This is to certify that the
thesis entitled

Exposure to P. aeruginosa and purified LPS alter CFTR-dependent ion conductance in cultured 2WT2 epithelial cells in a time and dose dependent fashion.

presented by

Michael D. Haenisch

has been accepted towards fulfillment
of the requirements for the

Master of Science degree in Physiology

Major Professor's Signature

5/12/09

Date

EXPOSURE TO P. AERUGINOSA AND PURIFIED LPS ALTER CFTR-DEPENDENT ION CONDUCTANCE IN CULTURED 2WT2 SWPITHELIAL CELLS IN A TIME AND DOSE DEPENDENT FASHION

By

Michael D. Haenisch

A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Physiology

2009

Abstract

EXPOSURE TO P. AERUGINOSA AND PURIFIED LPS ALTER CFTR-DEPENDENT ION CONDUCTANCE IN CULTURED 2WT2 WPITHELIAL CELLS IN A TIME AND DOSE DEPENDENT FASHION

By

Michael D. Haenisch

The most common heritable genetic disease in America, cystic fibrosis (CF), is caused by mutations in the CF transmembrane conductance regulator (CFTR), a chloride channel that interacts with and regulates a number of other proteins. Pseudomonas aeruginosa infects 80% of patients. It is not known how mutations in the chloride channel allow for colonization of the majority of patients by a single pathogen. The hypothesis that CFTR interacts with toll-like receptor 4 (TLR4) to phagocytise bacteria was tested. A competitive antagonist of TLR4 did not alter the rate of phagocytosis of P. aeruginosa by cultured epithelia; however, bacteria formed antibiotic resistant microcolonies in these experiments meaning the actual rate of phagocytosis was not measured. Cultured epithelia treated with P. aeruginosa had increased CFTR channel activity. Lipopolysaccharide (LPS) purified from P. aeruginosa also increased CFTR channel activity in a dose dependent manner; however, shorter exposure to LPS resulted in reduced CFTR channel activity. This data is consistent with a model in which CFTR is removed from the plasma membrane during phagocytosis of P. aeruginosa followed by recruitment of the channel to the membrane to replace channels removed during phagocytosis. More studies are needed to confirm this model, but this is the first report of a bacterial product causing a biphasic time-dependent and a dose-dependent alteration of CFTR channel activity.

Dedicated to everyone who gave me a shot: first second or otherwise.

TABLE OF CONTENTS

List of Figures

Key to symbols and abbreviations

CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
ΔF508	Deletion of phenylalanine at position 508
G542X	changed to at position 542
N1303K	changed to at position 1303
G551D	changed to at position 551
W1282X	changed to at position 1282
mRNA	Messenger ribonucleic acid
ATP	Adeninenucleotide triphosphate
PKA	Protein kinase A
cAMP	Cyclic adeninenucleotide monophosphate
ENaC	Epithelial sodium channel
AP-2	Adaptor protein 2
EBP50	Ezrin binding protein 50
ERM	Ezrin radixin moesin
ASF	Airway surface fluid
Ig	Imunoglobin
IL	Interleukin
TNF	Tumor necrosis factor
PAMP	Pathogen associated molecular pattern
TLR	Toll-like receptor
LPS	Lipopolysaccharide

APC	Antigen presenting cell
NFκB	Nuclear factor kappa B
°C	Degrees centigrade
MKLPS	Mutant pentaacylated lipopolysaccharide
MIC	Minimum inhibitory concentration
MOI	Multiplicity of infection
CFU	Colony forming units
S9	Human bronchial epithelial cells expressing normal CFTR
2WT2	C127 cells expressing normal CFTR
C127	Immortalized mouse epithelial cells
GFP	Green fluorescent protein
125-I	Iodine 125 (radioisotope)
IBMX	Isobutylmethylxanthine
AUC	Area under the curve
FBS	Fetal bovine serum
BSA	Bovine serum albumin
G418	Geneticin
EDTA	Ethylenediaminetetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
TSB	Tryptic soy broth
LB	Lysogeny broth
DMEM	Dulbeco's modified eagle medium
PBS	Phosphate buffered saline

ATCC	American type culture collection
SEM	Standard error of the mean
rpm	Revolutions per minute
OD600	Optical density at 600nm light
DAP	Diaminopimelic acid
r	Rate constant
R1	Percentage of total iodide remaining at time 1
R2	Percentage of total iodide remaining at time 2
t1	Time 1
t2	Time 2
ANOVA	Analysis of variance
min^-1	Per minute (unit of rate)
CPM	Counts per minute
min	Minute
ISE	Ion selective electrode
T	Threonine
R	Arginine
L	Leucine
Na+	Sodium ion
Cl-	Chloride ion
H2O	Water
TJ	Tight junction
N,K-pump	Sodium / potassium ATPase

man	Mannose
rha	Rhamnose
abe	Arabinose
gal	Galactose
P	Phosphate group
gln	Glucosamine
O	Oxygen
HN	Amino group
Ci	Curie
n	Number of replicates
MRSA	Methycilin resistant staphalococcus aureus

INTRODUCTION

Cystic fibrosis (CF) is the most common fatal genetic disease in the United States with an incidence of approximately 1 in 2,500 and a carrier frequency of about 1 in 20 individuals (Kerem *et al*, 1989; Welsh and Smith, 1995; Mateu *et al*, 2002). Without treatment, patients die in early childhood; however, with aggressive supportive therapy, patients now have a life expectancy over 30 years (Davis, 2006). The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), and to date over 1000 mutations have been identified that cause the disease (Mateu *et al*, 2002). A single mutation, a deletion of a phenylalanine residue at amino acid position 508 (ΔF508), accounts for 2/3 of global CF chromosomes, and only four other mutations, G542X, N1303K, G551D and W1282X, have frequencies over 1% (Mateu *et al*, 2002). The high prevalence a single gene causing this autosomal recessive disease has led many to speculate that heterozygotes bearing a single copy of the mutant allele may have had a protective advantage that caused the gene to be selected for, in much the same way as sickle cell anemia (Romeo *et al*, 1989; Pier, 1999). Although many questions still remain unanswered, research into this field has unearthed a wealth of information about cell and organismal physiology, and host-pathogen interactions.

HISTORY

First descriptions

CF was first described as a pathological condition based on autopsies done by Dorothy Andersen in 1938. She identified obstructed pancreatic ducts as a difference between what became known as cystic fibrosis of the pancreas and celiac syndrome (Andersen, 1938). In 1946, the disease was further described as an autosomal recessive disorder and in 1948 it was discovered that CF patients presented with abnormally high concentrations of sodium chloride in their sweat (Andersen and Hodges, 1946; diSantAgnese *et al*, 1953). This discovery laid the basis for the use of the sweat chloride test for the diagnosis of CF, allowing for the disease to be detected earlier in life and in patients with less severe phenotypes (Davis, 2006; Welsh and Smith, 1995). This allowed monitoring and treatment to begin earlier, thus improving the life expectancy of patients. Before the mutant gene was discovered, several groups described the epithelium from CF patients as having decreased chloride permeability and increased sodium reabsorption, two of which described chloride impermeability in respiratory epithelium, and another in sweat ducts (Quinton, 1983; Knowles *et al*, 1983; Boucher *et al*, 1986).

Discovery of the gene and molecular biology

When the gene was identified in 1989, it was named the cystic fibrosis transmembrane conductance regulator for its ability to regulate ion transport (Kerem *et al*, 1989; Riordan *et al*, 1989; Rommens *et al* 1989). A multi-group collaboration led to the discovery of a 180,000 base pair gene on the long arm of chromosome 7 with an open reading frame encoding a 1480 amino acid gene product. The group also identified the ΔF508 mutation (a deletion of three base pairs resulting in a deletion of a phenylalanine residue at position 508 in a predicted nucleotide binding domain) which accounted for 70% of mutations in mRNA transcripts isolated from CF patients (Rowe *et al*, 2005). Analysis of the amino acid sequence revealed two predicted membrane spanning domains, two nucleotide binding domains similar to those found in the ATP-binding cassette superfamily of proteins, and a central regulatory domain with phosphorylation sites for protein kinase A and protein kinase C (Figure 1) (Riordan *et al*, 1989).

The protein is highly expressed in the apical membrane of epithelial cells of the airway, sweat and pancreatic ducts, vas deferens, intestine and biliary tree (Davis, 2006). It is not surprising therefore that these are the areas and organs most effected by the disease, and many of the aspects of the disease can be explained by a secretory defect caused by lack of functional CFTR. Normally the protein is formed in the rough

endoplasmic reticulum where it is glycosylated and transported to the Golgi apparatus (Gelman and Kopito, 2002). The sugar residues are shortened at the Golgi apparatus and the protein trafficks to the plasma membrane (Gelman and Kopito, 2002). Here it is concentrated in lipid rafts and clathrin coated pits until it is removed from the membrane (Kowalski and Pier, 2004; Dudez *et al*, 2008; Bradbury *et al*, 1994; Weixel and Bradbury, 2001). CFTR is removed from the plasma membrane in a clathrin dependent manner, and the ΔF508 CFTR is sorted to early endosomes in a Rab5 dependent manner (Gentzsch *et al*, 2004). After removal to an endosome, ΔF508 CFTR is either sent to the golgi, degraded, or recycled back to the membrane (Gentzsch *et al*, 2004).

At the membrane, CFTR performs several functions. Its best described function is as a cyclic adenosine monophosphate (cAMP) activated chloride channel, but there is strong evidence that it also has the ability to interact with and regulate other proteins. Not only may CFTR regulate the activity of other ion channels such as the epithelial sodium channel (ENaC), it also appears to interact with the cytoskeleton, several other structural and trafficking proteins, and it has been suggested to regulate vesicle trafficking within the cell (Kunzelman, 2001; Gentzsch *et al*, 2004). While the body of evidence suggests that the first nucleotide binding domain of CFTR is important for its regulation of ENaC, the other interactions are believed to be mediated through a PDZ

domain interaction sequence found at the C-terminus (Schreiber *et al*, 1999; Rowe *et al*, 2005). This sequence was shown to interact with adaptor protein 2 (AP-2) and ezrin binding phosphoprotein 50 (EBP50) (Kunzelman, 2001; Gentzsch *et al*, 2004). AP-2 is involved in assembly of cargo into clathrin coated vesicles (Gentzsch *et al*, 2004; Conner and Schmid, 2003; Traub, 2003; Motley *et al*, 2003). EBP50, a member of the ezrin radixin moesin (ERM) family of proteins, has been shown to interact with polarized actin filaments to maintain formation of polarized cellular structures (Kunzelman, 2001; Morales *et al*, 2004).

CLINICAL PERSPECTIVE

Presentation and symptoms

The disease is characterized by elevated sweat chloride levels, pancreatic insufficiency, intestinal obstruction, congenital bilateral absence of the vas deferens, and lung disease with frequent microbial infections and bronchiectasis (Davis, 2006). A secretory defect explains many manifestations of the disease such as the failure of electrolyte transport responsible for the lack of resorption of sodium chloride from the sweat duct and the failure to secrete fluid into the duct of the pancreas leading to improper secretion from that organ, pancreatitis and digestive deficiencies (Davis, 2006).

Several hypotheses have attempted to explain how CF lung disease could also be caused by a secretory defect, but the situation in the lung is more controversial and will be discussed in a separate section.

The most pronounced feature of the disease, pancreatic insufficiency, typically becomes evident by 6 months of age due to failure to thrive or menconium ileus, an intestinal blockage (Lyczak *et al*, 2002). In this early stage, intestinal blockage is the most common cause of death while death due to respiratory infections and associated respiratory collapse becoming more common in older patients (Andersen, 1938; Davis, 2006).

The leading cause of death in CF patients is lung disease (Davis, 2006). Approximately 80-95% of CF patients become infected with *Pseudomonas aeruginosa* and die from sepsis or respiratory failure caused by chronic respiratory infections (Lyczack *et al*, 2002). Early in life, the lungs of CF patients become colonized with a narrow range of opportunistic microbial pathogens (Chmiel and Davis, 2003). The progression of these infections is curious because of its persistence and the narrow range and temporal progression of colonization by a few specific species (Figure 2). Immunity is clearly compromised although the reasons for this are a topic of debate. Despite vigorous use of antimicrobial treatments, patients continue to maintain or relapse into

infection (Chmiel and Davis, 2003). The mucus of the airways thickens and cannot be cleared properly (Davis, 2006). The damage caused by inflammation from chronic infection and the buildup of debris eventually causes clogging of small airways (Davis, 2006). Clinically, this progression leads to fatal incidence of sepsis or respiratory collapse (Davis, 2006). What is not well known is the mechanism by which defective CFTR allows for these frequent and persistent microbial infections.

Treatments

Although the pancreatic insufficiency can be treated with enzyme replacement therapy and nutritional supplementation, approximately 20% of children and 40% of adults with CF can be diagnosed as having nutritional failure (Davis, 2006). This leads to a generalized failure to thrive as well as several specific nutritional deficiencies. Despite supportive nutritional therapies, the pancreas is still damaged in CF patients which can lead to insulin dependent diabetes as the endocrine functions of this organ are impaired (Davis, 2006).

The goals of treatments for pulmonary complications are to eradicate bacteria, reduce inflammation, and clear the airways of mucus to enhance pulmonary function (Davis, 2006). Antibiotic chemotherapy is used to treat recurrent infections; however,

despite vigorous administration, many patients are unable to clear infections of *P. aeruginosa* and *B. cepacia* which are broadly resistant to antibiotics (Hart and Winstanley, 2002). It is accepted that the intense inflammation that occurs in the lungs of CF patients contributes to the decline in pulmonary function, but clinical trials have yet to demonstrate an anti-inflammatory treatment regimen that results in long-term improvements in pulmonary function (Balfour-Lynn and Dinwiddie, 1996). Clearance of mucus is achieved by a variety of techniques aimed at detaching mucus and lowering its viscosity. Chest percussion and forced coughing and breathing are the traditional techniques used for breaking up tightly adhered mucus, but work has been done to develop rapid chest compression vests and breathing apparatuses for more effective and widely available use (Welsh and Smith, 1995). Inhalation of nebulized DNase has been used to lower the viscosity of CF mucus and several attempts have been made to develop treatments with inhaled compounds that increase osmosis and thus lower mucus viscosity (Jaques *et al*, 2008).

The research community is also still trying to find ways to rescue CFTR before it is destroyed within the epithelial cell. CFTR with the ΔF508 mutation can still function as a chloride channel, but rarely gets a chance to do so since it is detected by molecular chaperones and sent to the proteosome for degradation (Sun *et al*, 2008). Many still hope

to find a compound that can be used to inhibit these chaperones and thus allow the ΔF508 CFTR to reach the plasma membrane where it could function normally. However, the loftiest treatment goal is the development of a gene therapy method that could be used to replace or complement the mutant gene, allowing cells to express wild-type protein (Wison, 1995). Work continues and treatments continue to improve, but it may still be some time until anything approaching a true cure is found.

INNATE IMMUNITY

The innate immune system comprises the elements of immunity that are constitutively present before the activation of adaptive immunity which involves activation of T and B lymphocytes and antibody production (Bals *et al*, 1999). This system is linked to adaptive immunity through its determination of antigen presentation (Fearon and Locksley, 1996).

In humans, approximately 10mL of fluid cover the surface area of the lung which is about the area of half a soccer field (Bals *et al*, 1999). This airway surface fluid (ASF) is comprised of a layer of mucus that is positioned on top of an aqueous layer. Secretions from mucus glands, goblet cells, clara cells, and type II pneumocytes comprise the upper layer and contain an array of antimicrobial compounds and peptides including defensins,

cathelicidins, lactoferrin, lysozyme, complement, secretory IgA, protease, and phospholipase A2 (Donaldson and Boucher, 2006; Bals *et al*, 1999; Hiemstra, 2007; Gerritsen, 2000). Microbes are captured in the overlying mucus layer and removed from the lung by movement of the mucus by cilia positioned on the underlying epithelium. This system is effective enough to maintain sterility in the lung despite colonization of the upper airway (Hart and Winstanley, 2002).

Epithelial cells, alveolar macrophages and neutrophils are considered part of the innate immune system (Gerritsen, 2000; Bals *et al*, 1999). They act not only to block or destroy pathogens, but also to activate and coordinate other aspects of the immune response (Unanue and Allen, 1987; Zhang *et al*, 2000). This later activity is mediated through the secretion of inflammatory cytokines such as interleukin-1 (IL-1), IL-8, IL-10, and tumor necrosis factor α (TNFα) (Zhang *et al*, 2000; Bals *et al*, 1999; Hart and Winstanley, 2002). Secretion of these compounds is associated with an influx of macrophages and neutrophils into the infected area (Zhang *et al*, 2000). Eventually adaptive immunity becomes activated and patients produce antibodies to *P. aeruginosa* (Unanue and Allen, 1987). Despite this response, patients fail to clear bacteria, but the reason why remains unclear.

Pathogen recognition in innate immunity is achieved through recognition of pathogen associated molecular patterns (PAMPs) by toll-like receptors (TLRs) of which 10 have been described in mammals (Akira, 2003). PAMPs include molecules such as bacterial flagella, peptidoglycan from gram-positive bacteria, lipopolysaccharide (LPS) from gram negative bacteria, and capsid proteins and double stranded RNA (Delgado *et al*, 2006). The prevalence of these molecules in many pathogens makes them logical molecules for early detection of microbes since it does not require the activation of antigen presenting cells (APC), antigen processing, or antibody production; although, TLRs are involved in APC activation (VanKooyk and Geijtenbeek, 2006).

In humans, TLRs are found in a variety of immune and nonimmune cell types including macrophages, dendritic cells, neutrophils, and epithelial cells (Hippenstiel *et al*, 2006). Once activated, TLRs activate a signal transduction cascade that leads to activation of NFκB, gene transcription and the secretion of inflammatory mediators and antimicrobial compounds (Hippenstiel *et al*, 2006). Although evidence is limited, there is evidence that TLRs help mediate phagocytosis and phagosome maturation in macrophages and it is required for clearance of *P. aeruginosa* from mouse lungs (Blander and Medzhitov, 2004; Chignard *et al*, 2008). There is also evidence to suggest that TLRs mediate apoptosis of epithelial cells that have phagocytised microbes (Ruckdeschel *et al*,

2004). Apoptosis and desquamation after phagocytosis of microbes by epithelial cells has been proposed as a method to kill, or capture and remove potential pathogens (Cannon *et al*, 2003).

THEORIES ON IMMUNODEFICIENCY

High salt / low fluid

Quinton was the first to suggest an ion transport defect in the lung (Quinton, 1983). It was thought that the absence of CFTR lead to impaired secretion of ions and thus impaired secretion of fluid of the ASF. This would explain the high viscosity of mucus in the CF lung since a failure to secrete fluid would lead to mucus dehydration (Boucher, 2007). In 1996, Welsh and colleagues proposed that the primary cause of CF lung disease was deactivation of endogenous antimicrobial compounds in the airway mucus caused by elevated sodium chloride concentrations, thus the primary defect would be failure to reabsorb chloride due to a lack of CFTR-mediated chloride transport similar to the situation in the sweat gland (Smith *et al*, 1996). However, *in vivo* measurements of electrolyte concentrations in the airway mucus and ASF are difficult due to their small volume and the patient's reflexes, and this remains one of the weak points in this theory (Hanrahan, 2000). One might also predict that a generalized immunodeficiency would

result in colonization with a broad range of pathogens, rather than the narrow range that is found in CF. An alternate theory was proposed shortly thereafter by Boucher and colleagues who contended that CFTR was responsible for regulating conductance through other ion channels (Matsui *et al*, 1998). They believed that the ability of CFTR to regulate the activity of the epithelial sodium chloride channel (ENaC) was critical for the maintenance of ASF volume and that the loss of this ability was responsible for increased sodium reabsorption and dehydration of the mucus causing subsequent loss of mucocilliary clearance (Figure 3). In either model, the underlying defect allows for colonization by microbes that damage tissue and trigger inflammation to further impede mucocilliary clearance and thus exacerbate the conditions that initially allowed for colonization (Wine, 1999).

Intracellular acidification

The hypothesis that CFTR regulates organellar pH and could thus effect a wide range of metabolic functions was an attempt to explain the pleiotropic presentation of CF patients and how a mutation in a chloride channel could result in seemingly unrelated pathologies. Some evidence does support this hypothesis, but most evidence in confined to cultured cells and the results of some very similar studies are contradictory (Dunn *et*

al, 1994; VanDyke *et al*, 1992). Several groups suggested that CFTR regulates the pH of Golgi stacks, and that defective regulation in this compartment could explain at least one of the defects seen in CF: increased adherance of *P. aeruginosa* (Barasch and al-Awqati, 1993; Chandy *et al* 2001). The hypothesis is that if Golgi pH is altered due to a defective CFTR, then glycoprotein biosynthesis in the Golgi may also be effected since the enzymes responsible are pH sensitive. This theory does agree with the finding that the glycolipid asialo-GM1 is enriched on CF epithelial cells (Soong *et al*, 2004). Furthermore, this molecule is a receptor for binding of *P. aeruginosa*, and is thought to help the bacteria colonize the host airway (Imundo *et al*, 1995). One group has noted defective killing of phagocytised *P. aeruginosa* by macrophages, and has linked this phenomenon to defective acidification of phagosomes (Di *et al*, 2006). Thus CFTR may effect pH regulation in more than one intracellular compartment, contributing to the pleitropic effects of mutations in CFTR.

Hyperinflammation

Some evidence suggests that the inflammatory mechanisms of the innate immune response are misregulated in CF. Both adult and infant airways of CF patients show elevated levels of macrophages and neutrophils and adults have elevated levels of IL-8,

IL-6 and TNF-α (Muhlebach *et al*, 2004). It has been proposed that the inflammatory response may be constitutively active, overly vigorous, or fail to attenuate properly. It has been shown in cell culture that secretion of IL-8 from non-CF epithelial cells in response to treatment with *P. aeruginosa* culture filtrates is attenuated with repeated stimulations, but the response fails to attenuate in CF cells (Wu *et al*, 2005).

Strategies to increase production of functional CFTR (transfection or incubation at 25°C) have been shown to reduce NF-κB activation and production of inflammatory cytokines such as IL-8 and TNF-α in immortalized cell lines and primary cell cultures (Kube *et al*, 2005; Vij *et al*, 2009; Venkatakrishnan *et al*, 2000). Data from primary cell culture is controversial since the results could be artifacts caused by chronic bacterial exposure. This has led several groups to investigate the inflammatory profile of naïve CF airways. One group reported increased inflammation and infiltration by neutrophils in xenografts from CF fetuses in severely immunocompromised mice compared to wild-type xenografts (Tirouvanziam *et al*, 2000; Tirouvanziam *et al*, 2002). Several studies have sampled the airways of young CF patients to monitor the levels of inflammatory mediators (Hubeau *et al*, 2001). The results of these studies are contradictory; however, it should be noted that measuring the concentration of cytokines from the ASF *in vivo* is difficult for the same reasons as measurement of ion concentrations. Sampling

techniques are invasive and can easily disrupt the system that is being sampled (Hanrahan, 2000).

CFTR acts as a receptor for phagocytosis

Several lines of evidence suggest that CFTR may play a more direct role in defending against pulmonary infections. Pier *et al* (1997) showed that a portion of the first extracellular loop of CFTR binds to lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* (Pier *et al*, 1996; Pier *et al*, 1997). The group proceeded to demonstrate that this interaction was necessary for phagocytosis of the bacteria, activation of NF-κB, and apoptosis of pulmonary epithelia (Kowalski and Pier, 2004; Schroeder *et al*, 2002). This model suggests that CFTR may act as a receptor for phagocytosis of invading bacteria by epithelial cells which constitute the first line of defense against pulmonary pathogens.

BACTERIA

Progression of colonization

Although Stapholococcus aureus and Hemophilus influenza occur early in the life of CF patients, little research has been done on their role in disease progression (Lyczak *et al*, 2002). It has been speculated that these pathogens may breach the defenses of the

lungs, weakening them enough to allow for colonization of more rarified pathogens later in the course of disease (Bals *et al*, 1999). As treatments improved in the late 60s and patients lived longer, *P. aeruginosa* emerged as the most common pathogen in CF patients (Lyczak *et al*, 2002). The emergence of this pathogen correlates with the founding of CF centers; however, it also correlates with an increase in life expectancy due to the use of effective antimicrobial chemotherapy (Figure 2) (Elborn *et al*, 1991). Patient to patient transmission of *P. aeruginosa* and other bacteria such as *B. cepacia* has been documented in CF centers; however, it is not clear if the establishment of these centers is the primary cause for the emergence of these pathogens as common infectious agents in the CF population, or their transmissibility is just a cautionary note for clinicians (Lyczak *et al*, 2002).

Pseudomonas aeruginosa

Approximately 80-90% of patients with CF become colonized by *P. aeruginosa*. *P. aeruginosa* is a gram negative flagellated rod shaped bacteria responsible for many nosocomial infections in immuno-compromised individuals (Sherertz and Sarubbi, 1983). A widely distributed microbe, *P. aeruginosa* can inhabit many substrates including the soil, rhizosphere, and the surfaces of medical devices and implants (Hart and Winstanley,

2002). It is metabolicly diverse and even has applications in bioremediation for toxic waste cleanup (Ganuli and Tripathy, 2002). Unfortunately this metabolic diversity has allowed the evolution of a natural resistance to several antibiotics (Carmeli *et al*, 1999). Like all gram negative bacteria, the outer membrane of *P. aeruginosa* contains the molecule lipopolysaccharide (LPS), a powerful activator of the immune and inflammatory response (Kronborg, 1995). The role of this molecule in CF has previously been studied for its ability to induce tissue inflammation, stimulate secretion of inflammatory cytokines, and its proposed role as an antigen for the innate immune system (Pier, 2007).

During the course of infection, *P. aeruginosa* can undergo several changes that increase its ability to persist in the host. The progression goes from strains with a smooth to rough then to a mucoid colony phenotype. These changes in colony morphology are caused by changes in the structure of their LPS and alginate production (see the section on LPS structure for more details on structural changes in the molecule). The conversion from smooth to rough colony morphology correlates with the loss of the O side chain which increases the pro-inflammatory activity of the molecule (Pier, 2007). This conversion has been shown to increase adherence to corneal epithelial cells (Fletcher *et al*, 1993).

Mucoid colonies produce alginate, a secreted polysaccharide that protects the microbes from killing by the host immune system by reducing the effectiveness of secreted antimicrobial compounds (Hart and Winstanley, 2002; Pier *et al*, 2001). Alginate production is commonly correlated with the formation of stable biofilms in which individuals cooperatively evade host defenses (Hart and Winstanley, 2002). Biofilm formation not only increases the resistance of *P. aeruginosa* to the host immune system, but also increases resistance to antibiotic chemotherapy (Hart and Winstanley, 2002). Formation of biofilms is mediated through quorum sensing, a type of bacterial intercellular communication, which allows bacteria to coordinate their defense against and attacks on the host (Smith and Iglewski, 2003).

Burkholderia cepacia

Burkholderia was first described in 1950 as the cause of soft rot in onions and was named Pseudomonas cepacia (Burkholder, 1950). These bacteria do bear many similarities to Pseudomonas sp in that they are gram negative rod shaped bacteria that are metabolically diverse, found in the soil and rhizoshpere, and are motile by means of flagella (Hart and Winstanley, 2002). However, genetic analysis has led to them being classified as a new genus, Burkholderia (Yabuuchi *et al*, 1992). *B. cepacia* was first

recognized as a common pathogen of CF patients in 1984 (Isles *et al*, 1984). Patients can present with symptoms ranging from subclincial infections to a rapid and fatal decline due to sepsis referred to as cepacia syndrome (Govan *et al*, 1996). Unlike *P. aeruginosa*, *B. cepacia* has a genome with 2-4 circular replicons with multiple insertion elements rather than the classical single circular chromosome (Rodley *et al*, 1995; Tyler *et al*, 1996). This confers a high degree of genetic plasticity to the bacteria, another feature that is common in *P. aeruginosa*, and which seems to be enhanced in isolates from chronically infected CF patients (Hart and Winstanley, 2002). This plasticity is thought to confer a survival advantage by allowing infectious strains to rapidly evolve to the harsh conditions found in the CF airway.

Infection by *B. cepacia* begins with pilus mediated attachment of bacteria either to airway epithelia or mucins (Sajjan *et al*, 2000; Sajjan *et al*, 1992). *B. cepacia* is naturally resistant to the host's antimicrobial peptides as well as a number of antibiotics, making clearance of the colonizing bacteria difficult (Baird *et al*, 1999; McClean and Callaghan, 2009). LPS from both *B. cepacia* and *P. aeruginosa* has been shown to induce inflammation in cultured cells and animal models, and serves other roles in the pathogenesis of these bacteria (Mahenthiralingam *et al*, 2005). Several genetic knockouts have shown that the core oligosaccharide in *B. cepacia* LPS is required for its

resistance to antimicrobial peptides (Mahenthiralingam *et al*, 2005; Cox and Wilkinson, 1991; Loutet *et al*, 2006). Further evasion of host defenses is achieved through quorum sensing and biofilm formation, much as is the case for *P. aeruginosa* (Tomlin *et al*, 2005). Vigorous antimicrobial chemotherapy is able to reduce colonization to the point that sputum cultures are negative for bacteria; however, cases of the same strain of *B. cepacia* returning to a patient after a negative sputum culture are not uncommon (Denis *et al*, 2007). It could be that the second infection occurs through reinnoculation by bacteria in the environment or on medical devices where *B. cepacia* has been shown to form antiseptic resistant biofilms; however, it has been postulated that the bacteria evades eradication and detection by invading host cells (Hart and Winstanley, 2002; Sajjan *et al*, 2006). *B. cepacia* has been shown to penetrate and survive within cultured macrophages and epithelial cells (Sajjan *et al* 2006; Saini *et al*, 1999; Burns *et al*, 1996; Pirone *et al*, 2008). This localization would protect bacteria during antibiotic administration and allow the strain to reemerge after treatment has stopped; however, this phenomenon is difficult to document *in vivo*.

Lipopolysaccharide

The outer leaflet of the outer membrane of gram-negative bacteria is composed completely of lipopolysaccharides (LPS) (Doerrler, 2006). These molecules have four structural components; the inner and outer core oligosaccharides, the O-antigen, and the lipid A (Figure 4) (Pier, 2007). The lipid A consists of 4-7 fatty acid residues linked to a diglucosamine. This portion of LPS varies in the number and species of fatty acids, and modifications to the diglucosamine such as addition of other sugar residues. Such modifications have been shown to alter not only bacterial behavior, but also the host response to the LPS (Pier, 2007; Backhed *et al*, 2003). The length and diversity of sugar residues of the inner and outer core oligosaccharides varies between strains (Pier, 2007; Backhed *et al*, 2003). The O-antigen is a highly variable sequence of sugars that can confer resistance to antimicrobial compounds, but is also important for immune recognition and is the basis of serotype classification of *P. aeruginosa* and *B. cepacia* (Mahenthiralingam *et al*, 2005; Backhed *et al*, 2003; Pier and Ames, 1984). The conversion from rough to smooth colony morphology correlates with an increased production of LPS that lacks the O-antigen (Pier, 2007). Patients chronically colonized by *P. aeruginosa* have high serum levels of IgG that is reactive to the O-antigen of LPS,

thus conversion to a smooth colony phenotype may protect *P. aeruginosa* from acquired immunity (Pier, 2000).

Immune activation by LPS can also be achieved through binding of the molecule to toll-like receptor 4 (TLR4) (Greene *et al*, 2005). The lipid A structure of LPS interacts with TLR4 and the potency of immune activation varies depending on its structure. It has been found that *P. aeruginosa* converts from the production of primarily penta-acylated lipid A to hexa-acylated lipid A during colonization of the lung (Pier, 2007). The hexa-acylated form of the lipid A is a more potent activator of inflammation and the immune response than the penta-acylated form and confers a higher degree of resistance to antimicrobial peptides (Pier, 2007). Penta-acylated MKLPS from the mutant Escherichia coli strain has even been shown to competitively antagonize activation of TLR4 signaling thus reducing activation of the inflammatory signal transduction cascade (Somerville *et al*, 1996). Certainly increased resistance to antimicrobial peptides confers an advantage, but the role of conversion to hexaacylated LPS and subsequent inflammation in *P. aeruginosa* colonization is still unclear.

THIS STUDY

Hypothesis and objectives

My work has been focused on host-pathogen interactions in the CF lung infections. Based on evidence from Gerald Pier's group at Harvard and the ability of CFTR to interact with many diverse proteins, its ability to regulate protein and vesicular trafficking, and the well known role for TLR4 in the response to LPS, I set out to test the hypothesis that both CFTR and TLR4 are both involved in phagocytosis of gram negative bacteria by epithelia. A possible model is shown in Figure 5. To test this model, I used an iodide efflux assay to measure the presence and activity of CFTR at the cell surface in response to treatment with *Pseudomonas aeruginosa* and LPS purified from *P. aeruginosa*. I predicted that if CFTR is involved in phagocytosis of *P. aeruginosa* through an interaction with LPS, then treatment of cells with either LPS or live bacteria would reduce the abundance of CFTR at the plasma membrane which would cause a decrease in CFTR activity observable by iodide efflux. Furthermore, if LPS is the antigen responsible for phagocytosis of *P. aeruginosa*, then addition of LPS should reduce the number of phagocytised bacteria quantified using the gentamicin protection assay.

Gentamicin protection assay

The gentamicin protection assay, also known as an invasion / adhesion assay, is capable of quantitatively differentiating between bacteria that are free living, adhered to host cells, or inside of host cells. This is possible because of the inability of the antibiotic gentamicin to cross eukaryotic cell membranes (Vaudaux and Waldvogel, 1979). This allows for killing of all extracellular bacteria without killing intracellular bacteria which represent phagocytised or invaded bacteria. This difference between phagocytised and invaded is usually subtle since many invasive bacteria are able to invade host cells by allowing themselves to be phagocytised and subsequently escaping into the cytosol or inhibiting maturation of the phagosome (High *et al*, 1992). Before this experiment can be run, several preliminary experiments must be run.

1) The minimum inhibitory concentration (MIC) of gentamicin for the bacteria must be determined.

2) The growth of the bacteria must be characterized to determine when log phase growth occurs and to correlate optical density to bacterial density to allow a consistent inoculum to be administered between experiments.

3) The sensitivity of the bacteria to reagents used in manipulating cell culture must be tested.

4) The density of eukaryotic cells grown to confluence must be determined to allow for the calculation of a consistent multiplicity of infection (MOI = CFU bacteria/eukaryotic cell) to allow for comparison to other work.

Immortalized human bronchial epithelial cells taken from a CF patient and transfected to express wild-type CFTR (S9 cells) were grown in 24-well plates and incubated with bacteria for 3 hours. After this, bacteria from one set of wells was used to quantify total bacteria, and the others were rinsed to remove any un-adhered bacteria. Gentamicin was then added to kill any extracellular bacteria. Removal of antibiotic followed by lysing of the host cells, dilution of samples and plating for CFUs allows for quantification of internalized bacteria. In one experiment, host cells were lysed prior to removal of antibiotic to confirm that bacteria remained susceptible to antibiotics. Cultures were treated with MKLPS, an underacylated form of LPS that has been shown to competitively inhibit TLR4 (Somerville *et al*, 1994). If TLR4 is involved in binding and phagocytosis of the bacteria, then treatement with MKLPS should have reduced the amount that were adhered and internalized; however, this was not observed.

To confirm that phagocytosis of bacteria actually occurred, the assay was repeated with *P. aeruginosa* PA01 labeled with green fluorescent protein (GFP) and human bronchial epithelial cells grown on glass coverslips. Observation of cultures after treatment with gentamicin revealed the presence of adherent, and apparently antibiotic resistant, microcolonies at the junctions between eukaryotic cells. This indicates that the quantification of phagocytised bacteria includes extracellular microbes in addition to intracellular microbes, and thus is not representative of phagocytosis and therefore cannot be considered conclusive evidence that the rate of phagocytosis is unaffected by treatment with MKLPS. It is likely that lysing of the host cells disrupts these resistant colonies and allows them to be exposed to antibiotics again, thus explaining why lysing of the cells before antibiotics are removed results in killing of bacteria.

Iodide efflux

Iodide can be used to study the activity of CFTR by measuring the rate at which it flows out of cells. Iodide will pass through CFTR, but little will pass through other chloride channels and exchangers (Long and Walsh. 1997). In this assay, cultured cells are loaded with iodide or radioactive 125-I by incubating cultures with a high concentration of sodium iodide or radionuclide for 1 hour. The buffer is then removed

and extracellular iodide is rinsed away with iodide-free buffer. Cultures are then incubated in iodide-free buffer for 1 minute after which the buffer is collected and replaced. During this time, iodide will flow out of the cells through activated CFTR. After establishing a stable baseline efflux rate, buffer is added containing forskolin and IBMX. Forskolin activates adenylate cyclase which produces cyclic adenosine monophosphate (cAMP) and IBMX inhibits phosphodiesterase, the enzyme that degrades cAMP (Seamon *et al*, 1981; Wang *et al* 1999). The resulting increase in cAMP concentrations will activate protein kinase A which has been shown to activate CFTR by phophorylation of its R-domain (Chappe *et al*, 2005). If CFTR is present at the plasma membrane, this will cause the rate at which iodide leaves the cells to increase. Thus the increase in efflux rate is due to an increase in CFTR channel activity, and the amount of channel activity is dependent upon not only the probability of the channel opening, but also it's abundance at the plasma membrane. Iodide in the samples can be measured using ion selective electrodes or with a scintillation or gamma-counter, and used to calculate efflux rates. Data can be analyzed by comparing peak efflux rates after stimulation with forskolin and IBMX, or by comparing the area under the curve (AUC) after treatment with the cAMP elevating agents forskolin and IBMX.

In this study, radioactive 125-I was found to be most effective for measuring CFTR activity. It was found that the iodide selective electrode was not sensitive enough to reliably detect the small amounts of iodide secreted from cell cultures during baseline efflux. Cultures were treated with variable concentrations of LPS or bacteria at different time points to observe the effect of the treatments on CFTR channel activity. Acute administration of LPS (at the same time as stimulation with forskolin and IBMX) was found to reduce CFTR channel activity compared to forskolin and IBMX alone, while sustained treatment for longer time periods increased activity in a dose-dependent manner. Similarly, sustained treatment with *P. aeruginosa* PA14 resulted in a modest increase in CFTR channel activity. This is the first report of a change in CFTR channel activity in response to treatment with LPS.

METHODS

Materials and reagents

Fetal bovine serum (FBS), bovine serum albumin (BSA), type IV human placental collagen, geneticin (G418), saponin, ethylenediaminetetraacetic acid (EDTA), sodium iodide, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), glucose, potassium chloride, sodium bicarbonate, forskolin, isobutylmethylxanthine (IBMX), tryptic soy, glycerol and lipopolysaccharides from *P. aeruginosa* serotype 10 were purchased from Sigma. Dulbecco's modified eagle medium (DMEM), gentamicin, PBS without calcium or magnesium chloride, PBS containing calcium and magnesium, 100X penicillin/streptomycin, streptomycin and tryspin/EDTA were purchased from GIBCO. Potassium nitrate, calcium nitrate, sodium nitrate, sodium hydroxide, sodium chloride, magnesium sulfate, sodium phosphate (monobasic) and calcium chloride were Purchased from JT Baker.

Cell culture

Murine mammary epithelial cells (C127) stably expressing wtCFTR under control of a bovine papiloma based vector, 2WT2 cells (Genzyme), and S9 cells, human bronchial epithelial cells from a patient with a ΔF508 and W1282X mutations complemented with wt human CFTR cDNA (ATCC), were routinely cultured in Dulbeco's Modified Eagle Medium supplemented with 10.0% fetal bovine serum, 100μg/mL penicillin, 100μg/mL streptomycin and in T25 culture flasks (Corning) coated with 0.10mg/mL collagen and 0.01mg/mL bovine serum albumin in a water saturated 5% carbon dioxide (Airgas) / 95% air atmosphere at 37.0°C. Culture media for 2WT2 cells also contained 100μg/mL geneticin (G418). Cultures were passed by rinsing with 1.0mL of 0.05% trypsin EDTA followed by 15 minutes incubation in 0.50mL of 0.25% trypsin EDTA. Growth media was added at the end of incubation and cells were seeded into 35mm dishes (Corning), glass cover slips (Fisher) or 24-well plates (Corning) coated with 0.10mg/mL collagen and 0.01mg/mL bovine serum albumin. For gentamicin protection assays, cells were grown in the absence of antibiotics. Cells were harvested for hemacytometer counts using the passaging protocol.

Bacterial Growth

Pseudomonas aeruginosa strains PA14 and PA01 were generously provided by Dr. Todd Ciche. For all experiments, 5.0mL overnight cultures of *P. aeruginosa* PA01 were inoculated in tryptic soy broth (TSB) or *P. aeruginosa* PA14 were inoculated in lysogeny broth (LB) (Acumedia) from a frozen stock and grown aerobically at 37.0°C with shaking at 135rpm in an incubator shaker (New Brunswick Scientific).

For growth curves, 10.0mL of respective media were inoculated with 100μL of overnight culture and grown as before. 800μL was removed at each time-point and used for serial dilutions and plating and to measure the OD600 using a Beckman DU520 spectrophotometer. Dilutions were spread on plates of LB with 1.5% agar (Difco) and grown overnight at 37°C. Plates were counted and used to calculate the bacterial density (CFU/mL) which was plotted against time and OD600 to determine when cultures entered log-phase growth and the concentration of bacteria at a given optical density.

For treatment of eukaryotic cells, a 10.0mL culture was inoculated with 100μL of overnight culture and grown for 6 hours. The OD600 was measured and used to calculate the volume of culture needed to obtain a multiplicity of infection (MOI) of 50 during each time-point of the efflux experiment, or for each well of a 24-well plate. For efflux experiments, the required volume was removed from the culture and cells were pelleted

by centrifugation at 3500rpm for 3.0 minutes in an Eppendorf 5415 centrifuge. The supernatant was discarded and cells were rinsed with efflux buffer and re-pelleted as before. Cells were re-suspended in efflux buffer to a density of 10^8CFU/mL. Bacteria culture was added directly to culture media for gentamicin protection assays.

The minimum inhibitory concentration (MIC) of gentamicin was determined by growing *P. aeruginosa* PA01 as described for the gentamicin protection assay. Bacteria were diluted in DMEM to the same density that would be used for the gentamicin protection assay and 500μL of suspension was added to 24-well plates with serially increasing concentrations of gentamicin added to groups of four wells. The plates were then incubated for 1.0 hour under the same conditions used in the gentamicin protection assay (see below). After incubation, three LB agar plates were spread with 200μL from each well and incubated overnight at 37°C. The MIC was defined as the concentration of gentamicin that resulted in no visible growth in all four wells of a given concentration in three replicates of the experiment. The concentration of gentamicin used in the gentamicin protection assay was 300μg/mL, 50μg/mL higher than the MIC.

The sensitivity of *P. aeruginosa* PA01 to 1% saponin and 21.2mM EDTA dissolved in calcium free PBS was determined by incubating bacteria suspended in DMEM in the working concentration of saponin solution for 15 minutes in a 24-well

plate similar to the treatment in the MIC experiment. Samples were serially diluted, plated, and incubated overnight at 37°C after which colonies were counted and compared to untreated controls.

GFP was stably expressed in *P. aeruginosa* PA01 using a triparental mating system to induce recombination of the gene into the chromosome. Donor cells, Escherichia coli bearing either pURR25 or pUX-BF13, were generously provided by Dr. Todd Ciche and were grown in LB containing 300μg/mL diaminopimelic acid (DAP) and 100μg/mL ampicillin (Roche), and recipient cells, *P. aeruginosa* PA01, were grown in LB as described previously. Overnight cultures were used to inoculated 10mL of respective media with 100μL of donor and recipient bacteria. E. coli strains were grown for 10.5 hours, and *P. aeruginosa* strains were grown for 3.5 hours, at which time all cultures had an OD600 greater than 1. Cells were pelleted by 2 minutes of centrifugation at 2400rpm, and washed twice with their respective media. Pellets were resuspended in the remaining fluid, mixed, the suspension was spotted on a plate containing LB and 300μg/mL DAP, and the plate was incubated overnight at 37°C. Cells were washed off of the plate with 1.5mL LB and washed twice by pelleting at 2400rpm for 2min and resuspending in 1.5mL LB. Bacteria were plated on LB agar containing 30μg/mL

kanamicin (Roche) and 60μg/mL streptomycin, and incubated overnight at 37°C. Transformants were streaked onto LB agar and used to make freezer stocks.

Gentamicin protection assay

S9 cells were grown to 100% confluence in 24-well plates in culture media lacking antibiotics. *P. aeruginosa* PA01 were suspended in DMEM to the cell density required to achieve an MOI of 100 and split into two tubes. MKLPS (Invivogen) was added to one tube to a concentration of 50μg/mL. A 500μL volume of suspension was added to each well and cultures were incubated for 3 hours in a water saturated 5% carbon dioxide / 95% air atmosphere at 37°C. After incubation, plates were removed and samples were collected to quantify total bacteria. The remaining wells were rinsed 5X in room temperature PBS (with calcium chloride). A microscope was used to confirm that cells were still adhered after rinsing. Half of the remaining cells were lysed in a solution of 1% saponin and 21.2mM EDTA dissolved in calcium free PBS (saponin solution) for 15min and collected, and the other half were incubated for 1 hour with 300μg/mL gentamicin in a water saturated 5% carbon dioxide/95% air atmosphere at 37°C. Cells were lysed as before and samples collected. Samples were serially diluted, plated on LB agar, and incubated overnight at 37°C. Colony counts were used to calculate the density

of total bacteria, adhered bacteria, and protected bacteria in each treatment. One experiment was performed in which cells were lysed before the removal of antibiotic.

For microscopy, S9 cells were grown on glass coverslips under normal conditions for the assay. GFP labeled *P. aeruginosa* PA01 was prepared as before to achieve an MOI of 1 and added to cultured cells. After rinsing or treatment with gentamicin, cultures were not lysed, but were viewed using fluorescent and differential interference contrast using a Leica DM5000 compound microscope (Leica Microsystems, Wetzlar, Germany) equipped with an X-cite 120 fluorescence illuminator (EXFO, Quebec, Canada), a Spot Pursuit charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI), and a GFP filter set (Leica) as described in Ciche *et al* (2008).

Iodide efflux

The ORION iodide selective electrode was calibrated with fresh sodium iodide standard solutions of 10mM, 1.0mM, 0.10mM, 0.01mM, and 0.001mM made in nitrate based efflux buffer as per the manufacturer's instructions. Nitrate based efflux buffer contains 3mM potassium nitrate, 2mM calcium nitrate, 20mM HEPES, 1mM glucose and either 136mM sodium iodide or 136mM sodium nitrate. To verify electrode function, serial dilutions were made from the same stock solution used to standardize the electrode

initially. These solutions were read with the meter and the ratio between the measured concentration and the actual concentration was calculated. Since the meter would read a trace concentration of iodide in distilled water rather than reporting a null concentration, the limit of detection had to be defined as the concentration below which the ratio between the measured concentration and the actual concentration was greater than 1.2, after which the deflection away from the actual concentration was sharp. Similar experiments were performed with the electrode being calibrated and measurements being made in standards prepared in distilled water and chloride based efflux buffer.

Experiments were performed in tandem with three cultures at a time. Each day an experiment was run, a replicate set of three cultures of control cells treated with only forskolin and IBMX was ran to standardize the results for comparison to treatments. To begin, growth media was removed and cultures were rinsed with efflux buffer containing 150mM sodium chloride, 5.4mM potassium chloride, 0.80mM magnesium sulfate, 10mM HEPES, 1.0mM sodium phosphate (monobasic), 1.8mM calcium chloride, and 1.0mM glucose. Cultures were incubated in 1.0mL of efflux buffer with 2μCi of 125-I (PerkinElmer) for 1-1.5 hours at 37°C. After loading with radionuclide, buffer was removed to a collection tube and cells were rinsed 4X with 1mL of 4°C efflux buffer. A 1mL volume of room temperature efflux buffer was added to each culture for one minute,

after which the buffer was removed to a collection tube and replaced with 1mL of room temperature efflux buffer. This process was repeated for 15 minutes. Beginning at 11min, buffer was replaced with efflux buffer containing 10μM forskolin and 100μM IBMX. After 15 minutes, 1mL of 2M NaOH was added for 10 minutes to lyse the cells. While cells were lysing, the next set of 3 dishes were rinsed and loaded with radionuclide as before. Effluxed 125-I in samples was counted with a Cobra II auto-gamma counter (Packard). In experiments where cells were treated with lipopolysaccharide (Sigma) or bacteria, the treatment was added to the efflux buffer at the concentration indicated for the entire period of sample replacement beginning after the 4 rinses with 4°C efflux buffer. A schematic of the time-course is shown below (figure 6).

After running control experiments, samples were only collected between 5min and 15min of the efflux experiment and peak efflux was measured as the fold change over baseline of each culture at 12min. All measurements of the area under the curve were taken from the start of the stimulus until time 13, just before values returned to basal levels. The efflux rate constant was calculated as described by Becq *et al* (2003). Briefly, the total radioactivity in the timecourse was summed (from the first sample replacement to that collected in the lysate) and used to calculate the percentage of intracellular iodide left at each time point. To calculate the rate constant for each time

point, the equation $r = [\ln(R1) - \ln(R2)] / (t1-t2)$ was used where r is the rate constant, R1 and R2 are the percentages of intracellular iodide left at time point t1 and t2 respectively.

Data analysis

Data analysis was performed using StatView (Abacus Inc). Single comparisons were made using an unpaired 2-tailed student's t-test, and multiple comparisons were made using ANOVA using the Bonferoni correction for post-hoc analysis. A p-value of ≤ 0.1 was considered significant.

RESULTS

TLR4 antagonist LPS does not alter recovery of *P. aeruginosa* in the gentamicin protection assay.

To determine if TLR4 is involved in phagocytosis of *P. aeruginosa*, a gentamicin protection assay was used to quantify bacteria that were internalized by human bronchial epithelial cells that express wild-type CFTR (S9 cells). Bacterial growth curves were performed to ensure reproducible production of bacterial inocula, and the minimum inhibitory concentration of gentamicin was determined to establish a concentration required for killing of extracellular bacteria. Lastly, phagocytosis was confirmed with fluorescent microscopy.

Cell counts, bacterial growth curves, gentamicin MIC, and treatment sensitivity.

Growth curve analysis was performed for *P. aeruginosa* strains PA01 and PA14 to allow for treatment of cultures with a consistent amount of bacteria in log phase growth. Growth curves for *P. aeruginosa* PA01 and PA14 are shown in Figures 7 and 8, and the correlation between bacterial cell density and optical density (OD600) is shown in figures 9 and 10. Both strains reached log phase growth by 6 hours and had a linear

absorbance between 0 and 1 OD600. An optical density of 1 correlated to approximately 10^9 colony forming units (CFU)/mL of bacteria. To calculate how many bacteria were required for a specific multiplicity of infection (MOI), S9 cells grown to confluency were counted using a hemacytometer. Confluent cultures of S9 cells in 24-well plates were found to have approximately 250,000 cells/well. Confluent cultures of 2WT2 epithelial cells grown in 35mm dishes were found to have approximately 950,000 cells/dish.

To determine what concentration of gentamicin was required to kill extracellular bacteria, the minimum inhibitory concentration (MIC) of gentamicin was determined by adding increasing concentrations of gentamicin to cultures of *P. aeruginosa* PA01. The MIC was found to be 250μg/mL, so 300μg/mL gentamicin was used in all experiments. Similar experiments were performed to confirm that other agents used to treat cultures in the course of the gentamicin protection assay (0.25% trypsin EDTA, 1% Triton X-100, phosphate buffered saline, 1% saponin with 21.2mM EDTA, and DMEM) did not have an effect on *P. aeruginosa* PA01 viability. Cultures of *P. aeruginosa* PA01 treated with trypsin EDTA had fewer bacteria than untreated controls; however, they were not effected by treatment with saponin and EDTA.

TLR4 antagonist LPS does not alter invasion or adhesion of P. aeruginosa PA01.

Quantification of media used to inoculate cells showed that S9 cultures were treated with 6*10^6CFU/mL *P. aeruginosa* PA01, or an MOI of about 13. During the incubation, bacteria roughly tripled to a total of about 2*10^7CFU/mL or an MOI of about 40. Bacteria adhered at a rate of 1% of the total inoculum (at the end of incubation) and about 0.03% of the total inoculum were protected from gentamicin. This means that on average, approximately 40% of S9 cells had a bacteria adhered to their surface, but only 1% had engulfed bacteria. No significant difference was seen in protection or adhesion between untreated controls and cultures treated with the TLR4 antagonist MKLPS (Figure 11). Lysis of cells before the removal of antibiotic resulted in no recovery of bacteria.

<u>*P. aeruginosa* PA01 forms antibiotic resistant colonies on epithelial cells.</u>

The continued susceptibility of bacteria to 300μg/mL gentamicin after incubation with S9 cells was confirmed by lysing epithelial cells in the presence of the antibiotic. No bacteria were recovered from this treatment. *P. aeruginosa* PA01 was labeled with GFP and used to inoculate S9 cells grown on glass coverslips at an MOI of one bacteria per epithelial cell. Similar examination after treatment with 300μg/mL gentamicin

revealed the formation of antibiotic resistant microcolonies at eukaryotic cell junctions (Figure 12).

Alterations in CFTR mediated efflux in response to treatment with bacteria.

An iodide efflux assay was used to monitor changes in the abundance and activity of CFTR at the plasma membrane in response to treatment with *P. aeruginosa* or purified LPS. Measurement of iodide in samples was used to calculate the rate at which the ion was secreted from cultured cells. This data was then used to calculate the efflux rate constant at each time point. By measuring the efflux as a rate constant (min^-1) instead of a rate (CPM/min), the analysis compensates for variability in the amount of iodide loaded between trials. If CFTR is involved in phagocytosis of the bacteria, then these treatments should result in depletion of CFTR at the plasma membrane and a corresponding decrease in the rate of iodide efflux. Two methods were employed for this experiment, one using an ion selective electrode to detect iodide released during the course of the experiment, and the other using radioactive 125-Iodide as a tracer.

The iodide selective electrode was not sensitive enough for detection of iodide efflux.

The ORION iodide selective electrode was not sensitive enough for use in ion efflux experiments. The limit of detection was determined experimentally using either a buffer containing chloride, nitrate, or with distilled water as described in the methods. Although the manufacturer states that the electrode can detect 50nM iodide, the meter was only able to reliably detect iodide down to 60μM in chloride buffer, and at 10-fold lower concentrations in either nitrate buffer or distilled water (Figure 13). Despite the use of nitrate buffer in efflux experiments, the baseline efflux readings for S9 cells were at or below the limit of detection of the meter when using the sample replacement technique. Stimulation with forskolin had no detectable effect on efflux rates in these cells (Figure 14). When the experiment was performed with 2WT2 cells, baseline efflux rates were similarly low, but forskolin was able to elicit a detectable increase in efflux rate (Figure 15).

Direct continuous measurement of iodide in the extracellular buffer without sample replacement was attempted by placing the meter directly in the buffer in the cell culture dish. This technique was problematic for several reasons. First, the solution needed to be agitated to facilitate diffusion of iodide throughout the buffer. Second, the meter can take anywhere from 20 seconds to 5 minutes to reach a stable reading, far too

slow for direct measurement of efflux from cultured cells. Third, the solution came to equilibrium between intracellular and extracellular iodide concentrations too quickly to allow for iodide to continue to efflux for the amount of time required to allow the meter to generate a stable reading or baseline efflux rate. Despite the ability to read higher concentrations of extracellular iodide during the efflux experiment, no detectable response was observed in response to forskolin (Figure 16).

Radionuclide efflux controls

In radionuclide efflux experiments, the initial efflux rate constant declined steadily from approximately 0.12min^-1 until stabilizing 5 minutes into the timecourse at about 0.05min^-1 after which the rate constant declined slightly over another 15 minutes to 0.03min^-1 (at 20 minutes into the timecourse). Treatment of cells with 10μM forskolin and 100μM IBMX at 10 minutes into the time-course stimulated an increase in the efflux rate beginning at 11 minutes, peaking 3.3 fold higher than baseline (a rate constant of 0.2min^-1) at 12 minutes, and decreasing steadily back to baseline by 14 minutes (4 minutes after addition of 10μM forskolin and 100μM IBMX). Acute treatment of cultures with 100μg/mL LPS alone at 10 minutes (the same time interval as previous treatment with 10μM forskolin and 100μM IBMX) had no effect on efflux rate

compared to untreated cultures. Figure 17 shows the calculated rate constants from unstimulated, 10μM forskolin and 100μM IBMX stimulated, and acute LPS stimulated cultures and Figure 18 shows these values normalized to the baseline of approximately 0.05min^-1.

Based on these results, samples were only collected between 5 minutes and 15 minutes of the efflux experiment and peak efflux was measured as the fold change over baseline of each culture at 12 minutes. All measurements of the area under the curve (AUC) were taken from the start of the stimulus until time 13, just before values returned to basal levels. Treatments were administered at two times: either acute (at 10 minutes into the efflux experiment until the end at 15 minutes) or continuous (beginning at the start of the efflux time-course and continuing until the end at 15 minutes).

P. aeruginosa LPS alters CFTR channel activity in a dose-dependent manner.

Cultures treated continuously with *P. aeruginosa* PA14 at an MOI of 100 showed a 25% increase in cAMP stimulated peak efflux and a 35% increase in AUC compared to control cultures (Figures 21 and 22). Cultures were treated with LPS from *P. aeruginosa*, to determine if LPS was responsible for the response observed with live bacteria. Continuous treatment of cultures with increasing concentrations of LPS resulted in an

increase in cAMP-stimulated peak efflux and AUC in a dose dependent fashion compared to controls treated only with cAMP elevators. This trend is shown in Figures 19 and 20. Cultures continuously treated with 50μg/mL LPS and had a 30% increase in cAMP stimulated peak efflux (3.25 fold compared to a 2.5 fold increase in control cultures) and a 60% increase in AUC. Cultures treated with 100μg/mL LPS had a 40% increase in cAMP stimulated peak efflux (3.5 fold compared to a 2.5 fold increase in control cultures) and an 85% increase in AUC. Cultures treated with 200μg/mL LPS had a 50% increase in cAMP stimulated peak efflux (3.75 fold compared to a 2.5 fold increase in control cultures) and a 110% increase in AUC.

P. aeruginosa LPS alters CFTR channel activity in a time-dependent manner.

To determine if alteration of CFTR channel activity by LPS from *P. aeruginosa* was time-dependent, experiments were performed in which cultures were not treated with LPS until 10 minutes into the time course of sample replacement (instead of continuous administration over the entire duration of sample replacement). As stated previously, acute treatment of cultures with 100μg/mL LPS alone (in place of 10μM forskolin and 100μM IBMX at 10 minutes) had no response that could be differentiated from untreated (no forskolin or IBMX) cultures (Figures 17 and 18). Cultures treated acutely with a

cocktail of 100μg/mL LPS, 10μM forskolin and 100μM IBMX at 10 minutes showed a 25% decrease in peak efflux that was not statistically significant, and a 40% reduction in area under the curve that was significantly different from cultures treated with cAMP elevating agents alone (Figure 23 and 24).

DISCUSSION

There is evidence to support the hypothesis that the cystic fibrosis transmembrane conductance regulator (CFTR) acts as a receptor for lipopolysaccharide (LPS) on the surface of gram-negative pathogens, and this interaction leads to phagocytosis of bacteria by epithelial cells (Pier *et al*, 1997; Schroeder *et al*, 2002). This interaction is thought to be important for clearance of *Pseudomonas aeruginosa* from the airway, and explains why cystic fibrosis (CF) patients without CFTR become infected with these bacteria and can not clear them from the airway. The idea that CFTR would serve as a receptor for LPS seems odd since toll-like receptor 4 (TLR4) is a known receptor for LPS found on pulmonary epithelial cells that has been shown to be involved in phagocytosis of gram negative bacteria and activation of the immune response (Blander and Medzhitov. 2004). Since both CFTR and TLR4 seem to be involved in the cell's response to *P. aeruginosa*, I hypothesized that these proteins form a multi-protein complex that assembles to facilitate phagocytosis of bacteria.

I tested this hypothesis using two experimental models: a gentamicin protection assay, and an ion efflux assay. The gentamicin protection assay allows for the quantification of intracellular (phagocytised) bacteria, and the iodide efflux assay allows

for a measurement of CFTR channel activity at the plasma membrane. Based on my model in which CFTR and TLR4 both participate in LPS-mediated phagocytosis of *P. aeruginosa*, I predicted that treatment with MKLPS, a competitive antagonist of TLR4, would reduce the quantity of bacteria phagocytised during the gentamicin protection assay. I would also predict that treatment of cells with either purified LPS, or live bacteria would result in a decrease in CFTR channel activity at the plasma membrane that could be detected with the ion efflux assay.

P. aeruginosa strain PA01 adheres to respiratory epithelial cells but is not frequently phagocytised.

Human bronchial epithelial cells that stably express wt-human CFTR (S9 cells) were infected with *P. aeruginosa* and then treated to kill extracellular bacteria. The remaining bacteria were then presumed to be intracellular (phagocytised). To quantify extracellular adherence of bacteria to the epithelial cells, several samples were treated to remove unadhered bacteria by rinsing with PBS, but were not exposed to the antibiotic to kill extracellular bacteria.

A small amount of *P. aeruginosa* PA01 was found to adhere to pulmonary epithelial cells after 3 hours of incubation. This result is consistent with the

characterization of the bacteria as an adherent pathogen (Taminiau *et al*, 2002). Although the frequency of adherence was small, the inoculum used in these experiments was large (MOI of 50), and the small frequency of adhesion could be skewed lower due to depletion of binding sites by the large number of bacteria added. It is also possible that a longer incubation time could increase the frequency of adherence.

The amount of bacteria protected from gentamicin was much smaller than that of adhesion and is consistent with previous reports of the intracellular occurrence of this pathogen (Darling and Evans, 2001; Pier *et al*, 1997). Several studies have concluded that *P. aeruginosa* is an invasive pathogen; however, the *in vivo* significance of the small frequencies of invasion seen in these studies has not been determined. Just because a small fraction of bacteria are found to invade host cells during a gentamicin protection assay does not mean that invasion of cells is important for colonization and infection by the bacterium. It is possible that invasion by a small fraction of bacteria allows enough individuals to evade the host immune system and re-emerge periodically to sustain infection despite a strong immune response. However, it is also possible that the results of these experiments are artifacts. It may be that a small amount of intracellular bacteria are phagocytised by the host cell, but that the endosomes have not yet matured enough to kill them. The large inoculums used in these experiments (MOI of 100) more closely

resemble the situation when infection has already been well established and the host immune system overcome. The results of these conditions may therefore be more representative of how pathogens damage a host when infection has been established rather than how bacteria behave to initially colonize and maintain infection in a host. Both situations are relevant to the treatment of CF, but the underlying question of how early colonization is established is more relevant to the underlying defect in the CF lung.

MKLPS does not effect the rate of adhesion or phagocytosis.

MKLPS is an underacylated form of LPS from an E. coli mutant. This molecule has been shown to bind to, but not activate TLR4, thus it acts as a competitive inhibitor of TLR4 signaling. Pre-treatment of bronchial epithelia cells (S9) with MKLPS beginning 15 minutes before the addition of bacteria and continuing over the 3 hour period of incubation had no effect on the rate of adhesion or phagocytosis of *P. aeruginosa* strain PA01. This would seem to indicate that under the conditions tested in this study, TLR4 is not necessary for binding or phagocytosis of *P. aeruginosa* to epithelial cells; however, with the rate of invasion being as low as it was, it is possible that any difference was lost to noise. This result is not consistent with Pier *et al* (1997) since his group showed that LPS reduced the rate of phagocytosis of *P. aeruginosa*;

however it does agree with other studies that have shown *P. aeruginosa* to have a low frequency of invasion (phagocytosis) (Darling and Evans, 2001). It is possible that *P. aeruginosa* was able to bind to other receptors at the cell surface at a high enough frequency to mask the effect of TLR4 blockade (Figure 25). For example, TLR5 is a receptor for bacterial flagella and asialo-GM1 glycolipids have been studied for their ability to bind pili and promote adherance of *P. aeruginosa* (Saiman and Prince, 1993; Ramphal *et al*, 2008). It is possible that several or all of these receptors are involved in phagocytosis of *P. aeruginosa.* This would mirror the work done by Chignard *et al* (2008), who initially concluded that TLR5 was not involved in the host response to *P. aeruginosa* because of evidence from a TLR5 knockout mouse. However, in a later paper when the TLR5 knockout mice were crossed with TLR4 knockout mice, it was shown that both receptors are involved in the host response to *P. aeruginosa.* This redundancy that is built into the immune system complicates elucidation of the mechanisms responsible for host immune response.

P. aeruginosa PA01 forms antibiotic resistant microcolonies on pulmonary epithelial cells.

S9 cells grown on glass coverslips were inoculated with GFP-labeled *P. aeruginosa* strain PA01, treated to kill extracellular bacteria using the same techniques as the gentamicin protection assay, and viewed under a differential interference contrast fluorescent microscope. Observation of GFP-labeled *P. aeruginosa* after gentamicin treatment of inoculated S9 cells revealed the presence of extracellular bacterial microcolonies at the junctions between epithelial cells. This pattern of growth of *P. aeruginosa* has been observed in lungs from CF transplant patients and in animals infected in the laboratory (de Bentzmann *et al*, 1996; Haubler, 2003). However, this pattern has not been noted in the context of a gentamicin protection assay. The presence of extracellular bacteria after treatment with gentamicin indicates that these bacteria were resistant to the treatment and calls into question not only the results of the gentamicin protection assay performed in this study, but the results of several decades of experiments in which this assay has been used to quantify invasion / phagocytosis of *P. aeruginosa* and *B. cepacia* (Pier, 1996; Sajjan *et al*, 2006; Pirone *et al*, 2008). Since the presence of extracellular bacteria would increase the apparent frequency of invasion in these experiments, the resulting uncertainty about where those colonies came from makes that

data unreliable without performing control experiments in which microscopy is used to confirm effective killing of extracellular bacteria. I have not read a single paper in which this control was performed as part of a gentamicin protection assay which may explain why my results are different from those of Gerald Pier (1997). A common control is to lyse host cells before removal of antibiotic to confirm that the remaining bacteria are still susceptible to killing by the antibiotic. My results confirmed that lysis of epithelial cells before removal of gentamicin resulted in death of all remaining bacteria; however, if biofilm formation was necessary for antibiotic resistance, then lysing host cells would disrupt the biofilm rendering the bacteria susceptible to antibiotics again. Thus microscopy is the only way to definitively confirm antibiotic susceptibility in this assay.

The ORION iodide selective electrode can detect changes in cAMP stimulated iodide efflux in cells that overexpress CFTR, but may not be sensitive enough to accurately detect baseline readings in these cells or others expressing physiologically relevant levels of CFTR.

One method for measuring CFTR channel activity at the plasma membrane is with an ion efflux assay. In this experimental system, cultured cells are "loaded" by incubation in buffer with sodium iodide in place of sodium chloride, or in buffer

containing small amounts of radioactive 125-I. Repeated replacement of the buffer with iodide-free buffer allows intracellular iodide to efflux from the cells, which it does through CFTR, thus the rate of ion efflux is proportional to CFTR channel activity (Becq *et al*, 2003). Iodide can be quantified using ion selective electrodes, or with any means of detecting gamma radiation, depending on which technique is used.

Newer studies have used ion selective electrodes to measure iodide concentrations in the iodide efflux assay since this saves the hazard of working with radioactivity. According to the manufacturer, the ORION iodide selective electrode (ISE) should be able to detect 50nM iodide; however, in our lab, the ISE was not accurate below 60μM and was found to be unreliable below concentrations of 6μM, almost 100-fold higher than the reported limit listed by the manufacturer. However, rather than reading 0, the ISE continues to give readings that are greater than the actual concentration of iodide when measuring samples that are below its limit of detection. This is problematic in efflux studies since it can create an artificially elevated baseline efflux rate. The baseline values measured on the ISE are at or just below the experimentally determined limit of detection for the meter. When the concentration of iodide drops below the limit of detection of the meter, the ISE will show a higher concentration of iodide than what is really there, thus elevating and leveling the baseline efflux reading.

Depending on what cells type is used (cells that overexpress CFTR, or cells that express physiologically relevant levels of CFTR), stimulation of cells with cAMP elevating agents may be able to raise efflux rates above the lower limit of detection of the ISE. No detectable change in cAMP stimulated efflux rates was ever observed in S9 cells using this technique; however, the response from 2WT2 cells which overexpress CFTR was detectable using the ISE. Still, if future work is to be done with this model of instrument, trials with iodide concentrations below 10μM should be excluded from analysis.

Sustained exposure to LPS during the entire period of sample replacement causes a dose-dependent increase in CFTR channel activity.

Efflux rates were measured in 2WT2 epithelial cells using the radionuclide efflux technique. These cells were used in this experiment because they over-express human CFTR, giving them a robust response to cAMP elevating agents in the iodide efflux assay. Treatment of cells with LPS or live bacteria was either acute (10 minutes into sample replacement) or sustained (over the entire duration of sample replacement), but treatment with the cAMP elevating agents forskolin and IBMX was always acute.

Sustained treatment of cultures by the addition of a range of LPS concentrations to the sample replacement buffer resulted in a significant dose-dependent increases in cAMP stimulated efflux rate. The same result was obtained, although to a lesser extent, when cultures were treated with live *P. aeruginosa* PA14. These results are the opposite of those predicted by my hypothesis. If LPS is the antigen responsible for CFTR mediated phagocytosis of *P. aeruginosa*, then treatment of cultures with LPS should have reduced the abundance of CFTR at the plasma membrane resulting in a concomitant reduction in cAMP stimulated efflux rate.

The results of these experiments could be caused by increasing the activity of CFTR at the plasma membrane, or by increasing the abundance of CFTR at the plasma membrane. An increase in activity could be due to an increased probability of opening or a decrease in channel inactivation. The abundance of CFTR at the plasma membrane is determined by the rate at which the protein is removed and the rate at which it is added. If this is the reason for the increase in CFTR activity, then all that can be concluded from the efflux data is that the ratio of the removal rate to the recruitment rate decreased.

Acute treatment with LPS reduces CFTR channel activity.

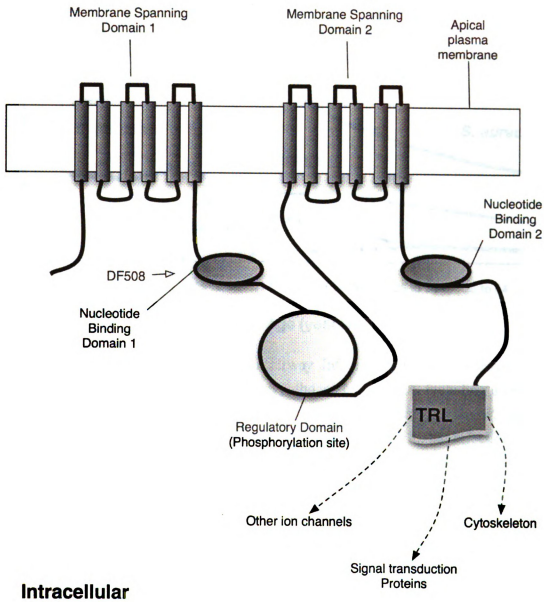
It is possible that treatment with LPS activates a signaling mechanism that increases the abundance of CFTR at the plasma membrane to replace molecules removed through phagocytosis. In this case, changes in CFTR channel activity in response to LPS treatment might show time dependence. To test this hypothesis, cells were treated acutely (10 minutes after the start of sample replacement, rather than over the entire duration of sample replacement) with a cocktail of LPS and cAMP elevating agents and compared to cultures treated with cAMP elevating agents alone. Acute treatment of cultures with a cocktail of LPS, forskolin and IBMX resulted in a significant reduction in AUC, but not in peak efflux when compared to cultures treated with just forskolin and IBMX. This indicates that CFTR channel activity is decreased in response to LPS, consistent with the hypothesis that CFTR is involved in LPS mediated phagocytosis. The biphasic nature of this response is also consistent with the presence of a mechanism to replace CFTR after it is removed as a result of LPS mediated phagocytosis.

The idea that CFTR can be removed as part of a phagocytic event in this short amount of time is not inconsistent with previous reports. Phagocytosis has been shown to begin within as little as 45 seconds of stimulation isolated macrophages, and can be completed in as little as 5 minutes (Matsumura *et al*, 2006). This conclusion also agrees

with the work done by Pier *et al* (1997) who showed not only greater rates of phagocytosis of *P. aeruginosa* in cells expressing wild-type CFTR compared to those expressing the ΔF508 CFTR, but also showed that phagocytosis could be reduced by the addition of purified LPS or a peptide identical to the first extracellular loop of CFTR.

Figure 1: Proposed structure of the CFTR showing its major functional domains. The N-terminus is on the left. Membrane spanning domains 1 and 2 are thought to form a pore that allows the passage of chloride ions, and membrane-spanning domain 2 is the site of protein glycosylation at its first extracellular loop. Binding of ATP at nucleotide binding domains 1 and 2 increases the channel's open probability. The location of the ΔF508 mutation is shown in nucleotide binding domain 1. Channel activation is regulated through phosphorylation of the central R-domain. The PDZ domain interacting motif (TRL or threonine, arginine, leucine) is shown at the C-terminus of the protein interacting with a PDZ domain that links the protein to other cellular components. Taken from Rowe *et al* (2001).

Extracellular



Intracellular

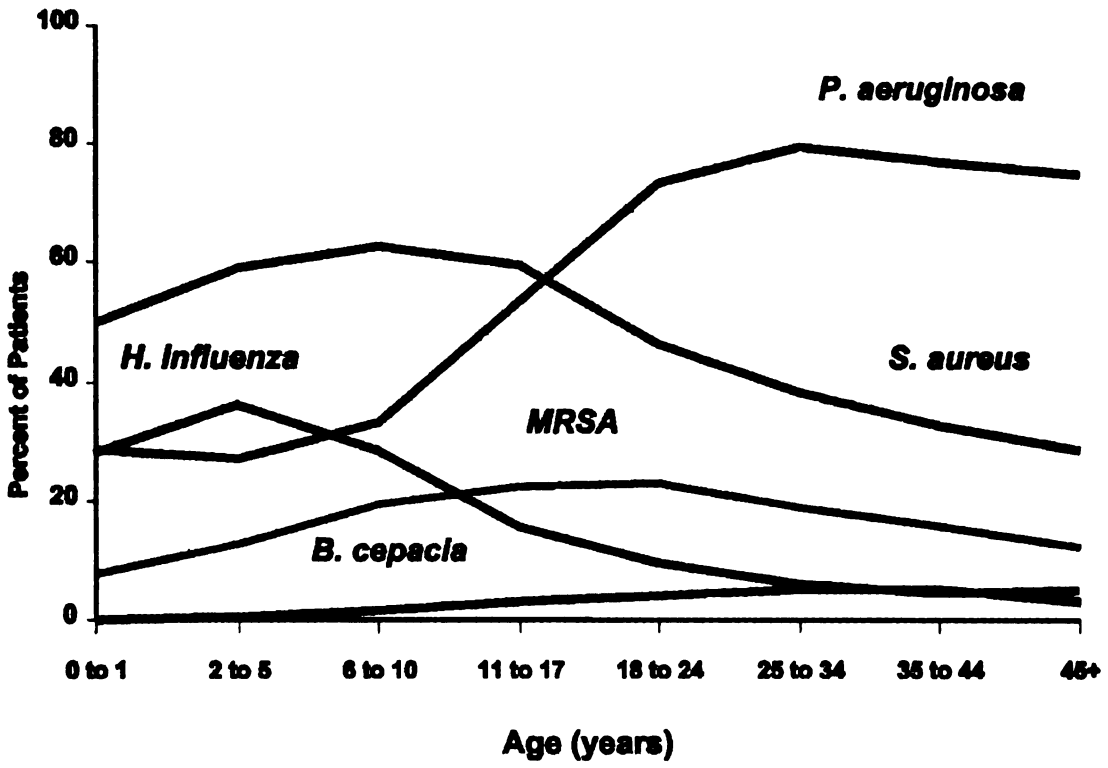
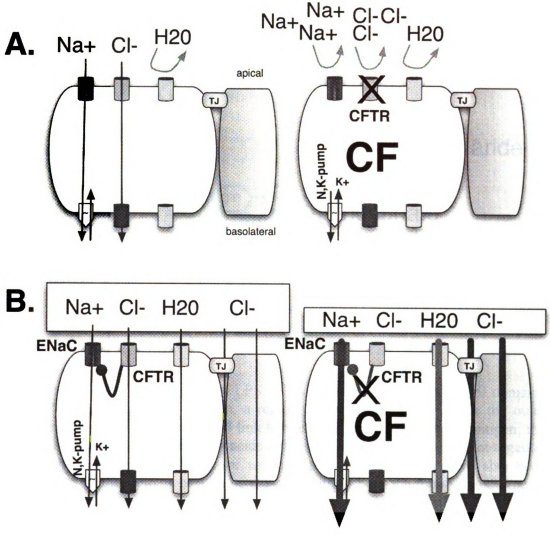


Figure 2: Age correlated incidence of airway infections in cystic fibrosis airways. Staphalococcus aureus is the most common pathogen early in life, including infections by methycilin resistant *S. aureus*, but is soon surpassed in frequency by *Pseudomonas aeruginosa*. *Haemophilus influenza* is more common early in life, but infections by other organisms like *Burkholderia cepacia* increase in frequency later in life. Taken from the Cystic Fibrosis Foundation (2005).

Figure 3: Depiction of two theories explaining how alterations in the ASL could lead to chronic bacterial infections. The high salt hypothesis shown in panel A postulates that airway epithelium are impermeable to water (H2O) and that chloride (Cl-) must move through CFTR to be reabsorbed. Since the sodium potassium (K+) ATPase (N,K-pump) maintains a constant gradient favoring the reabsorption of sodium, the amount of sodium that actually is reabsorbed is regulated by the amount of chloride that can be absorbed. Lack of functional CFTR causes prevents reabsorption of chloride thus keeping the concentration of sodium (Na+) chloride in the ASL higher than normal, a situation which has been shown to inactivate antimicrobial compounds. Panel B shows the low volume hypothesis. In this model, chloride can transit from the apical to basolateral suface by CFTR-independent mechanisms like tight junctions (TJ) between cells. However, the loss of CFTR-mediated reduction in sodium currents through the epithelial sodium channel (ENaC) results in hyperabsorption on water, resulting in a reduced ASL volume and impaired mucociliary transport. Taken from Wine (1999).


A.
Na+
Cl-
H20
apical
TJ
basolateral
Na+
Na+
Na+
Cl-
Cl-
Cl-
H20
CFTR
CF
N,K-pump
K+
B.
Na+ Cl- H20 Cl-
ENaC
CFTR
TJ
N,K-pump
K+
CF

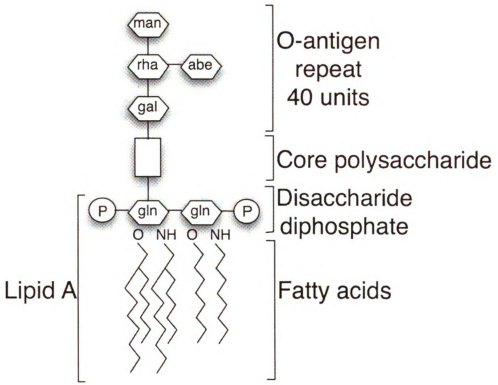


Figure 4: Conserved structure of LPS. This figure shows the conserved domains of LPS, including the O-antigen, a region of variable sugar residues; the outer core polysaccharide, a conserved link between the lipid A and the O-antigen; the core disaccharide to which fatty acids are linked; and the fatty acids that together with the core disaccharide make up the lipid A. Variations have also been described in the lipid A portion of the molecule in both the number and type of fatty acids. Taken From Fox (2009).

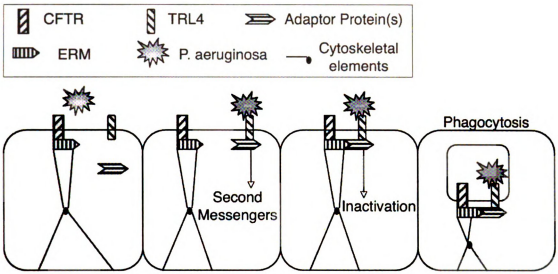


Figure 5: Model of a possible interaction between CFTR and TLR4 during phagocytosis of *P. aeruginosa*. *P. aeruginosa* is thought to interact with TLR4 through LPS located in its outer membrane. This activates signal transduction pathways that activate inflammation. I propose that the bacteria / receptor complex then forms a complex with CFTR to recruit cytoskeletal components and to lead to inactivation of the inflammatory signal. The complex is also able to phagocytise the bacterium.

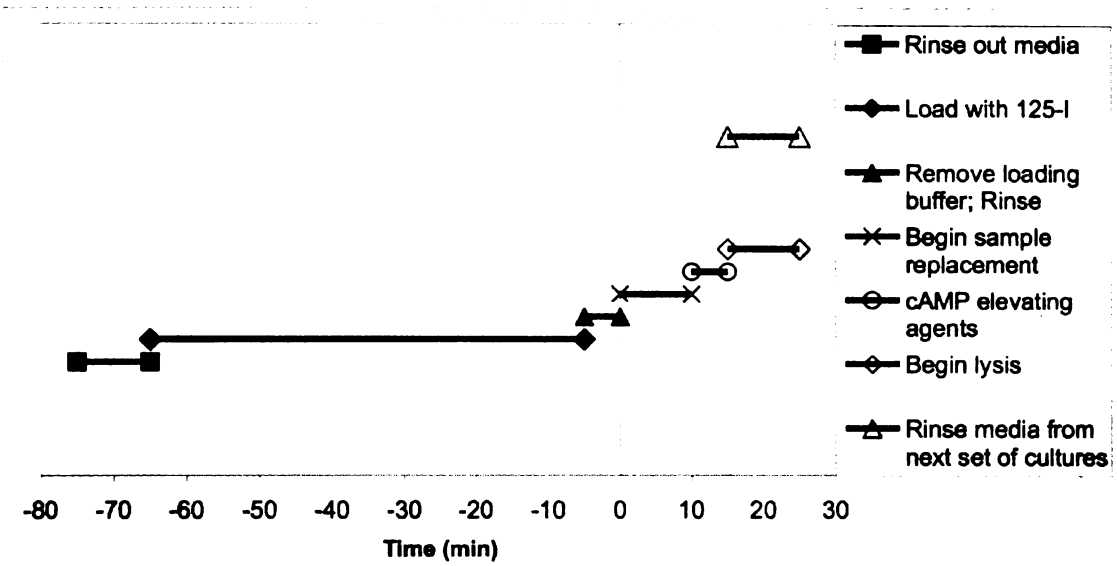


Figure 6: Timecourse of efflux experiments. Efflux experiments were run with sets of three 35mm dishes of 250,000 cultured 2WT2 cells at a time. First, growth media was removed and cultures were rinsed with efflux buffer. Next, cultures were incubated in efflux buffer containing 2μCi of 125-I for one hour. After incubation, the loading buffer was removed and cultures were rinsed four times with efflux buffer. Over the duration of sample replacement beginning at time 0, cultures were incubated in 1mL of efflux buffer for one minute intervals, after which the buffer was removed and replaced with fresh buffer. Application of cAMP elevating agents was achieved through sample replacement with buffer containing the cAMP elevating agents forskolin and IBMX beginning at 10 minutes into sample replacement. After five minutes of treatment with cAMP elevating agents, cultures were lysed by incubation in 1M sodium hydroxide for fifteen minutes. During lysis, the efflux experiment was set up in the next set of three 35mm dishes by removing growth media and repeating the treatment outlined above.

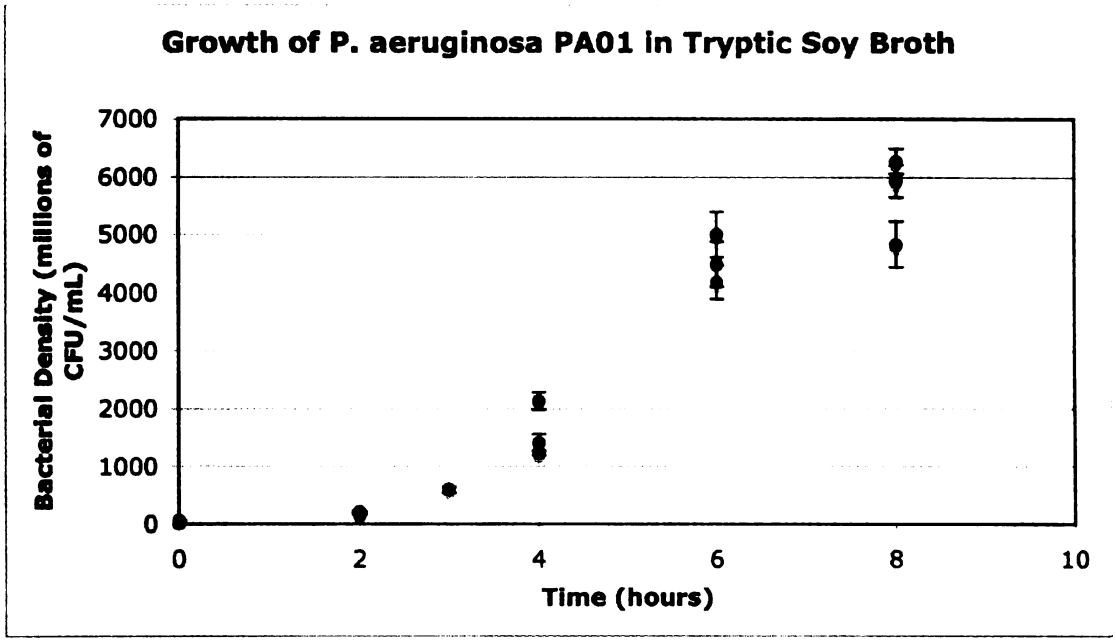


Figure 7: Growth curve of *P. aeruginosa* PA01 grown in tryptic soy broth showing entry into log phase growth. Samples were taken from 10mL cultures of *P. aeruginosa* PA01 that had been inoculated with 100μL from an overnight culture. Samples were diluted and plated on LB agar and incubated overnight. Colonies were counted and used to calculate bacterial cell density in relation to time. The growth curve shows entry into log-phase growth by 6 hours. Data are presented as mean ± SEM (n = 3 to 6).

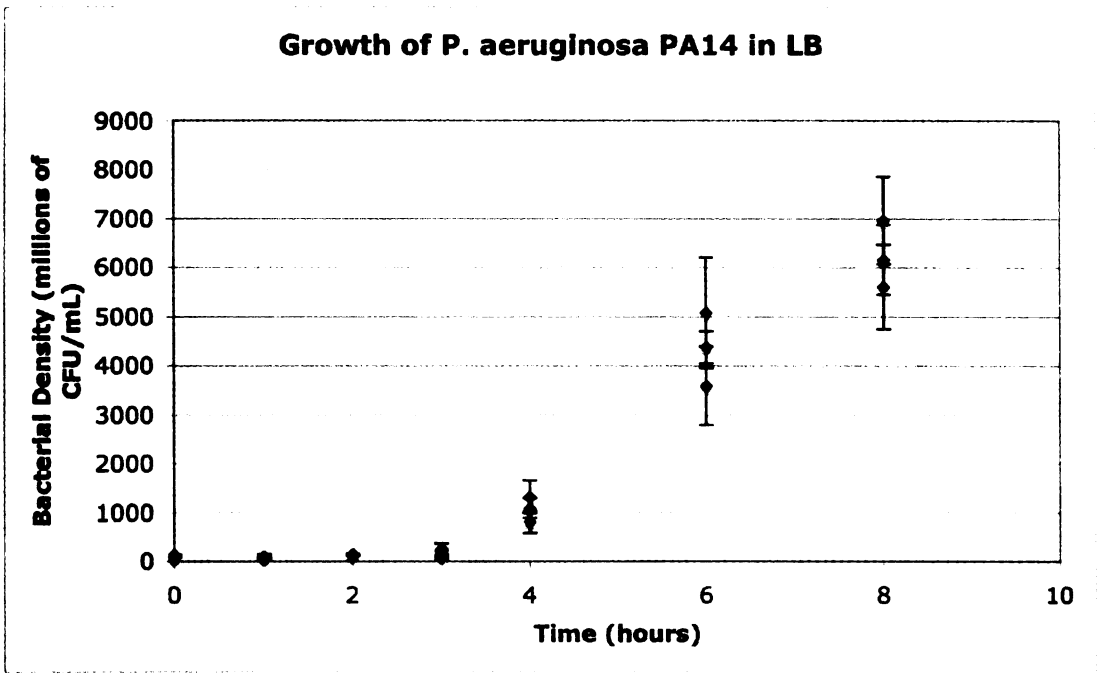


Figure 8: Growth curve of *P. aeruginosa* PA14 grown in LB showing entry into log phase growth. Samples were taken from 10mL cultures of *P. aeruginosa* PA14 that had been inoculated with 100μL from an overnight culture. Samples were diluted and plated on LB agar and incubated overnight. Colonies were counted and used to calculate bacterial cell density in relation to time. The growth curve shows entry into log-phase growth by 6 hours. Data are presented as mean ± SEM (n = 3 to 6).

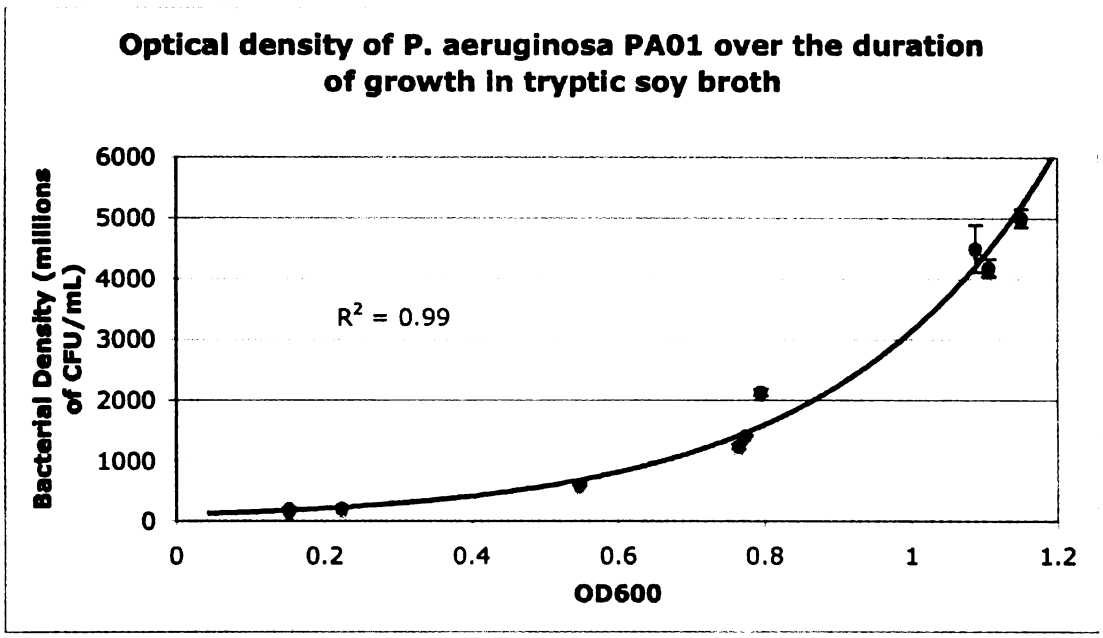


Figure 9: Optical density correlates to cell density in *P. aeruginosa* PA01. Samples were taken from 10mL cultures of *P. aeruginosa* PA01 that had been inoculated with 100μL from an overnight culture. The optical density of samples was measured at 600nm, and aliquots were diluted and plated on LB agar and incubated overnight. Colonies were counted and used to calculate bacterial cell density in relation to OD600. The equation of the best fit line was used to calculate the volume of bacteria needed to inoculate eukaryotic cell cultures. Data are presented as mean ± SEM (n = 3 to 6).

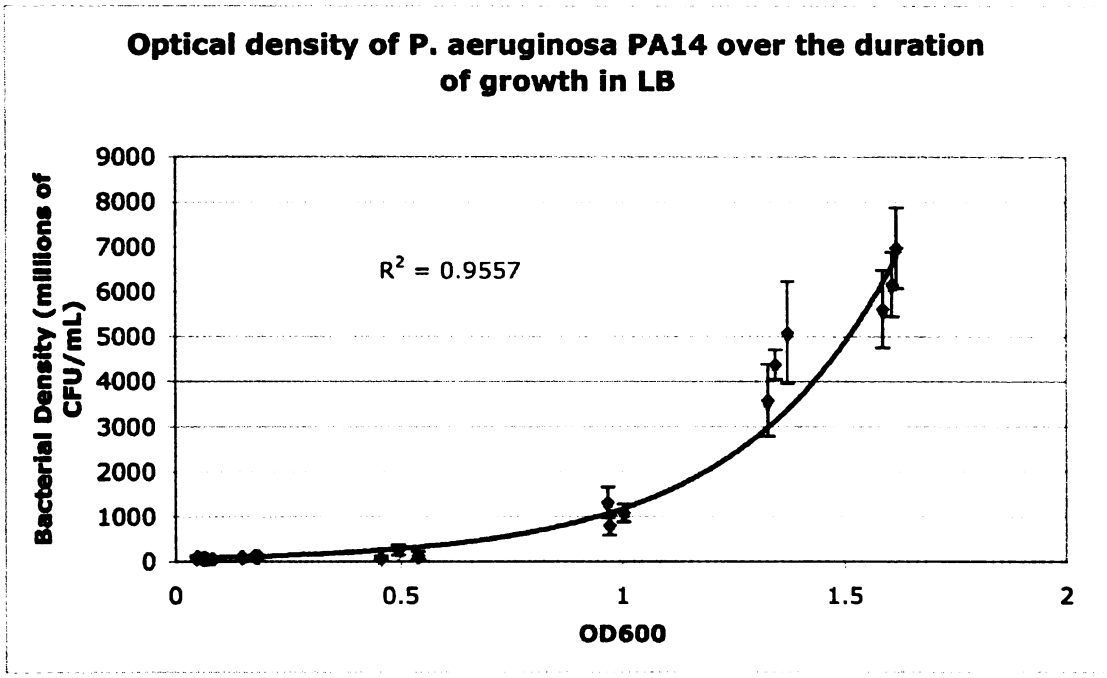


Figure 10: Optical density correlates to cell density in *P. aeruginosa* PA14. Samples were taken from 10mL cultures of *P. aeruginosa* PA14 that had been inoculated with 100μL from an overnight culture. The optical density of samples was measured at 600nm, and aliquots were diluted and plated on LB agar and incubated overnight. Colonies were counted and used to calculate bacterial cell density in relation to OD600. The equation of the best fit line was used to calculate the volume of bacteria needed to inoculate eukaryotic cell cultures. Data are presented as mean ± SEM (n = 3 to 6).

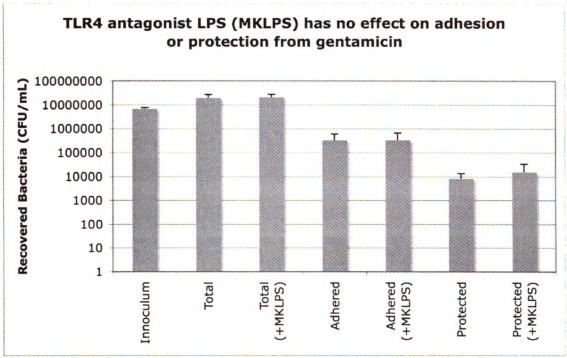


Figure 11: Treatment with the TLR4 antagonist MKLPS had no effect on recovery of total bacteria, adhered bacteria, or phagocytised (protected) bacteria. Immortalized human bronchial epithelial cells taken from a CF patient and transfected with wtCFTR (S9 cells) were grown to confluence in 24-well plates. Cultures were inoculated with 10 bacteria per epithelial cell and incubated for 3 hours. After incubation, cultures were treated to isolate total bacteria, adhered bacteria, and phagocytised (protected) bacteria. No significant difference was detected between the recovery rates of either set of bacteria in the presence of MKLPS. Data are presented as mean ± standard deviation (n = 41 to 56).

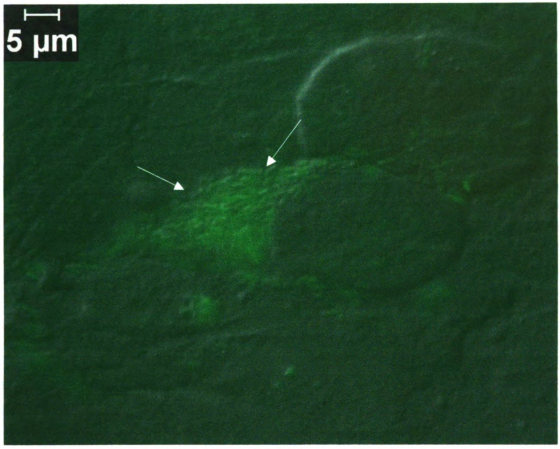


Figure 12: Merged image of fluorescent and differential interference contrast photograph of S9 epithelial cells incubated for 3 hours with an MOI of 1 GFP-labeled *P. aeruginosa* PA01 per S9 cell followed by treatment with 300µg/mL gentamicin for 1 hour. Extracellular bacteria were observed (arrows) adhered to the junctions between epithelial cells in antibiotic resistant microcolonies. Images in this thesis are presented in color.

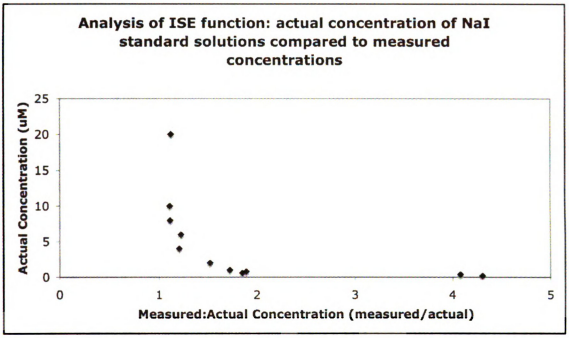


Figure 13: Analysis of ISE function when reading standards made in distilled water. The ORION iodide selective electrode (ISE) was calibrated with standards prepared in distilled water as per the manufacturer's instructions. A dilution series of standard sodium iodide solutions was prepared and the concentrations measured using the ISE. The ratios between the actual concentrations of sodium iodide and the measured concentrations of sodium iodide were calculated and show that the meter loses sensitivity at concentrations below 5μM sodium iodide. (n = 1)

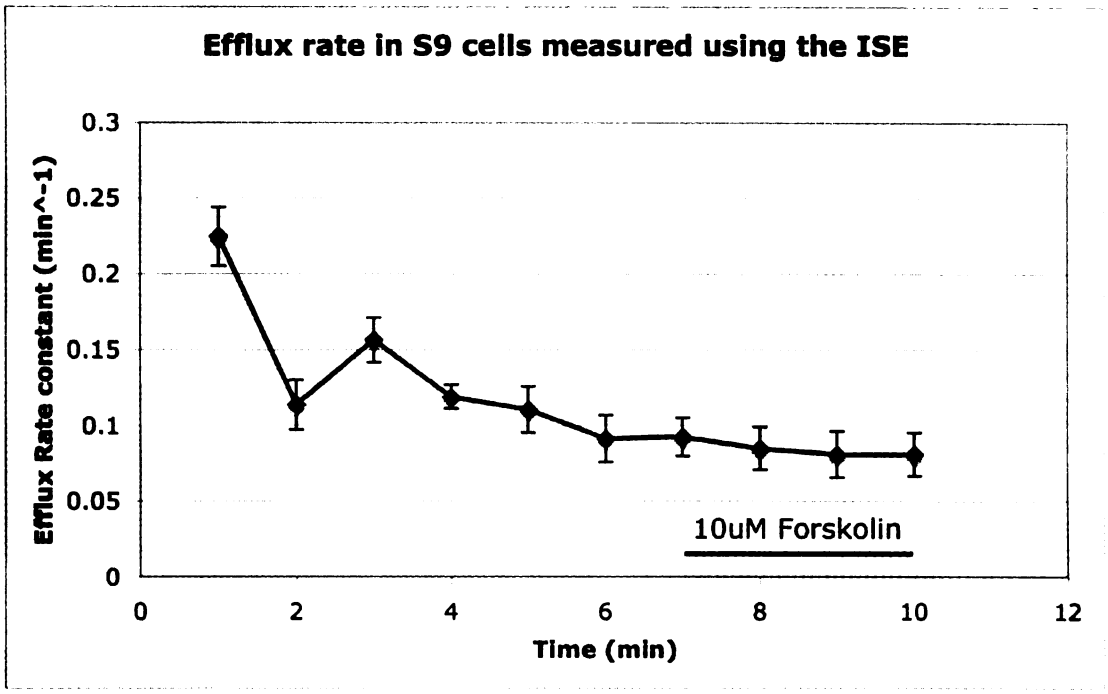


Figure 14: Efflux rate constant in S9 human bronchial epithelial cells stimulated with 10μM forskolin measured using the ISE. The ORION iodide selective electrode (ISE) was used to read samples from efflux experiments run with S9 cells using the sample replacement technique. Forskolin was unable to stimulate a detectable increase in efflux using this technique. Values are mean ± standard deviation (n = 4).

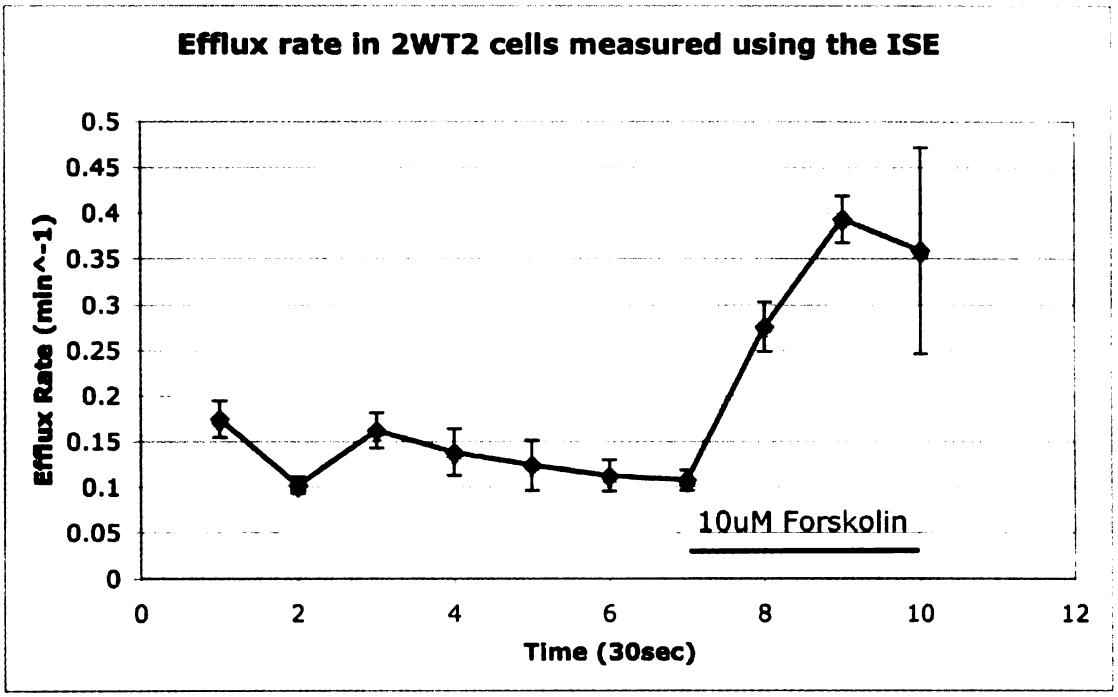


Figure 15: Efflux rate constant in 2WT2 mouse mammary epithelial cells stimulated with 10μM forskolin measured using the ISE. The ORION iodide selective electrode (ISE) was used to read samples from efflux experiments run with 2WT2 cells using the sample replacement technique. Forskolin stimulated an approximately 4-fold increase in efflux using this technique. Values are mean ± standard deviation (n = 4).

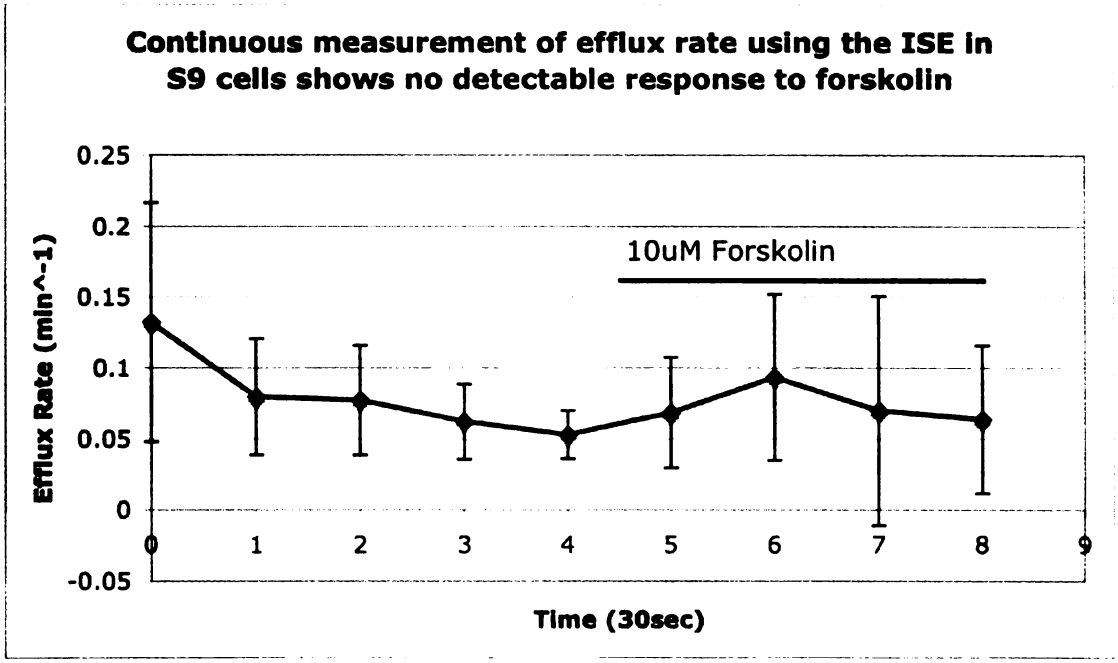


Figure 16: Efflux rate constant in S9 human bronchial epithelial cells stimulated with 10μM forskolin measured using the ISE. The ORION iodide selective electrode (ISE) was used to directly monitor the concentration of iodide in the extracellular buffer in cultured S9 cells. Forskolin was unable to stimulate a detectable increase in efflux using this technique. Values are mean ± standard deviation (n = 6).

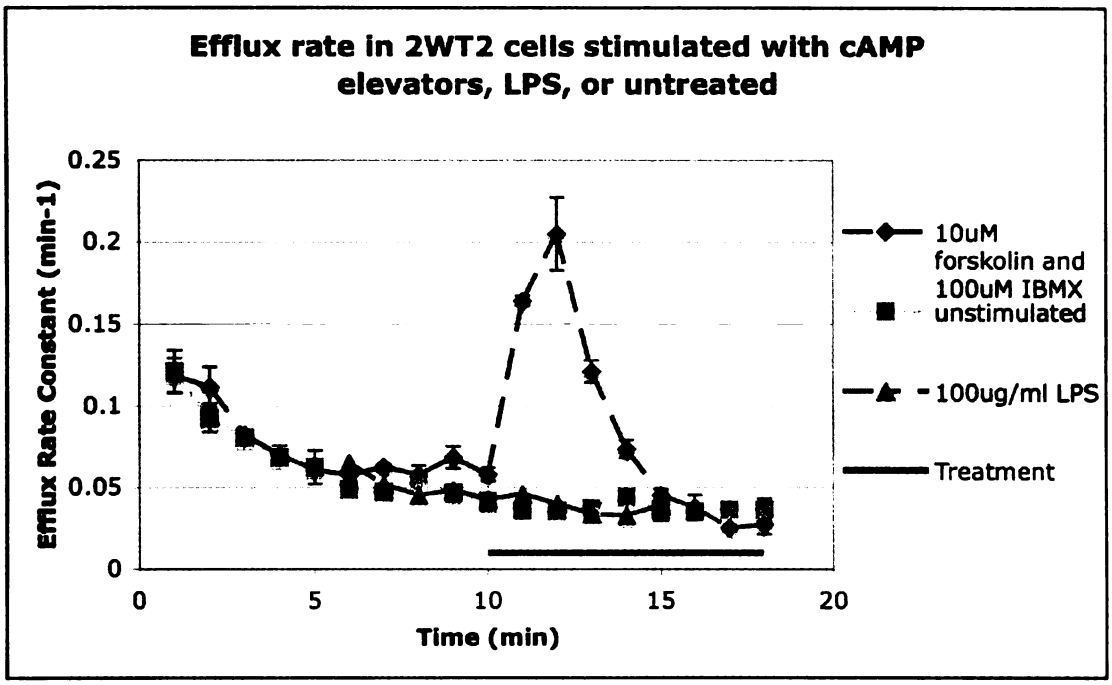


Figure 17: Efflux rate constant in 2WT2 mouse mammary epithelial cells stimulated with 10μM forskolin measured using radionuclide tracer technique. Experiments were done with cultured 2WT2 cells which were unstimulated, treated with 10μM forskolin and 100μM IBMX, or treated with 100μg/mL LPS over the duration indicated. Only cultures treated with the cAMP elevating agents forskolin annd IBMX showed a change in efflux rate. Values are mean ± SEM of the efflux rate constant (n = 3).

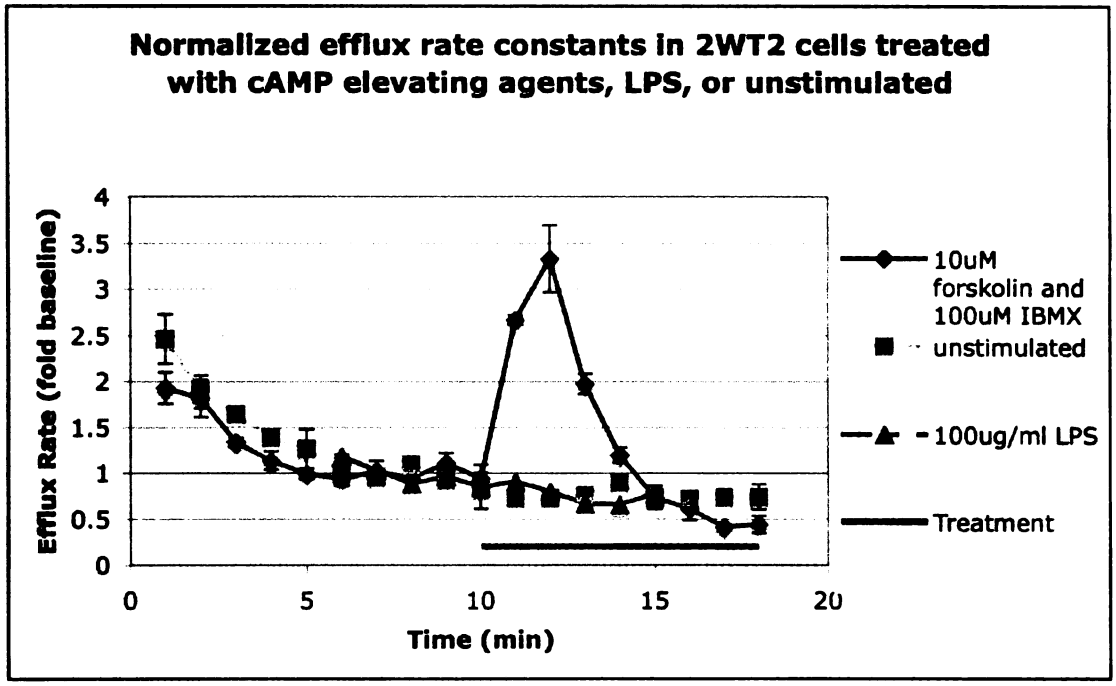


Figure 18: Normalized efflux rate constant in 2WT2 mouse mammary epithelial cells stimulated with 10μM forskolin measured using radionuclide tracer technique. Efflux experiments were done with cultured 2WT2 cells which were unstimulated, treated with 10μM forskolin and 100μM IBMX, or treated with 100μg/mL LPS over the duration indicated. Only cultures treated with the cAMP elevating agents forskolin annd IBMX showed a change in efflux rate. Values are mean ± SEM of the efflux rate constant normalized to the baseline efflux rate (n = 3).

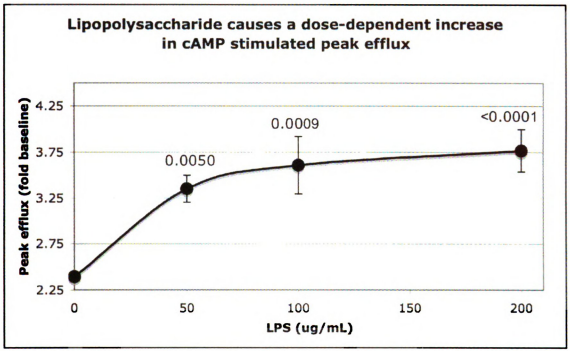


Figure 19: Dose response curve of CFTR mediated peak efflux in response to increasing concentrations of LPS. Cultured 2WT2 cells were treated with the concentration of LPS indicated for the entire duration of sample replacement. Values represent the average peak efflux normalized to baseline in response to treatment with the cAMP elevating agents forskolin and IBMX which were administered from 10 minutes into sample replacement until the end of the experiment. The numbers above each point indicate the p-value compared to cultures treated with only cAMP elevating agents. Values are mean ± SEM (n = 3 to 6).

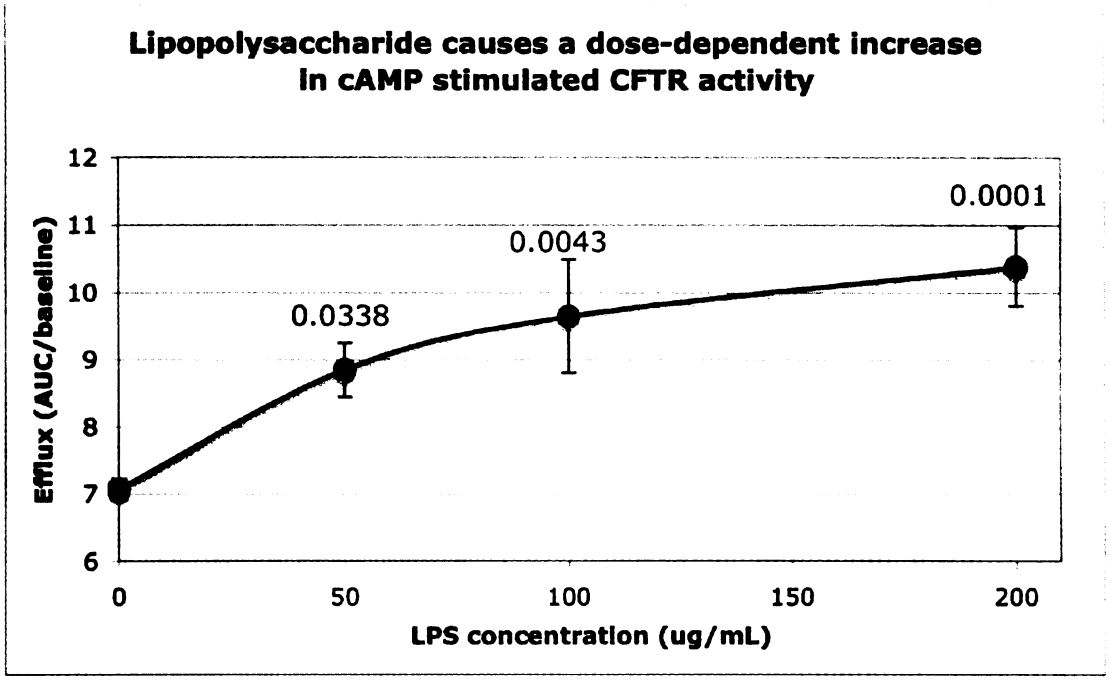


Figure 20: Dose response curve of CFTR mediated iodide efflux area under the curve in response to increasing concentrations of LPS. Cultured 2WT2 cells were treated with the concentration of LPS indicated for the entire duration of sample replacement. Values represent the average AUC normalized to baseline in response to treatment with the cAMP elevating agents forskolin and IBMX which were administered from 10 minutes into sample replacement until the end of the experiment. The numbers above each point indicate the p-value compared to cultures treated with only cAMP elevating agents. Values are mean ± SEM (n = 3 to 6). 0.0629 bt 200 and 50

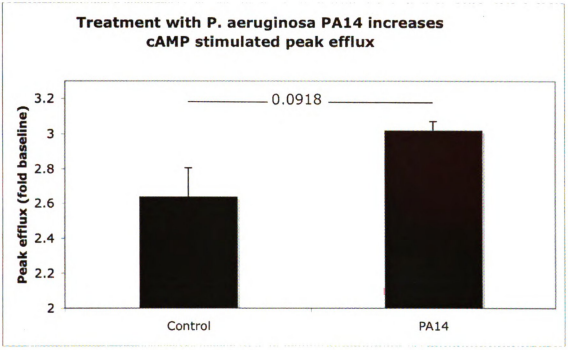


Figure 21: Increase in CFTR mediated peak efflux in response to treatment with *P. aeruginosa* PA14. Cultured 2WT2 cells were treated at an MOI of 100 bacteria / epithelial cell for the entire duration of sample replacement. Values represent the average peak efflux normalized to baseline in response to treatment with the cAMP elevating agents forskolin and IBMX which were administered from 10 minutes into sample replacement until the end of the experiment. The numbers above each point indicate the p-value compared to cultures treated with only cAMP elevating agents. Values are mean ± SEM (n = 3).

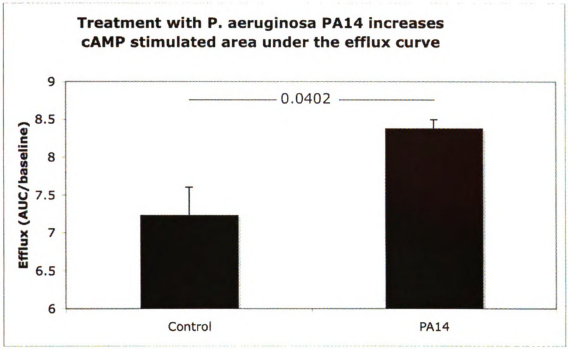


Figure 22: Increase in CFTR mediated iodide efflux area under the curve in response to treatment with *P. aeruginosa* PA14. Cultured 2WT2 cells were treated at an MOI of 100 bacteria / epithelial cell for the entire duration of sample replacement. Values represent the average AUC normalized to baseline in response to treatment with the cAMP elevating agents forskolin and IBMX which were administered from 10 minutes into sample replacement until the end of the experiment. Values are mean ± SEM (n = 3).

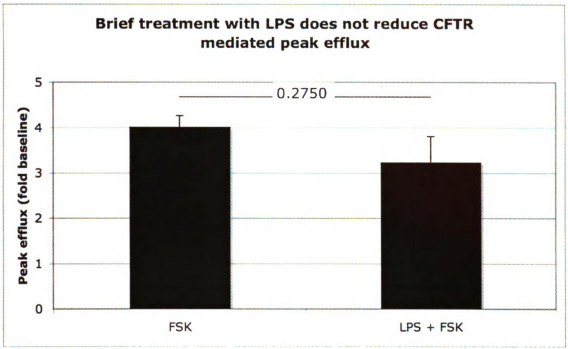


Figure 23: No significant difference in CFTR mediated peak efflux was detected in response to brief treatment with 100μg/mL LPS. Cultured 2WT2 cells were treated with a cocktail of 100μg/mL LPS, 10μM forskolin and 100μM IBMX beginning 10 minutes into sample replacement until the end of the experiment. Values represent the average peak efflux normalized to baseline in response to treatment with the cocktail. Values are mean ± SEM (n = 3).

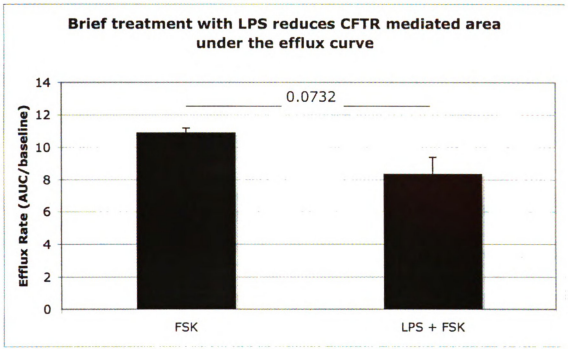


Figure 24: Decreased CFTR mediated iodide efflux area under the efflux curve was detected in response to brief treatment with 100μg/mL LPS. Cultured 2WT2 cells were treated with a cocktail of 100μg/mL LPS, 10μM forskolin and 100μM IBMX beginning 10 minutes into sample replacement until the end of the experiment. Values represent the average AUC normalized to baseline in response to treatment with the cocktail. Values are mean ± SEM (n = 3).

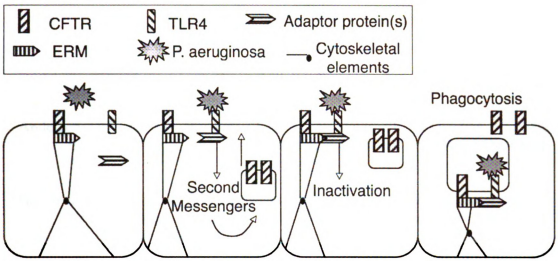


Figure: 25 Model of a possible interaction between CFTR and TLRs during phagocytosis of *P. aeruginosa*. *P. aeruginosa* is thought to interact with TLR4 through LPS located in its outer membrane and with TLR5 through flagella. This activates signal transduction pathways that activate inflammation. I propose that the bacteria / receptor complex then forms a complex with CFTR to recruit cytoskeletal components and to lead to inactivation of the inflammatory signal. One signal that is generated is responsible for recruiting CFTR to the membrane to replish channels removed during the phagocytic event. The complex is also able to phagocytise the bacterium.

REFERENCES

Akira S. 2003. Toll-like receptor signaling. JBC. 278-40: 38105-38108

Andersen DH. 1938. Cystic fibrosis of the pancreas and its relation to celiac disease. Am J Dis Child. 56: 344

Andersen DH and RG Hodges. 1946. Celiac syndrome v. genetics of cystic fibrosis of the pancreas with a consideration of etiology. Am J Dis Child. 72:62

Backhed F, S Normark, EKH Schweda, S Oscarson, A Richter-Dahlfors. 2003. Structural requirements for TLR4-mediated LPS signalling: a biological role for LPS modifications. Microbes and Infection. 5: 1057–1063

Baird RM, H Brown, AW Smith, ML Watson. 1999. Burkholderia cepacia is resistant to the antimicrobial activity of airway epithelial cells. Immunopharmacology 44: 267–272

Balfour-Lynn IM, R Dinwiddie. 1996. Role of corticosteroids in cystic fibrosis lung disease. Journal of the Royal Society of Medicine. 89: 8-13

Bals R, DJ Weiner, JM Wilson. 1999. The innate immune system in cystic fibrosis lung disease. JCI. 103-3: 303-307

Barasch J, Q Al-Awqati. 1993. Defective acidification of the biosynthetic pathway in cystic fibrosis. J Cell Sci Suppl. 17: 229-233

Becq F, C Auzanneau, C Norez, R Derand, L Bulteau-Pignoux. 2003. Radiotracer flux method to study CFTR channel activity: regulation, pharmacology and drug discovery. JOURNAL?

Blander JM, R Medzhitov. 2004. Regulation of phagosome maturation by signals from toll-like receptors. Science. 304: 1014-1018

Boucher RC, MJ Stutts, MR Knowles, L Cantley, JT Gatzy. 1986. Na+ transport in cystic fibrosis respiratory epithelia: abnormal basal rate and response to adenylate cyclase activation. J Clin Invest. 78: 1245-1252

Boucher RC. 2007. Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. Annual review of medicine. 58: 157-170

Bradbury NA, JA Cohn, CJ Venglarik, RJ Bridges. 1994. Biochemical and biophysical identification of cystic fibrosis transmembrane conductance regulator chloride channels as components of endocytic clathrin-coated vesicles. The Journal of Biological Chemistry. 269-11: 8296-8302

Burkholder WH. 1950. Sour skin, a bacterial rot of onion bulbs. Phytopathology. 40: 115–7

Burns JL, M Jonas, EY Chi, DK Clark, A Berger, A Griffith. 1996. Invasion of respiratory epithelial cells by Burkholderia (Pseudomonas) cepacia. Infect. Immun.64-10: 4054-4059

Cannon CL, MP Kowalski, KS Stopak, GB Pier. 2003. Pseudomonas aeruginosa–Induced Apoptosis Is Defective in Respiratory Epithelial Cells Expressing Mutant Cystic Fibrosis Transmembrane Conductance Regulator. Am J Respir Cell Mol Biol. 29: 188–197

Carmeli Y, N Troillet, GM Eliopoulos, MH Samore. 1999. Emergence of antibiotic- resistant Pseudomonas aeruginosa: comparison of risks associated with different antipseudomonal agents. Antimicrobial agents and chemotherapy. 43-6: 1379- 1382

Chandy G, M Grabe, HPH Moore, TE Machen. 2001. Proton leak in regulation of golgi pH in respiratory epithelial cells. Am J Cell Physiol. 281: C908-C921

Chappe V, T Irvine, J Liao, A Evagelidis, JW Hanrahan. 2005. Phosphorylation of CFTR by PKA promotes binding of the regulatory domain. The EMBO Journal. 24: 2730–2740

Chmiel JF, PB Davis. 2003. State of the art: why do the lungs of patients with cystic fibrosis become infected and why can't they clear the infection? Respiratory Research. 4: 8-20

Ciche TA, K Kim, B Kaufmann-Daszczuk, KCQ Nguyen, DH Hall. 2008. Cell Invasion and Matricide during Photorhabdus luminescens Transmission by Heterorhabditis bacteriophora Nematodes. Applied and Environmental Microbiology. 74-8: 2275– 2287

Conner SD, SL Schmid. 2003. Differential requirements for AP-2 in clathrin-mediated endocytosis. JCB. 162-5: 773-779

Cox AD. SG Wilkinson. 1991. Ionizing groups in lipopolysaccharides of Pseudomonas cepacia in relation to antibiotic resistance. Molecular Microbiology. 5-3: 641-646

Cystic Fibrosis Foundation. 2005. Patient Registry Annual Data Report, Bethesda, Maryland

Darling KE, TJ Evans. 2001. Novel Non-CFTR-Dependent Pseudomonas aeruginosa Epithelial Internalization Results in Intracellular Bacterial Replication and Transmigration without Host Cell Death. Abstr Intersci Conf Antimicrob Agents Chemother Intersci Conf Antimicrob Agents Chemother. Dec 16-19; 41: abstract no. B-973

Davis PD. 2006. Cystic fibrosis since 1938. Am J Respir Crit Care Med. 173: 475-482

de Bentzmann S, P Roger, F Dupuit, O Bajolet-Laudinat, C Fuchey, MC Plotkowski, E Puchelle. 1996. Asialo GM1 is a receptor for Pseudomonas aeruginosa adherence to regenerating respiratory epithelial cells. Infect. Immun. 64-5: 1582-1588

Delgado MA, JF Poschet, V Deretic. 2006. Nonclassical pathway of Pseudomonas aeruginosa DNA-induced interleukin-8 secretion in cystic fibrosis airway epithelial cells. Infection and immunity. 74-5: 2975-2984

Denis M, K Ramotar, K Vandemheen, E Tullis, W Ferris, F Chan, C Lee, R Slinger, SD Aaron. 2007. Infection With Burkholderia cepacia Complex Bacteria and Pulmonary Exacerbations of Cystic Fibrosis. Chest. 131: 1188-1196

Di A, ME Brown, LV Deriy, C Li, FL Szeto, Y Chen, P Huang, J Tong, AP Naren, V Bindokas, HC Palfrey, DJ Nelson. 2006. CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. Nature Cell Biology. 8-9: 933-944

di Sant'Agnese PA, RC Darling, GA Perera, E Shea. 1953. Abnormal electrolyte composition of sweat in cystic fibrosis of the pancreas. Clinical significance and relationship to disease. Pediatrics. 12: 549-563

Doerrler WT. 2006. Lipid trafficking to the outer membrane of Gram-negative bacteria. Molecular Microbiology. 60-3: 542–552

Donaldson SH, RC Boucher. 2006. Pathophysiology of Cystic Fibrosis. Ann Nestlé. 64: 101–109

Dudez T, F Borot, S Huang, BR Kwak, M Bacchetta, M Ollero, BA Stanton, M Chanson. 2008. CFTR in a lipid raft-TNFR1 complex modulates gap junctional intercellular communication and IL-8 secretion. Biomchimica et Biophysica Acta. 1783: 779- 788

Dunn KW, J Park, CE Semrad, DL Gelman, T Shevell, TE McGraw. 1994. Regulation of endocytic trafficking and acidification are independent of the cystic fibrosis transmembrane regulator. The Journal of Biological Chemistry. 269-7: 5336- 5345

Elborn JS, DJ Shale, JR Britton. 1991. Cystic fibrosis: current survival and population estimates to the year 2000. Thorax. 46: 881-885

Fearon DT, RM Locksley. 1996. The instructive role of innate immunity in the acquired immune response. Science. 272-5258: 50-54

Fletcher EL, SMJ Fleiszig, NA Brennnan. 1993. Lipopolysaccharide in adherence of Pseudomonas aeruginosa to the cornea and contact lenses. Invest Ophthalmol Vis Sci. 34: 1930-1936

Fox A. Bacteriology – Chapter Four: Cell envelope, spores and macromolecular biosynthesis. Microbiology and immunology online. Accessed 5/5/09

Ganguli A, AK Tripathy. 2002. Bioremediation of toxic chromium from electroplating effluent by chromate-reducing Pseudomonas aeruginosa A2Chr in two bioreactors. Appl Microbiol Biotechnol. 58: 416-420

Gelman MS, RR Kopito. 2002. Rescuing protein conformation: prospects for pharmacological therapy in cystic fibrosis. JCI. 110: 1591-1597

Gentzsch M, XB Chang, L Cui, Y Wu, VV Ozols, A Choudhury, RE Pagano, JR Riordan. 2004. Endocytic trafficking routes of wild type and dF508 cystic fibrosis transmembrane conductance regulator. Molecular Biology of The Cell. 15: 2684- 2696

Gerritsen J. 2000. Host defence mechanisms of the respiratory system. Peadiatric respiratory reviews. 1: 128–134

Greene, Carroll, Smith, Taggart, Devaney, Griffin, O'Neill, McElvaney. 2005. TLR- induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. Journal of Immmunology. 174: 1638-1646

Govan JRW, JE Hughes, P Vandamme. 1996. Burkholderia cepacia: medical, taxonomic and ecological issues. J. Med. Microbiol. 45: 395-407

Hanrahan JW. 2000. Airway plumbing. JCI. 105: 1343-1344

Hart AC, C Winstanley. 2002. Persistant and aggressive bacteria in the lungs of cystic fibrosis children. British Medical Bulletin. 61: 81-96

Haubler S, I Ziegler, A Löttel, F Götz, M Rohde, D Wehmhöhner, S Saravanamuthu, B Tümmler, I Steinmetz. 2003. Highly adherent small-colony variants of Pseudomonas aeruginosa in cystic fibrosis lung infection. J Med Microbiol. 52: 295-301

Hiemstra PS. 2007. Antimicrobial peptides in the real world: implications for cystic fibrosis. Eur Respir J. 29: 617–618

High N, J Mounier, MC Prevost, PJ Sansonetti. 1992. IpaB of Shigella flexneri causes entry into epithelial cells and escape from the phagocytic vacuole. The EMBO Journal. 11-5: 1991-1999

Hippenstiel S, B Opitz, B Schmeck, N Suttorp. 2006. Lung epithelium as a sentinel and effector system in pneumonia= molecular mechanisms of pathogen recognition and signal transduction. Respiratory Research. 7-97

Hubeau C, E Puchelle, D Gaillard. 2001. Distinct pattern of immune cell population in the lung of human fetuses with cystic fibrosis. J Allergy clin immunol. 108: 524- 529

Imundo L, J Barasch, A Prince, Q Al-Awqati. 1995. Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface. PNAS. 92: 3019-3023

Isles A, I Maclusky, M Corey, R Gold, C Prober, P Fleming, H Levison. 1984. Pseudomonas cepacia infection in cystic fibrosis: an emerging problem. J Pediatr. 104-2: 206-210

Jaques A, E Daviskas, JA Turnton, K McKay, P Cooper, RG Stirling, CF Robertson, PTP Bye, PN LeSouef, B Shadbolt, SD Anderson, B Charlton. 2008. Inhaled mannitol improves lung function in cystic fibrosis. Chest. 133: 1388-1396

Kerem B, JM Rommens, JA Buchanan, D Markiewicz, TK Cox, A Chakravarti, M Buchwald, LC Tsui. 1989. Identification of the cystic fibrosis gene: genetic analysis. Science. 245: 1073-1080

Knowles MR, MJ Stutts, A Spock, N Fischer, JT Gatzy, RC Boucher. 1983. Abnormal ion permeation through cystic fibrosis respiratory epithelium. Science. 221: 1067- 1070

Kowalski MP, GB Pier. 2004. Localization of cystic fibrosis transmembrane conductance regulator to lipid rafts of epithelial cells is required for Pseudomonas aeruginosa- induced cellular activation. The Journal of Immmunology. 172: 418-425

Kronborg G. 1995. Lipopolysaccharide (LPS), LPS-immune complexes and cytokines as inducers of pulmonary inflammation in patients with cystic fibrosis and chronic Pseudomonas aeruginosa lung infection. APMIS Suppl. 50: 1-30

Kube DM, D Fletcher, PB Davis. 2005. Relation of exaggerated cytokine responses of CF airway epithelial cells to PA01 adherence. Respiratory Research. 6-69

Kunzelman K. 2001. CFTR: interacting with everything? News Physiol. Sci. 16: 167-170

Long KL, KB Walsh. 1997. Iodide efflux measurements with an iodide-selective electrode: A non-radioactive procedure for monitoring cellular chloride transport. Methods in Cell Science. 19: 207–212

Loutet SA, RS Flannagan, C Kooi, PA Sokol, MA Valvano. 2006. A Complete Lipopolysaccharide Inner Core Oligosaccharide Is Required for Resistance of Burkholderia cenocepacia to Antimicrobial Peptides and Bacterial Survival In Vivo. Journal of Bacteriology. 188-6: 2073–2080

Lyczak JB, CL Cannon, GB Pier. 2002. Lung infections associated with cystic fibrosis. Clinical Microbiology Reviews. April 2002: 194-222

Mahenthiralingam E, TA Urban, JB Goldberg. 2005. The multifarious, multireplicon burkholderia cepacia complex. Nature. 3: 144-156

Mateu E, F Calafell, MD Ramos, T Casals, J Bertranpetit. 2002. Can a place of origin of the main cystic fibrosis mutations be identified? Am J Hum Genet. 70: 257-264

Matsui H, BR Grubb, R Tarran, SH Randell, JT Gatzy, CW Davis, RC Boucher. 1998. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airway disease. Cell. 95: 1005-1015

Matsumura K, K Orita, Y, Wakamoto, K Yasuda. 2006. Phagocytic response to fully controlled plural stimulation of antigens on macrophage using on-chip microcultivation system. Journal of Nanobiotechnology. 4:7

McClean S, M Callaghan. 2009. Burkholderia cepacia complex: epithelial cell–pathogen confrontations and potential for therapeutic intervention. Journal of Medical Microbiology. 58: 1–12

Morales FC, Y Takahashi, EL Krelmann, MM Georgescu. 2004. Ezrin-radixin-moesin (ERM)-binding phosphoprotein 50 organizes ERM proteins at the apical membrane of polarized epithelia. PNAS. 101-51: 17705-17710

Motley A, NA Bright, MNJ Seaman, MS Robinson. 2003. Clathrin-mediated endocytosis in AP-2-depleted cells. JCB. 162-5: 909-918

Muhlebach MS, W Reed, TL Noah. 2004. Quantitative cytokine gene expression in CF airway. Pediatr Pulmonol. 37-5: 393-399

Pier GB, P Ames. 1984. Mediation of the killing of rough, mucoid isolates of Pseudomonas aeruginosa from patients with cystic fibrosis by the alternative pathway of complement. J Infect Dis. 150-2: 223-8

Pier GB, M Grout, TS Zaidi, JC Olsen, LG Johnson, JR Yankaskas, JB Goldberg. 1996. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. Science. 271: 64-67

Pier GB, M Grout, TS Zaidi. 1997. Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of Pseudomonas aeruginosa from the lung. PNAS. 94: 12088-12093

Pier GB. 1999. Evolution of the ΔF508 CFTR mutation: Response. Trends in microbiology. 56-58

Pier GB. 2000. Peptides, Pseudomonas aeruginosa, polysaccharides and lipopolysaccharides – players in the predicament of cystic fibrosis patients. Trends in Microbiology. 8-6: 247-250

Pier GB, F Coleman, M Grout, M Franklin, DE Ohman. 2001. Role of alginate O acetylation in resistance of mucoid Pseudomonas aeruginosa to apsonic phagocytosis. Infection and Immunity. 69-3: 1895-1901

Pier G. 2007. Pseudomonas aeruginosa lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. Int. J Med Microbiol. 297(5): 277-295.

Pirone L, A Bragonzi, A Farcomeni, M Paroni, C Auriche, M Conese, L Chiarini, C Dalmastri, A Bevivino, F Ascenzioni. 2008. Burkholderia cenocepacia strains isolated from cystic fibrosis patients are apparently more invasive and more virulent than rhizosphere strains. Environmental Microbiology. 10-10: 2773 – 2784

Quinton PM. 1983. Chloride impermeability in cystic fibrosis. Nature. 301: 421-422

Ramphal R, V Balloy, J Jyot, Amrisha Verma, M Si-Tahar, M Chignard, 2008. Control of Pseudomonas aeruginosa in the Lung Requires the Recognition of Either Lipopolysaccharide or Flagellin. The Journal of Immunology. 181: 586-592

Riordan JR, JM Rommens, B Kerem, N Alon, R Rozmahel, Z Grzelczak, J Zielenski, S Lok, N Plavsic, JL Chou, ML Drumm, MC Iannuzzi, FS Collins, LC. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science. 245: 1066-1073

Rodley PD, U Romling, B Tummler. 1995. A physical genome map of the Burkholderia cepacia type strain. Molecular Microbiology. 17-1: 57-67

Romeo G, M Devoto, LJV Galietta. 1989. Why is the cystic fibrosis gene so frequent? Human genetics. 84: 1-5

Rommens JM, MC Iannuzzi, B Kerem, ML Drumm, G Melmer, M Dean, R Rozmahel, JL Cole, D Kennedy, N Hidaka, M Zsiga, M Buchwald, JR Riordan, LC Tsui, FS Collins. 1989. Identification of the Cystic Fibrosis Gene: Chromosome Walking and Jumping. Science. 245: 1059-1065

Rowe SM, S Miller, EJ Sorcher. 2005. Cystic fibrosis: mechanisms of disease. NEJM. 352: 1992-2001

Ruckdeschel K, G Pfaffinger, R Haase, A Sing, H Weighardt, G Hacker, B Holzmann, J Heesemann. 2004. Singaling of apoptosis through TLRs critically involves toll/IL-1 receptor domain-containing adapter inducing IFN-B, but not MyD88, in bacteria-infected murine macrophages. The Journal of Immunology. 173: 3320- 3328

Saiman L, A Prince. 1993. Pseudomonas aeruginosa pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. J Clin Invest. 92-4: 1875-80

Saini LS, SB Galsworthy, MA John, MA Valvano. 1999. Intracellular survival of Burkholderia cepacia complex isolates in the presence of macrophage cell activation. Microbiology. 145: 3465-3475

Sajjan US, MC, MA Karmali, JF Forstner. 1992. Binding of Pseudomonas cepacia to Normal Human Intestinal Mucin and Respiratory Mucin from Patients with Cystic Fibrosis. J. Clin. Invest. 89: 648-656

Sajjan U, Wu Y, Kent G, Forstner J. 2000. Preferential adherence of cable-piliated burkholderia cepacia to respiratory epithelia of CF knockout mice and human cystic fibrosis lung explants. J Med Microbiol. 49-10: 875-885.

Sajjan US, JH Yang, MB Hershenson, JJ LiPuma. 2006. Intracellular trafficking and replication of Burkholderia cenocepacia in human cystic fibrosis airway epithelial cells. Cell Microbiol. 8-9: 1456-1466

Seamon KB, W Padgett, JW Daly. 1981. Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. PNAS. 78-6: 3363-3367

Sherertz RJ, FA Sarubbi. 1983. A three year study of nosocomial infections associated with Pseudomonas aeruginosa. Journal of clinical microbiology. July 1983: 160- 164

Schreiber R, A Hopf, M Mall, R Greger, K Kunzelmann. 1999. The first-nucleotide binding domain of the cystic-fibrosis transmembrane conductance regulator is important for inhibition of the epithelial Na+ channel. PNAS. 96: 5310–5315

Schroeder TH, MM Lee, PW Yacono, CL Cannon, AA Gerceker, DE Golan, GB Pier. 2002. CFTR is a pattern recognition molecule that extracts Pseudomonas aeruginosa LPS from the outer membrane into epithelial cells and activates NF- κB translocation. PNAS. 99: 6907-6912

Smith JJ, SM Travis, IP Greenberf, MJ Welsh. 1996 Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell. 85: 229-236

Smith RS, BH Iglewski. 2003. Pseudomonas aeruginosa quorum sensing as a potential antimicrobial target. JCI. 112: 1460-1465

Somerville JE, L Cassiano, B Bainbridge, MD Cunningham, RP Darveau. 1996. A novel Escherichia coli lipid A mutant that produces an antiinflammatory lipopolysaccharide. J Clin Invest. 97-2: 359–365

Soong G, B Reddy, S Sokol, R Adamo, A Prince. 2004. TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. JCI. 113: 1482-1489

Sun F, Z Mi, SB Condliffe, CA Bertrand, X Gong, X Lu, R Zhang, JD Latoche, JM Pilewski, PD Robbins, RA Frizzell. 2008. Chaperone displacement from mutant cystic fibrosis transmembrane conductance regulator restores its function in human airway epithelia. FASEB. 22-9: 3225-3263

Taminiau B, M Daykin, S Swift, ML Boschiroli, A Tibor, P Lestrate, X De Bolle, D O'Callaghan, P Williams, JJ Letesson. 2002. Identification of a Quorum-Sensing Signal Molecule in the Facultative Intracellular Pathogen Brucella melitensis. Infection and Immunity. 70-6: 3004–3011

Tirouvanziam R, S de Bentzmann, C Hubeau, J Hinrasky, J Jacquot, B Peault, E Puchelle. 2000. Inflammmation and infection in naïve human cystic fibrosis airway grafts. Am J Respir Cell Mol Biol. 23: 121-127

Tirouvanziam R, I Khazaal, B Peault. 2002. Primary inflammation in human cystic fibrosis small airways. Am J Lung Cell Mol Physiol. 283: L445-451

Tomlin KL, RJ Malott, G Ramage, DG Storey, PA Soko, H Ceri. 2005. Quorum-Sensing Mutations Affect Attachment and Stability of Burkholderia cenocepacia Biofilms. Applied and environmental microbiology. 71-9: 5208-5218

Traub LM. 2003. Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. JCB. 163-2: 203-208

Tyler SD, KR Rozee, WM Johnson. 1996. Identification of IS1356, a New Insertion Sequence, and Its Association with IS402 in Epidemic Strains of Burkholderia cepacia Infecting Cystic Fibrosis Patients. Journal of Clinical Microbiology. 34-7: 1610–1616

Unanue ER, PM Allen. 1987. The Basis for the Immunoregulatory Role of Macrophages and Other Accessory Cells. Science. 236-4801: 551-557

VanDyke RW, KV Root, JH Schreiber, JM Wilson. 1992. Role of CFTR in lysosomal acidification. Biochemical and Biophysical Research Communications. 184-1: 300-305

VanKooykY, TBH Geijtenbeek. 2006. Toll-like receptors keep antigen sorting on the right track. Immunity. 10/25/06: 525-527

Vaudaux P, FA Waldvogel. 1979. Gentamicin Antibacterial Activity in the Presence of Human Polymorphonuclear Leukocytes. Antimicrobial agents and chemotherapy. Dec: 743-749

Venkatakrishnan A, AA Stecenko, G King, TR Blackwell, KL Brigham, JW Christman, TS Blackwell. 2000. Exagerated activation of nuclear factor-kB and altered IkB-B processing in cystic fibrosis bronchial epithelial cells.

Vij N, S Mazur, PL Zeitlin. 2009. CFTR Is a Negative Regulator of NFkB Mediated Innate Immune Response. PLoS ONE. 4-2: e4664

Wang Z, EJ Bilsky, D Wang, F Porreca, W Sadée. 1999. 3-Isobutyl-1-methylxanthine inhibits basal μ-opioid receptor phosphorylation and reverses acute morphine tolerance and dependence in mice. European Journal of Pharmacology. 371-1: 1-9

Weixel KM, NA Bradbury. 2001. Endocytic adaptor complexes bind the C-terminal domain of CFTR. Eur J Physiol. 443. S70-S74

Welsh MJ, JJ Smith. 1995. Cystic fibrosis. Sci Am. 273: 52-59

Wilson JM. 1995. Gene therapy for cystic fibrosis. JCI. 96: 2547-2554

Wine JJ. 1999. The genesis of cystic fibrosis lung disease. JCI. 103: 309-312

Wu Q, L Zhong, MW Verghese, SH Randell. 2005. Airway epithelial cell tolerance to Pseudomonas aeruginosa. Respiratory Research. 6-26

Yabuuchi E, Y Kosako, H Oyaizu, I Yano, H Hotta, Y Hashimoto, T Ezaki, M Arakawa. 1992. Proposal of Burkholderia gen. nov. and transfer of seven species of the genus Pseudomonas homology group II to the new genus, with the type species Burkholderia cepacia (Palleroni and Holmes 1981) comb. nov. Microbiol Immunol. 36-12: 1251-1275

Zhang P, WR Summer, GJ Bagby, S Nelson. 2000. Innate immunity and pulmonary host defense. Immunological Reviews. 173: 39–51