table 4.2 & 4.3). An inflammatory signature was found in CF cells compared to non-CF cells under unstimulated conditions, which was enhanced after flagellin stimulation (Table 4.2-4.4). Together, an enhanced inflammatory signalling pattern in conjunction with ER stress and general stress response pathways was returned by the analysis of the gene expression arrays.

4.3.2 The Activation of the p38 MAP Kinase is Prolonged and Enhanced in CF Cells Compared to Non-CF Cells

In 2010 Bérubé et al. reported an enhanced and prolonged activation of the p38 MAP kinase and ERK in CF cells (CuFi) compared to non-CF cells (NuLi) when stimulated with *P. aeruginosa* [195]. Furthermore, p38 MAP kinase has been implicated in promoting the mRNA stability of pro-inflammatory genes such as *IL-8* in CF cells [196], potentially leading to increased and prolonged immune responses. Based on this literature and the results from the gene expression array analysis, the activity of p38 MAP kinase was evaluated in response to flagellin stimulation in IB3-1 compared to C38 cells by Western blot. As shown in Figure 4.2 a & b, flagellin induced an increased and prolonged phosphorylation of p38 MAP kinase in CF cells. When CF cells were treated with the specific p38 MAP kinase inhibitor, SB203580 (4μM), the exaggerated pro-inflammatory immune response was significantly reduced (Figure 4.2c). These data support the results from the gene expression array analysis suggesting that p38 MAP kinase activity plays a significant role in the pro-inflammatory immune response in CF cells.
4.3.3 ER Stress Induction Leads to Up-Regulation of ER Stress Markers and p38-Mediated IL-6 Production

Misfolding of proteins leads to ER stress due to the formation of protein aggregates, which in turn can lead to cell stress and inflammation. The response to ER stress is mediated by the UPR and its signalling pathways, namely the IRE-1α – XBP-1, the PERK – eIF2α and the ATF6 pathways. To determine whether ER stress is inducible in CF and non-CF airway cells, IB3-1 and C38 cells were treated with 20μg/ml tunicamycin (Tm), a glycosylation inhibitor which leads to the aggregation of proteins in the ER, and the commonly accepted ER stress markers XBP-1 and Grp78 (Glucose-regulated protein, 78kDa) were monitored [208, 223]. As shown in Figure 4.3 a & b, tunicamycin increased the expression of ER stress markers in both cell lines over a 24hs time-course. Furthermore, 24hs of tunicamycin treatment induced a robust IL-6 response in both cell lines, indicating the potent inflammatory outcome of ER stress. Interestingly, the ER stress induced IL-6 production seemed to be mediated by p38 MAP kinase as pre-treatment of the cell lines with a low concentration of SB203580 (0.8μM) abrogated the IL-6 response to tunicamycin (Figure 4.3 c & d).
4.3.4 The UPR is Enhanced in CF Cells at Baseline

To confirm the expression data from the gene expression array experiments and previous reports of increased ER stress markers in CF cells [200, 201, 213], the induction of XBP-1 (XBP-1s and XBP-1u), Grp78 and CHOP at baseline in C38 and IB3-1 cells was measured using qPCR. While XBP-1 is over-expressed in IB3-1 cells, CHOP down-stream of the PERK – eIF2α pathway was down-regulated at baseline (Figure 4.4). Grp78 was not differentially expressed in IB3-1 cells compared to C38 cells. These results indicate the activation of certain arms of the
UPR, suggesting the existence of ER stress. This is in agreement with previous reports of increased ER stress markers in conjunction with the expression of mutated CFTR [201]. Furthermore, the unexpected inverse relationship between XBP-1s and CHOP expression reflects a possible dysregulation of these pathways.

Figure 4.4: The XBP-1 Arm of The UPR is Up-Regulated at Baseline in CF Cells.
ER stress and UPR markers XBP-1s, XBP-1u, Grp78 and CHOP were measured by qPCR and expression levels in CF cells were compared to non-CF cells at unstimulated conditions. SEM with 95% confidence interval (CI) is shown.

4.3.5 Pre-Existing ER Stress Potentiates the CF Inflammatory Response to Flagellin

Considering the results from Figure 4.3 & 4.4, the question arises whether baseline UPR activation contributes to the potentiated immune response to flagellin displayed by CF cells. To test this hypothesis the level of ER stress was artificially increased by pre-incubating IB3-1 cells with tunicamycin (20μg/ml) for 6hs. Subsequent stimulation with flagellin (100ng/ml) resulted in an additive increase of IL-6 cytokine production (Figure 4.5 a & b). Since ER stress can induce a pro-
inflammatory immune response, this suggests that existing and increasing perturbation of the ER can potentiate the inflammatory immune response to flagellin.

**Figure 4.5: Tunicamycin-Induced ER Stress Augments the IL-6 Response to Flagellin in CF and Non-CF Airway Epithelial Cells.**

IB3-1 (a) and C38 (b) cells were incubated with 20μg/ml tunicamycin for 6hs prior to flagellin (100ng/ml) stimulation. IL-6 protein concentrations were determined after 18hs by ELISA. Statistical analysis was performed using one-way ANOVA using Bonferroni correction for multiple comparisons. *p<0.05; ***p<0.001.
Table 4.2: GO ORA for Biological Process by DE Genes in IB3-1 Over C38 at Unstimulated Conditions.

<table>
<thead>
<tr>
<th>GOBPID</th>
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<td>GO:0032874</td>
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<th>Count</th>
<th>Size</th>
<th>%</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0080134</td>
<td>0.016</td>
<td>2</td>
<td>3</td>
<td>66.6</td>
<td>Regulation of response to stress</td>
</tr>
<tr>
<td>GO:0070374</td>
<td>0.016</td>
<td>2</td>
<td>3</td>
<td>66.6</td>
<td>Positive regulation of ERK1 and ERK2 cascade</td>
</tr>
<tr>
<td>GO:0000165</td>
<td>0.033</td>
<td>5</td>
<td>24</td>
<td>20.8</td>
<td>MAPKKK cascade</td>
</tr>
</tbody>
</table>
Table 4.3: GO ORA for Biological Process by DE Genes in IB3-1 Over C38 After Flagellin Stimulation.

### GO ORA BP ALL only under flagellin stimulation

<table>
<thead>
<tr>
<th>GOBPID</th>
<th>P-value</th>
<th>Count</th>
<th>Size</th>
<th>%</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0030970</td>
<td>3.92E-04</td>
<td>4</td>
<td>4</td>
<td>100</td>
<td>Retrograde protein transport, ER to cytosol</td>
</tr>
<tr>
<td>GO:0034612</td>
<td>0.003</td>
<td>6</td>
<td>12</td>
<td>50</td>
<td>Response to tumor necrosis factor</td>
</tr>
<tr>
<td>GO:0006983</td>
<td>0.027</td>
<td>4</td>
<td>9</td>
<td>44.4</td>
<td>ER overload response</td>
</tr>
<tr>
<td>GO:0009306</td>
<td>0.031</td>
<td>6</td>
<td>18</td>
<td>33.3</td>
<td>Protein secretion</td>
</tr>
</tbody>
</table>

### GO ORA BP FC>1 only under flagellin stimulation

<table>
<thead>
<tr>
<th>GOBPID</th>
<th>P-value</th>
<th>Count</th>
<th>Size</th>
<th>%</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0032469</td>
<td>0.030</td>
<td>2</td>
<td>4</td>
<td>50</td>
<td>Endoplasmic reticulum calcium ion homeostasis</td>
</tr>
<tr>
<td>GO:0050850</td>
<td>0.006</td>
<td>4</td>
<td>11</td>
<td>36.3</td>
<td>Positive regulation of calcium-mediated signaling</td>
</tr>
<tr>
<td>GO:0002764</td>
<td>0.025</td>
<td>3</td>
<td>9</td>
<td>33.3</td>
<td>Immune response-regulating signal transduction</td>
</tr>
<tr>
<td>GO:0045089</td>
<td>0.006</td>
<td>5</td>
<td>17</td>
<td>29.4</td>
<td>Positive regulation of innate immune response</td>
</tr>
<tr>
<td>GO:0002221</td>
<td>0.044</td>
<td>3</td>
<td>11</td>
<td>27.3</td>
<td>Pattern recognition receptor signaling pathway</td>
</tr>
<tr>
<td>GO:0000187</td>
<td>0.044</td>
<td>7</td>
<td>44</td>
<td>15.9</td>
<td>Activation of MAPK activity</td>
</tr>
<tr>
<td>GO:0043123</td>
<td>0.039</td>
<td>12</td>
<td>91</td>
<td>13.2</td>
<td>Positive regulation of I-kappaB kinase/NF-kappaB cascade</td>
</tr>
</tbody>
</table>
Table 4.4: Transcription FactorsReturned by TF ORA at Baseline and After Flagellin Stimulation.

### TF ORA ALL only returned when unstimulated

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>P-value</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CREB1</td>
<td>0.05</td>
<td>Involved in inflammation</td>
</tr>
<tr>
<td>CEBPD</td>
<td>0.004</td>
<td>Dimerization partner with CHOP</td>
</tr>
<tr>
<td>CEBPE</td>
<td>0.009</td>
<td>Dimerization partner with CHOP</td>
</tr>
<tr>
<td>DDIT3</td>
<td>0.007</td>
<td>CHOP</td>
</tr>
<tr>
<td>NFYA</td>
<td>0.01</td>
<td>Dimerization partner with ATF6, XBP-1</td>
</tr>
<tr>
<td>RELA</td>
<td>0.01</td>
<td>NF-κB</td>
</tr>
<tr>
<td>RELB</td>
<td>0.05</td>
<td>NF-κB</td>
</tr>
</tbody>
</table>

### TF ORA ALL only returned under flagellin stimulation

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>P-value</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOSL1</td>
<td>0.03</td>
<td>AP-1</td>
</tr>
<tr>
<td>FOSL2</td>
<td>0.03</td>
<td>AP-1</td>
</tr>
<tr>
<td>JUN</td>
<td>0.004</td>
<td>AP-1</td>
</tr>
<tr>
<td>TFAP2A</td>
<td>0.03</td>
<td>AP-2</td>
</tr>
<tr>
<td>RELA</td>
<td>&lt;0.001</td>
<td>NF-κB</td>
</tr>
<tr>
<td>REL</td>
<td>0.04</td>
<td>C-Rel</td>
</tr>
<tr>
<td>CEBPD</td>
<td>&lt;0.001</td>
<td>Dimerization partner with CHOP</td>
</tr>
<tr>
<td>CEBPE</td>
<td>0.01</td>
<td>Dimerization partner with CHOP</td>
</tr>
<tr>
<td>DDIT3</td>
<td>0.007</td>
<td>CHOP</td>
</tr>
<tr>
<td>NFYA</td>
<td>0.007</td>
<td>Dimerization partner with ATF6, XBP-1</td>
</tr>
<tr>
<td>ATF6</td>
<td>0.007</td>
<td>ER stress sensor</td>
</tr>
<tr>
<td>XBP1</td>
<td>0.02</td>
<td>UPR transcription factor</td>
</tr>
</tbody>
</table>
4.4 Discussion

Inflammatory lung disease is the major cause of morbidity and mortality in CF. Even though studies using global anti-inflammatories have shown their use to be beneficial for CF patients, the clinical up-take is low due to unacceptable side effects such as growth impairment, weight gain and risk of intestinal bleeding [174]. This 'proof of concept' evidence, however, has initiated many studies to understand the specific pathways mediating the increased inflammatory response to bacterial infection in CF and effectively target inflammation while avoiding unwanted side effects. This thesis and other groups have generated evidence underlining the importance of innate immune receptors, such as TLRs, in mediating inflammatory responses to *P. aeruginosa* and their role in CF [95, 99, 192]. However, the mechanism underlying the increased immune response in CF remains unknown.

This study attempted to identify pathways involved in the increased pro-inflammatory cytokine production in response to flagellin in the CF respiratory cell line IB3-1 compared with its control cell line C38. To understand the potential dysregulation of signalling events in CF versus non-CF airway cells, gene expression arrays were conducted on both cell lines at baseline and after 2hs of flagellin stimulation. Using complementary bioinformatics approaches established a comprehensive overview of transcription factors (TFs), signalling pathways and gene ontology (GO) terms over-represented by the differentially expressed (DE) genes in CF and non-CF cells. The results reflected a general theme of activated stress response mechanisms in the CF cell line that was already present at baseline, suggesting an increased level of disturbance of cellular mechanisms. In particular, ER stress response mechanisms and the UPR were indicated in the GO and TF ORA (Table 4.2-4.4), which is in agreement with previous hypotheses suggesting an increased level of ER stress potentially due to misfolded CFTR [201, 224]. In addition to these results, the data analysis confirmed an expected immunological signature represented by NF-κB and AP-1 signalling, as well as pro-inflammatory MAP kinase pathways.
Numerous studies have shown that the pro-inflammatory response to infection is dysregulated in the CF airways. The mere absence of CFTR can induce increased NF-κB signalling [225, 226], supporting the concept that CF cells are at least more responsive to inflammatory stimuli if not already inflamed in the absence of infection. Although several studies have contributed important data explaining the induction and transmission of the pro-inflammatory immune response in CF [95, 157, 196], it remains unclear what sensitizes the CF airway epithelium to produce more inflammatory cytokines than non-CF airway cells. The stress response signature returned by the gene expression arrays, prompted the investigation of the kinases involved in the transduction of the pro-inflammatory signal. In particular, the experiments in this chapter focused on p38 MAP kinase which plays a well-established role in the TLR signalling cascade [194]. Interestingly, IB3-1 cells showed a markedly increased and prolonged activation of p38 MAP kinase after stimulation with flagellin when compared to C38 control cells. This observation confirms a previous report by Bérubé et al. showing the same phenomenon in a different set of CF (CuFi) and non-CF (NuLi) cell lines [195]. Treatment of IB3-1 with the specific p38 MAP kinase inhibitor, SB203580, normalized the increased pro-inflammatory immune response in CF cells (Figure 4.2c). These data along with the reports from the literature show that p38 MAP kinase signalling is dysregulated in CF cells, leading to an increased immune response to flagellin stimulation. However, how and why p38 MAP kinase activity is dysregulated remains unknown.

Given the role of p38 MAP kinase in CF signalling and the activated ER stress response mechanisms reflected in the gene array analysis, the aim was to determine whether there is a potential link between ER stress and the pro-inflammatory signalling mediated by p38 MAP kinase. Several reports in the literature have identified ER stress as a likely contributor to inflammatory conditions as well as implicating the UPR as being involved in the production of pro-inflammatory cytokines [227]. In fact, Martino et al. has shown that the UPR (typically activated by ER stress) plays a role in the inflammatory cytokine
production in CF cells. Over-expression of a dominant negative form of \textit{XBP-1s} (\textit{dnXBP-1s}) reduced the immune response to an inflammatory CF sputum preparation [213, 228]. Although the signalling networks activated by the imbalance of the ER homeostasis are very complex, evidence suggests that the UPR can augment NF-\textit{kB} activity by several different mechanisms [229]. To test whether ER stress can lead to inflammation, CF and non-CF airway cells were stimulated with tunicamycin. As expected, tunicamycin was able to induce the UPR as reflected by the time-dependent increase in expression of \textit{XBP-1s} and \textit{Grp78}. At the same time, tunicamycin induced potent pro-inflammatory cytokine production in both cell lines. Intriguingly, the tunicamycin-induced IL-6 production was completely inhibited by a low dose of the p38 MAP kinase inhibitor SB203580 (Figure 4.3), suggesting that ER stress-induced inflammation is mediated by p38 MAP kinase.

These findings raised the question whether ER stress is up-regulated in CF cells at baseline. The ER is a sensitive organelle which is responsible for the folding and distribution of proteins. Although many disease causing mutations exist, only the expression of the class II mutation \textit{\textDelta F508 CFTR} has been reported to result in the up-regulation of ER stress markers [199, 201]; however, other class II mutants are likely to have similar effects. Furthermore, it is arguable whether the retention of CFTR alone is sufficient to cause ER stress or whether the lack of the chloride channel function alone (all classes of \textit{CFTR} mutations) can also disturb the proteostasis. The comparison of the expression of \textit{XBP-1s} in CF to non-CF airway cells confirmed significantly increased expression levels at baseline, indicating the activation of the XBP-1 arm of the UPR in CF cells at resting conditions. At the same time, \textit{CHOP} expression was decreased. While the cross-talk between UPR pathways required for the successful resolution of ER stress is poorly understood, these data indicate a potential dysregulation of these signalling cascades. Furthermore, this finding is peculiar as CHOP has been implicated in ER stress mediated IL-8 production in intestinal epithelial cells (IECs) [211]. The study demonstrates that CHOP-mediated C/EBP-\textbeta inhibition leads to decreased \textit{PPAR-\gamma}
expression and thus the lack of NF-κB inhibition [211]. This is interesting as PPAR-γ down-regulation has been reported in the context of CF. In this study, Perez and colleagues suggest that the lack of PPAR-γ expression results in increased levels of NF-κB activity [230]. In theory, the present data indicating a decreased CHOP expression in the CF cells contradicts a mechanistic link along the CHOP – PPAR-γ – NF-κB axis at baseline. However, in depth experiments are needed to further determine the role of CHOP in CF inflammatory responses at unstimulated conditions and after stimulation with flagellin and CF pathogens.

The UPR results in the activation of ER stress relief mechanisms such as ERAD which is mediated by the 26S proteasome [231] and autophagy [232-234]. If the UPR pathways are ineffective these important efflux mechanisms are not appropriately activated leading to further increased perturbation of ER homeostasis. Interestingly, protein degradation mechanisms have been shown to be dysregulated in pulmonary inflammatory conditions. In CF, induction of autophagy has been reported to be defective, and strategies to re-establish this important cellular function normalized the inflammatory phenotype of CF cells [214]. Furthermore, the TF NRF-2, which controls an anti-oxidant response and is activated upon oxidative stress, has been shown to be decreased and thus responsible for the lack of proteasome activity in COPD, leading to elevated levels of ER stress [205]. Activation of NRF-2 with the small triterpenoid CDDO relieves the increased pro-inflammatory immune response in CF cells [212], indicating a potentially inadequate level of proteasome activity. Given this evidence, in conjunction with the folding mutation in the CFTR gene, it is likely that CF cells experience an increased level of stress on the ER at resting conditions.

As this role of ER homeostasis and the UPR becomes evident in the CF inflammatory response, I hypothesize that the baseline activation of the UPR
potentially sensitizes the CF cells to infectious stimuli. To test this hypothesis, ER stress was increased in CF cells by incubating them with tunicamycin for 6hs. Subsequent stimulation with flagellin resulted in a potentiated immune response in the CF cells. Although this response did not seem to be synergistic but rather additive, it supports the hypothesis that baseline UPR activity potentially worsens the outcome of pro-inflammatory signalling to flagellin. The fact that p38 inhibition decreases both ER stress-induced and TLR5-mediated inflammation, indicates that both pathways utilize p38 MAP kinase to induce IL-6 production. The data in Figure 4.4 suggest that the UPR is activated at baseline in CF cells, potentially due to increased ER stress. Thus increased ER stress levels may lead to the additional signal resulting in the prolonged and increased activation of p38 MAP kinase in CF airway epithelial cells. However, further experiments determining the level of ER stress in CF cells and the detailed signalling mechanism leading to the sensitization of the CF airway epithelium to infections with *P. aeruginosa* are needed to substantiate these findings.

Taken together, this study and reports from the literature indicate that it is likely that ER stress is present at baseline in CF cells. While this study design does not explain the reason for increased UPR activity, it provides insight into the mechanisms of increased inflammatory responses to TLR5 stimulation. The data presented in this chapter suggest p38 MAP kinase as a potential molecular link between ER stress and pro-inflammatory signalling, leading to the excessive immune response seen in CF airway epithelial cells after flagellin stimulation. Further experiments investigating the cross-talk between the UPR pathways and their activation, will contribute to the understanding of the role of proteostasis in CF inflammatory responses. Finally, once the mechanism underlying the activated ER stress response is understood, strategies to restore ER homeostasis and alleviating ER stress may prove useful in controlling the excessive pro-inflammatory immune response in the CF airways.
5. Conclusions and Future Directions

Chronic CF airway disease is characterized by a vicious cycle of pulmonary obstruction, infection and inflammation. As a result, lung insufficiency ultimately leads to the necessity of a lung transplant or death. Although the current median age of survival in the Canadian CF population is 47 years [3], the long and frequent hospitalizations are a significant burden on the patient, the social and economic environment, and the health care system. To increase life quality and life expectancy, it is crucial to find new therapies to treat CF lung disease. A multitude of therapeutic approaches such as mucolytics, physiotherapy and antibiotics are in use, however no effective option to treat the inflammatory component of CF lung disease is currently available.

While CF affects many organs, lung disease is the major cause of morbidity and mortality. The origin of the uncontrolled inflammation remains elusive to date, and the host-pathogen interactions between CF pathogens such as \textit{P. aeruginosa} and the airway epithelium are poorly understood. The number of studies highlighting the importance of PRRs as the first line of contact between the pathogen and the host, was the motivation to investigate the role of TLRs expressed by CF and non-CF respiratory cells [98-100, 105, 157, 194]. Using complementary model systems, the present thesis identifies TLR5 as the receptor recognizing and mediating much of the exaggerated immune response to \textit{P. aeruginosa}, adding important evidence to how excessive inflammatory responses are mediated by CF airway cells. Initially, LPS was predicted to be the major driver of the TLR-mediated inflammation to \textit{P. aeruginosa}. However, as discussed in Chapter 2, evidence suggests that TLR4 co-receptor molecules are minimally expressed in the human lung, thus reducing TLR4 activity [133-135]. Data derived from TLR KO mice, further underscore the complexity of \textit{P. aeruginosa} recognition by the airways. Together, these studies reveal that an orchestrated engagement of several TLRs is likely to be required to effectively protect the host and clear the pathogen. While \textit{P. aeruginosa} expresses
a variety of TLR ligands, the data presented in Chapter 2 and 3 strongly suggest that immortalized CF airway cells rely on TLR5 to mediate a pro-inflammatory immune response (Figure 2.7, Figure 3.1). On the contrary, a non-redundant role of TLR5 in recognizing flagellated bacteria seems biologically impractical, since failure of this mode of recognition could potentially lead to unopposed pulmonary infections. To confidently rule out possible contributions from other TLRs to host responses, experiments using primary cells derived from CF lung samples are needed. It is possible that these experiments will prove that a collaborative engagement of several TLRs is needed to mount an appropriate immune response leading to host protection and removal of the pathogen.

Although the data shown in Chapter 2 and the multiple lines of evidence from the literature underscore the role of TLR5 in innate immune recognition of \( P. \ aeruginosa \), it is not known whether TLR5 contributes to CF disease severity in vivo. Genetic variants (such as SNPs) capable of modifying the outcome of a disease provide a powerful strategy to investigate whether certain genes and proteins are important in the context of a disease condition. Interestingly, adult patients carrying a SNP rendering TLR5 mostly inactive (45.5-76.3% decreased flagellin responsiveness, Figure 3.2) have a significantly higher ideal body weight further supporting the role of TLR5 signalling in the pathophysiology of CF. Although the relationship between metabolism and inflammation is poorly understood, this study indicates a significant role of TLR5 in CF disease. While these findings contribute novel evidence that strategies to inhibit TLR5 may improve the disease outcome of CF patients, replication studies are needed to validate the effect of TLR5 variants on CF disease severity.

In addition to its role in pro-inflammatory signalling, it was recently suggested that TLR5 has important cytoprotective functions. Indeed, Vijay-Kumar et al. hypothesized that flagellin-mediated TLR5 activation protected mice against
numerous insults including infection, radiation and chemical challenge [235]. Another study supports these findings as activation of TLR5 and NF-κB signalling protected mice from lethal doses of radiation (sufficient to induce severe acute radiation syndromes) by inhibiting apoptosis and inducing survival [236]. This protective and homeostatic function of TLR5 is also reflected by its importance in gut epithelial homeostasis [237] and thus needs to be carefully considered when designing an anti-inflammatory therapy against TLR5 signalling.

The third part of the present thesis aimed to understand the molecular events leading to the dysregulated and inappropriate immune response in the CF lung. Global gene expression arrays combined with network analysis were used to establish a profile of potentially dysregulated signalling mechanisms in the CF airway cells (Figure 4.1, Tables 4.2-4.4). Globally, the experiments identified a signature of elevated stress levels in CF cells. While stress can originate from a multitude of sources, different strategies of over-representation analysis implicated up-regulation of ER stress mechanisms and MAP kinase pathways in CF compared to non-CF cells. The cell based experiments represented in Figure 4.2 confirmed these data and demonstrated dysregulation of the p38 MAP kinase, an observation that has recently been reported using a different model system [195]. In addition to p38 MAP kinase dysregulation, the XBP-1 arm of the UPR was upregulated while the CHOP expression was markedly decreased in CF cells at baseline. These findings indicated that ER stress is potentially existent at baseline while the UPR is differentially activated. Further induction of ER stress using tunicamycin led to significantly increased pro-inflammatory signalling. Given these observations, the data shown in Figure 4.3 identifies p38 MAP kinase as a protein potentially linking intrinsic ER stress signalling to inflammatory pathways, leading to increased IL-6 production. These results provide evidence that the potential perturbation of the ER (evident by activation of the UPR) leads to the inherent dysregulation of signalling events in CF airway epithelial cells resulting in increased pro-inflammatory immune responses.
Cells organized into a biologically functional unit have to maintain a homeostatic equilibrium to perform their highly specific tasks. Cellular homeostasis is dependent on functional proteins – the ‘working units’ of the cell. Maintaining a functional proteome (proteostasis) relies on correct and efficient protein production, folding, and degradation [238]. The balanced regulation of these events creates a network of molecular functions, which together comprise a functional cellular system. This network is presumably dynamic and agile in order to balance disturbances caused by insults to cellular functions. Thus the perturbation of proteostasis activates different UPR pathways with the goal to re-establish the ‘steady state’ of the network by carefully adjusting protein production, folding and degradation [239]. The interplay of these proteostatic control pathways is crucial in maintaining cellular homeostasis. Prolonged challenge or failure of any of the response mechanisms can compromise the ability of the cell to cope with stress conditions appropriately, potentially leading to cellular malfunctions [238]. If the cell is unable to return to a healthy state again, it may undergo apoptosis to minimize further damage [202].

Proteostasis is an emerging area of research interest. The decline of proteostatic control, in combination with intrinsic genetic (i.e. SNPs or mutations) or environmental (infection) stress factors, potentially leads to increased susceptibility to diseases, offering an interesting explanation for the often late onset of certain disease conditions [202]. Thus, the age related inability to clear oxidized protein aggregates can lead to the destabilization of cellular homeostasis, which is associated with a variety of neurodegenerative diseases and type II diabetes [202, 238, 240]. Interestingly, insulin resistance, a component of the metabolic syndrome often leading to type II diabetes, is associated with perturbed proteostasis, indicating a strong causative relationship between the imbalance of the ER and disease onset and severity [241].
Since proteostasis is controlled by a large and complex signalling network which has been shown to exhibit cross-talk with pro-inflammatory signalling pathways [242], imbalance in proteostasis may result in inflammatory responses. Indeed, recent evidence suggests that the accumulation of protein aggregates can augment inflammation and NF-κB activity in the context of aging [203] and pulmonary conditions such as COPD. The airway inflammation seen in COPD is associated with low NRF-2 activity, decreased proteasome activity, high levels of oxidative stress, ER stress, and increased apoptosis [205, 243], all events that are potentially associated with an imbalance of ER homeostasis. In contrast, activation of NRF-2 in COPD, leads to increased proteasome activity, decreased apoptosis and resolved ER stress [205]. Interestingly, the age-related decline in homeostatic functions, in conjunction with the presence of environmental pollutants (i.e. cigarette smoke), significantly worsened the pathology of COPD [204]. Taken together, these studies indicate that the accumulation of environmental or intrinsic burdens on the cell can impede the mechanisms necessary to maintain functionality and lead to an inflammatory phenotype.

While it is not known whether restoration of proteostasis resolves inflammation in COPD, parallels can be drawn to CF. It has been shown that NRF-2 is also dysfunctional in CF, resulting in the reduced ability of cells to deal with oxidative stress-induced inflammation [244]. Furthermore, important mechanisms involved in proteostasis, such as autophagy, are down-regulated [214], as is the expression of regulatory subunits of the proteasome [200]. Intriguingly, induction of NRF-2 or autophagy leads to a decrease in inflammation [212, 214, 244, 245], indicating that potential disruption of proteostasis contributes to the inflammatory phenotype seen in CF airway cells. In light of these findings, the folding defect and loss of chloride channel function caused by CFTR mutations, potentially present additional stress factors contributing to the disruption of the ER homeostasis leading to inappropriate signalling events.
The question arises whether any cell expressing mutant-\textit{CFTR} experiences ER stress and thus displays a hyper-inflammatory phenotype? Of note, low levels of ER stress do not necessary induce inflammation. It is more likely that a combination of endogenous and exogenous stresses accumulate to result in ER stress-mediated pro-inflammatory signalling events. The hypothesis that potential ER stress is the reason for a hyper-inflammatory phenotype in CF needs to be further investigated and verified in primary cell models (see below). However, given the data generated from CF patient-derived PBMCs (Figure 2.4) and the growing body of literature suggesting ER stress as pro-inflammatory signal, indicates, that cells other than AECs potentially experience the same phenomenon. It is not known whether activation of the UPR potentially contributes to the manifestation of CF in other epithelial tissues such as the intestine or the pancreatic duct. Intriguingly, ER stress and malfunctions in the UPR are strongly associated with inflammatory bowel disease (IBD) [246]. The intestine is constantly challenged with microbes which live in a balanced symbiosis with the human host. Therefore, intestinal homeostasis and epithelial cell integrity is essential in protecting the host from infection. When homeostatic mechanisms such as the UPR fail, inflammatory phenotypes are likely to emerge. Thus, mice deficient in IRE1 or mice exhibiting decreased activity of the ATF6-pathway, show increased susceptibility to DSS-induced colitis [247-250]. Furthermore, SNPs in the \textit{XBP-1} gene as well as \textit{ORMDL3}, a gene involved in the regulation of the PERK pathway and Ca\textsuperscript{2+}-signalling, are associated with Crohn's disease, ulcerative colitis and asthma [247, 249, 251, 252]. These studies indicate that genetic variants rendering ER stress responses dysfunctional can, possibly due to the additional burden of infectious signalling, result in inflammatory phenotypes. Intestinal inflammation can be observed in CF patients, but only few cases of IBD have been reported [253]. Nevertheless, with the increasing life expectancy, IBD cases in CF are expected to increase [253]. In summary, it can be concluded that the expression of mutant-\textit{CFTR} presents an endogenous stress burden onto the cell which, in combination with other, possibly environmental stresses can lead to inflammatory responses.
Taken together, this thesis and recent reports from the literature indicate an emerging paradigm that reflects the complex relationship between maintaining cellular functions during external and internal stress, and subsequent cellular behaviour including host responses. If the adverse conditions increase and eventually exceed the capability to cope, the cell may undergo apoptosis to prevent further damage. However, between a healthy state and controlled cell death is a wide and dynamic range of stress levels with a multitude of phenotypic outcomes (Figure 5.1). While stress may originate from senescence, protein dysfunctions (due to mutations) or environmental challenges (such as infections) all may result in a certain level of destabilization of the cell’s functionality. In CF, many studies have indicated mechanisms that potentially result in an increased burden on the ER, sensitizing the cell to further adverse events. The data showing a potentiated inflammatory response to flagellin in the presence of ER stress (Chapter 4) support this hypothesis. It suggests that increased burdens (loss of chloride channel function, folding defect, chronic infections) on the cellular homeostatic control alters the response to environmental signals such as chronic infection with *P. aeruginosa*, leading to uncontrolled and inappropriate immune responses.

In the present thesis, ER homeostasis and TLR5 signalling were identified as two mechanisms to target for treatment of CF lung disease. Based on the data shown in Chapter 2 and 3, interfering with flagellin-driven TLR5 signalling is an attractive option to control *P. aeruginosa*-induced inflammation. While it is hard to alter flagellin levels in the CF lung, it is likely to be more feasible to target TLR5 directly, for example by using a nebulized neutralizing antibody. However, it is not known whether the mucopurolent and proteolytic environment in the airways might interfere with successful delivery. Thus, development of a small molecule inhibitor against TLR5 signalling might prove more efficient. Furthermore, as described in Chapter 3, it is likely that neutralizing TLR5 activity may improve important clinical outcome measures such as BMI. While this approach is very specific and is likely to avoid unwanted side effects seen when using global anti-inflammatories, it may only be
effective in reducing inflammation triggered by flagellated bacteria. Nevertheless, given the prevalence and clinical significance of the two most common flagellated pathogens in CF (*P. aeruginosa* and species of the BCC), this approach may prove useful in improving lung disease.

**Figure 5.1: Proteostasis – Disease Paradigm.**

In contrast to inhibiting TLR signalling, modifying the ER stress response has received considerable attention as a therapeutic target in the context of a variety of diseases such as cancer and diabetes. Thus, the implication of the UPR in metabolic functions, suggests these pathways as attractive therapeutic targets and treatment options for insulin resistance and diabetes [241]. Furthermore, activation of the UPR is believed to contribute significantly to the survival of tumor cells under hypoxic conditions, indicating an important role of ER stress in tumorgenesis [254, 255]. These novel insights have prompted several studies investigating UPR inhibitors and ER stress modulators as cancer therapeutics [256-258]. This knowledge provides a substantial foundation for the development of potential new therapies. The implication of ER stress response mechanisms in inflammation and infection presents an attractive point of intervention, since a broad spectrum of infectious and inflammatory conditions could be targeted by ER stress modulators [202, 203, 227]. However, in appreciation of the complexity of ER stress signalling and its regulatory role in cellular functions, it is important to gain a more comprehensive understanding of the functions of each pathway involved in the ER
response and UPR. Taken together, while a significant amount of research is necessary to verify potential drug targets, both approaches present attractive options for anti-inflammatory therapy in CF.

**Future Directions**

In order to validate the therapeutic targets identified in the present studies, several mechanistic questions need to be answered. As it is suggested that ER stress contributes to the increased TLR5-mediated immune response to *P. aeruginosa*, the signalling events leading to this outcome need to be understood in further detail. Thus, the individual contribution of each UPR pathway to inflammation in CF needs to be understood. Determining the activation of each arm of the UPR in CF cells, would provide valuable information about the orchestrated response of these pathways and, since each pathway has different functional outcomes, may indicate what triggers ER stress in CF cells. Furthermore, inhibition of each arm of the UPR individually while monitoring inflammatory responses will show which pathway plays a role in the excessive inflammation seen in CF cells. In addition, simultaneous measurement of the activity of the other UPR pathways will indicate whether these pathways communicate with each other. If data derived from these experiments substantiate the hypothesis that ER stress is induced and the UPR is dysregulated in CF cells, strategies to alter UPR activity may prove useful in controlling inflammation. Thus, monitoring inflammation as well as ER stress markers while treating CF cells with UPR manipulating compounds such as salubrinal [259], may provide novel insight in the treatment options of CF airway inflammation. Once these details are elucidated, we can hypothesize whether specific inhibition or activation of one or more UPR pathways can restore ER homeostasis and thus decrease inflammation.

While several partial links between proteostatic control and inflammation have been established in CF [212, 214, 230], none is directly associated with ER stress. The
decrease of inflammation upon NRF-2 activation in CF cells [212], is potentially mediated by the proteasome-dependent relief of ROS-induced ER stress. Furthermore, it has been shown that activation of autophagy decreases inflammation in CF [214]. Thus, the activation of autophagy may lead to a more balanced ER homeostasis with the secondary effect of normalizing inflammatory responses. Finally, recently it was reported that incubation of CF cells at 27°C results in increased CFTR surface expression [260] and decreased inflammatory responses to P. aeruginosa diffusible material [103]. Conducting these same experiments and monitoring expression of ER stress markers will validate whether ER stress contributes to the increased inflammation in CF as well as add evidence that expression of mutated CFTR can lead to disturbances in ER homeostasis.

While model systems such as immortalized cell lines are useful in investigating biochemical mechanisms leading to the hyper-inflammatory phenotype, primary cells resemble most closely the in vivo situation of CF and are therefore a desirable model system. Given access to primary CF lung tissue, I propose several experiments to validate the in vitro findings of this thesis:

a) **Expression of TLRs in human CF lung tissue.**

Immunostaining of CF lung tissue samples for TLRs would generate important information on what TLRs are expressed in the CF lung at baseline and during infection.

b) **Validation of the role of TLRs in mediating the immune response to P. aeruginosa.**

Stimulation of primary CF airway epithelial cells with purified TLR ligands will indicate which TLRs are mediating the immune response in CF airways. Neutralization or silencing of TLRs expressed in the epithelial cells and subsequent stimulation with CF pathogens (and purified TLR ligands), will determine which TLRs mediate inflammatory responses in CF airways.
These experiments are essential before any therapeutic strategy of TLR inhibition should be pursued.

c) **Validation of the presence of ER stress and its role in CF airway inflammation.**

CF primary airway epithelial cells as well as lung tissue sections will be useful to verify the presence of ER stress. Histological staining for common ER stress markers and UPR proteins such as XBP-1s, CHOP or ubiquitinated proteins within the ER, will prove whether expression of mutant CFTR leads to the perturbation of proteostasis and activation of ER stress responses.

d) **Therapeutic value of manipulating ER stress response mechanisms.**

Finally, primary epithelial cells present an attractive model to investigate whether manipulation of one or more specific UPR pathways is useful in controlling pulmonary inflammatory responses to *P. aeruginosa*. In addition to inflammatory markers, monitoring ER stress markers will indicate whether manipulation of UPR pathways can restore proteostasis in CF cells.

In summary, it is essential to determine the level of perturbation of the proteostasis and the trigger activating the UPR in CF. Furthermore, validation of the role of the UPR in inflammation is important to identify specific targets for anti-inflammatory therapy.

CF is an orphan disease in that compared to other illnesses, such as cancer or diabetes, only a small number of people are affected. However, as an inflammatory disorder, the potential parallels with other inflammatory diseases ensures that new insights into CF inflammation stand to benefit a much broader population by serving as a basis for research and development of novel anti-inflammatory therapies. In conclusion, I identified TLR5 as an important mediator of pro-inflammatory signals to *P. aeruginosa* and validated the importance of TLR5 in CF disease severity.
Furthermore, the data presented in this thesis provide evidence that ER stress signalling is potentially responsible for the observed increased inflammatory response to pathogens such *P. aeruginosa* in CF. Thus, strategies to inhibit either TLR5, the receptor mediating much of the inflammatory response to *P. aeruginosa*, or modify ER homeostatic signalling may prove useful in alleviating pulmonary inflammation in patients suffering from CF lung disease.


