Discriminating Bacterial Derived Products by Airway Epithelial Cells via p38 Mitogen Activated Protein Kinase

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ABBREVIATIONS & SYMBOLS

ΔF508	Three-nucleotide deletion at the 508th codon in CFTR causing the deletion of a phenylalanine residue
µL	Microliter
µm	Micrometer
µM	Micromolar
3OC12-HSL	N-3-oxo-dodecanoyl-homoserine lactone
5-LOX	Archidonate 5-lipooxygenase
A	Adenine
AEC	Airway Epithelial Cells
AlgT	An activator of alginate production
ANOVA	Analysis of Variance
AP-1	Activator protein 1
ASK1	Apoptosis signal-regulating kinase 1
ATCC	American Type Culture Collection
ATF	Activating transcription factors
ATF2	Activator of Transcription 2
ATP	Adenosine tri-phosphate
BAL	Bronchial alveolar lavage
BEAS-2B	Immortalized human bronchial epithelial cells
bFGF	Basal Fibroblast Growth Factor
BiFC	Bimolecular fluorescence complementation
Bio.	Biofilm
BIRB0796	p38 MAPK inhibitor
BSA	Bovine Serum Albumin
C	Cytosine
C4-HSL	N-butanoyl-homoserine lactone
$CaCl_2$	Calcium Chloride
CARD	Caspase recruitment domain
CC10	Clara Cells 10kd Protein

cDNA	Complimentary deoxyribonucleic acid
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis transmembrane conductance regulator
Ci	Curri
Cl^-	Chloride
cm	Centimeters
CM	Conditioned media
CnT17	Airway epithelium Medium, Cell-n-Tec
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CpG	Cystine-phosphate-Guanine
CSF	Colony-stimulating factors
CSF-1	Colony Stimulating Factor 1
CTL	C-type lectins
CuFi	Airway epithelial cell line derived from lung of a 14-year-old female patient with cystic fibrosis homozygous for the CFTR∆F508 mutation
CXCL8	Chemokine C-X-C motif (IL-8)
DED	Death effector domain
DMEM	Dulbecco's Modified Essential Medium
Dnase	DNA cutting enzyme
dsDNA	Double stranded Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
eIF4F	Eukaryotic initiation factor 4F
ELISA	Enzyme linked immunosorbtion assay
EPS	Exopolymeric substances
ERK	Extracellular signal-regulated protein kinases
FBS	Foetal bovine serum
$FeSO_4 \cdot 7H_2O$	Iron(II) Sulfate heptahydrate

Flg	Flagellin
G	Guanine
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPCR	G-protein coupled receptors
GRO	Growth Regulated Oncogene
GST	Glutathione S-transferases
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric acid
hr	Hour
HSL	Homoserine lactones
Hsp70	Heat shock protein 70
IC50	Concentration for 50% inhibition
IFN	Interferons
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IKK	IκB Kinase
IL	Interleukin
IL-R	Interleukin Receptor
iNOS	Inducible nitric oxide synthase
IRAK	IL-1R-associated kinase
IRF	Interferon Regulator Factors
JNK	c-Jun N-terminal kinase
K63-pUb	Lysine 62 polyubiquitin chains
kV	Kilovolts
LasI	Las QS system inducer
LasR	Las QS system receptor
LBP	LPS binding protein
LGP2	Laboratory of genetics and physiology gene 2
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeats

LTA	Lipoteichoic acid
LUBAC	Linear Ubiquitin Assembly Complex
M	Molar
MAP2K or MKK	MAPK kinases
MAP3K	MAPK Kinase Kinases
MAPK	Mitogen activated protein kinase
MAPKAP	Mitogen activated protein kinase
MAPKAPK2	MAPK activated protein kinase-2
MBP	Microtubule associated protein
MCP	Monocyte Chemotactic Protein
M-CSF	Macrophage Colony Stimulating Factor
MDA5	Melanoma differentiation associate protein 5
MEKK3	MAPK/ERK kinase kinase 3
$MgCl_2$	Magnesium Chloride
min	Minutes
mL	Millilitre
mm	Millimetre
mM	Millimolar
MNK	MAPK-interacting kinase
mRNA	Messenger ribonucleic acid
MSD	Membrane spanning domains
MSK	Mitogen and stress activated kinases
Muc.	Mucoid
mucA	Mucoid supressing gene in P. aeruginosa
Myd88	Myeloid differentiation primary-response protein
MβCD	Methyl-β-cyclodextrin
nAb	Neutralizing antibody
NACHT	Nucleotide-binding and oligomerization
NBD	Nucleotide binding domain
NEMO	NfκB essential modifier
NLR	NOD-like receptors

NLS	Nuclear localization signal
nm	Nanometer
nM	Nanomolar
NO	Nitrogen Oxide
NOD	Nucleotide-binding oligomerization domain
NuLi	Human airway epithelial cell line derived from a normal lung of a 36-year-old male patient
^{o}C	Degrees Celsius
OD	Optical Density
PQS	*P. aeruginosa* Quinolone Signaling
P/S	100U penicillin G and 100 μg/mL streptomycin
PA	*Pseudomonas aeruginosa*
PACF508	A stable mucoid clinical isolate from the sputum of a patient with CF
PAMPs	Pathogen Associated Molecular Patterns
PAO1	Laboratory strain of *Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
PCL	Periciliary Layer
PDGF	Platelet derived growth factor
Pep	Peptone
pH	Presence of Hydrogen
Plk.	Planktonic
PRR	Pathogen Recognition Receptors
PsaDM	*P. aeruginosa* diffusible material
PVC	Polyvinyl Chloride
qPCR	Quantitative real time polymerase chain reaction
QS	Quorum Sensing
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
RHD	Rel homology domain
RhlI	Rhl QS inducer
RhlR	Rhl QS system Receptor
RIG	Retinoic acid inducible gene
RIP-1	Receptor-interacting protein 1

RLU	Relative luciferase units
RPM	Rotations per minute
RSK	Ribosomal S6 kinases
RSV	Respiratory Syntical Virus
s	Seconds
Sap1	SRF accessory protein 1
SAPK	Stress activated protein kinase
SCF	Stem Cell Factor
SCFM	Synthetic Cystic Fibrosis Media
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Small interfering Ribonucleic acid
SP-A	Surfactant Protein
ssRNA	Single stranded Ribonucleic acid
T	Thymine
TAB	TAK1 binding protein
TAK1	TGFβ-activated kinase 1
TAK1	Transforming growth factor β activated kinase 1
TFs	Transcription factors
TGF	Transforming Growth Factor
Thr	Threonine
TIR	Toll-IL1R domains
TLR	Toll-like Receptors
TNF	Tumor necrosis factor
TPL2	Tumour progression locus 2
TRAF6	Tumor necrosis factor receptor–associated factor-6
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain–containing adaptor inducing IFN-β
Tyr	Tyrosine
U	Uracil
v/v	Volume to volume

VEGF	Vascular endothelial growth factor
w/v	Weight to volume
WT	Wild type
xg	Times gravity

ABSTRACT

The airway epithelium represents the first point of contact between inhaled pathogens and the host environment. Typically the host exhibits a number of innate immune defenses, including efficient mucociliary clearance of pathogens that act to keep the lungs a relatively sterile environment. When these defenses are compromised however, infections can occur in the lung and cause increased inflammatory responses. The airway epithelium itself can recognize pathogenic material to induce an inflammatory response. Patients that have Cystic Fibrosis (CF) show impaired mucociliary clearance and are more susceptible to respiratory infections. In fact, the majority of patients become chronically infected with *Pseudomonas aeruginosa*, a gram negative, opportunistic pathogen, by their teenage years. The presence of a chronic infection leads to increased inflammation in the lungs, increased morbidity and ultimately leading to respiratory failure, which is the leading cause mortality in these patients.

The chronic infections observed in these CF patients exhibit a number of phenotypic changes that have not been extensively studied for their ability to initiate an immune response in the airway epithelium. Chronic infections are believed to be established by bacteria that form

biofilms, which exhibit striking phenotypic differences to their planktonic, free swimming counterparts. The majority of existing studies looking at and identifying virulence determinants of *P. aeruginosa* on the airway epithelium have been done using purified ligands prevalent on planktonic bacteria. In this thesis I use a biofilm model to identify the differences in activation of airway epithelial cells to planktonic and biofilm derived materials.

Additionally, the majority of *P. aeruginosa* strains isolated from chronically infected patients have adapted to exhibit a mucoid phenotype. The expression of a mucoid phenotype is protective in the CF environment, however various potential virulence determinants can also be differentially regulated. I will examine how this switch to a mucoid phenotype impacts activation of inflammatory mediators in airway epithelial cells.

Taken together, the approaches I use in this thesis better identify the determinants that are important in regulating inflammatory mediator activation in airway epithelial. By understanding the key pathways involved in host defense, new methods in detecting key virulence determinants can be developed.

ABRÉGÉ

L'épithélium des voies respiratoires représente le premier point de contact entre les agents pathogènes inhalés et l'environnement hôte. Typiquement l'hôte présente un certain nombre de défenses immunitaires innées, y compris la clairance mucociliaire efficace des agents pathogènes qui agissent pour maintenir les poumons d'un environnement relativement stérile. Lorsque ces moyens de défense sont compromises cependant, les infections peuvent se produire dans les poumons et provoquer une augmentation des réponses inflammatoires. L'épithélium des voies aériennes lui-même peut reconnaître matériau pathogène pour induire une réponse inflammatoire. Les patients qui ont de la fibrose kystique (CF) montrent une altération de la clairance mucociliaire et sont plus sensibles aux infections respiratoires. En fait, la majorité des patients deviennent chroniquement infectés par *Pseudomonas aeruginosa*, un Gram négatif, pathogène opportuniste, par leurs années d'adolescence. La présence d'une infection chronique conduit à une inflammation accrue dans les poumons, l'augmentation de la morbidité et conduisant finalement à une insuffisance respiratoire, qui est la principale cause de mortalité chez ces patients.

Les infections chroniques observées chez ces patients atteints de mucoviscidose présentent un certain nombre de modifications phénotypiques qui n'ont pas été largement étudiés pour leur capacité à déclencher une réponse immunitaire dans l'épithélium des voies aériennes. Les infections chroniques sont soupçonnés d'être établie par des bactéries qui forment des biofilms, qui présentent des différences frappantes phénotypiques à leur planctoniques, sans majorité piscine counterparts.The des études existantes qui cherchent à identifier et déterminants de la virulence de *P. aeruginosa* sur l'épithélium des voies respiratoires ont été réalisées en utilisant purifiée ligands répandue sur les bactéries planctoniques. Dans cette thèse, j'utilise un modèle de biofilm à identifier les différences dans l'activation des cellules épithéliales des voies aériennes à planctoniques et des matériaux dérivés du biofilm.

De plus, la majorité des souches de *P. aeruginosa* isolés à partir de patients chroniquement infectés sont adaptés à présenter un phénotype mucoïde. L'expression d'un phénotype mucoïde est dans l'environnement protecteur CF, cependant différents déterminants de virulence potentiels peuvent également être régulés de manière différentielle. Je vais examiner comment ce passage à un impact de phénotype mucoïde

activation de médiateurs inflammatoires dans les cellules épithéliales des voies respiratoires.

Pris ensemble, les approches que j'utilise dans cette thèse mieux identifier les déterminants qui sont importants dans la régulation de l'activation des médiateurs inflammatoires des voies aériennes épithéliales. En comprenant les principales voies impliquées dans la défense de l'hôte, de nouvelles méthodes de détection de déterminants de virulence clés peuvent être développées.

CHAPTER 1: INTRODUCTION

Airway Epithelial Cells

General Features of the Airway epithelium as a Physical Barrier:

The human respiratory tract consists of a population of diverse cell types that compose a mucosal layer separating the external environment from the internal milieu.[190, 382] Together, these cells are termed airway epithelial cells (AEC) and they represent the first point of contact between inhaled pathogens and pollutants while maintaining an important physiochemical and immunobiological barrier. The morphology of the airway epithelium is defined by the types of cells present in the region, with each cell type and region displaying distinct histological characteristics.[471] The epithelium of the large airways and the proximal lower respiratory track is dominated by the presence of ciliated cells, interspersed with a small percentage of basal and goblet cells present.[471] Collectively, these cells constitute a single continuous pseudo-stratified layer composed of ciliated cells on the luminal (apical) side and basal cells on the basal side. Each cell type establishes contact with the underlying basement membrane, forming a single, contiguous layer. [212, 312, 471] Mucus, secreted onto the lumen of the epithelium by the goblet and serous cells, forms a physical barrier. The overall effect of the

arrangement of the airway epithelial produces a barrier that is impermeable to infectious agents and debris, while also limiting and regulating ionic diffusion.[239, 471] The polarization of the airway epithelium allows for tight control and regulation of water transport and ionic composition of the fluid layer on top of the epithelial cells, which is crucial for proper functioning of the AEC.

Airway Epithelium: Mucociliary Clearance

In healthy individuals the presence of a thin mucosal layer on top of a hydrated periciliary layer (PCL), which envelopes the ciliated cells, allows for effective clearance of pathogen and debris through a movement known as mucociliary clearance. [291, 471, 511] While measurements for the thickness of the PCL vary, an approximate range of 10-45 μm is common.[489] This layer enables the complete extension of the cilia, which is between 7 and 8 μm in length.[438] Located on-top of the PCL is a thin layer of hydrated mucus termed viscoelastic mucus, which acts to moisten air and absorb harmful particles and pathogens.[17, 241] The mucus is composed of free proteins, including lactoferin and lysozyme, carbohydrate-rich glycoproteins known as mucin, as well as cellular debris and water.[17, 442] The relative amounts of each component help determine the viscosity of the mucus layer.[17]

The presence of mucin defines many of the properties of the mucus. A high molecular weight glycoprotein, mucin is responsible for much of the 'stickiness' of the mucus and helps trap pathogens via non-specific interactions. Pathogens also have a variety of receptors that bind to the carbohydrates in the mucin, further contributing to their entrapment within the layer. Proper clearance of the mucus layer is therefore vital for protection of the airway epithelium from pathogens.

Both the depth and the viscosity of the PCL layer are critical for proper mucus transport form the respiratory tract.[17, 241, 401, 471]. The cilium of ciliated epithelial cells extends through the PCL, and it is the tip of the cilium that comes into contact with the gel like interface of the PCL/mucus boundary.[241] The tip of each cilium has a barb-like structure that grabs and propels the mucus only during the forward movement of the cilium (and not during the back stroke.)[17, 471] In addition, proteins found at the base of each cilium provide orientation, allowing the cilium to bend in only one direction. Cell to cell communication via the gap junctions in the epithelial cells permit coordinated beating of each cilium, resulting in the efficient propulsion of mucus in one common direction. [241, 401, 471] Figure 1 represents the key features of the airway epithelium.

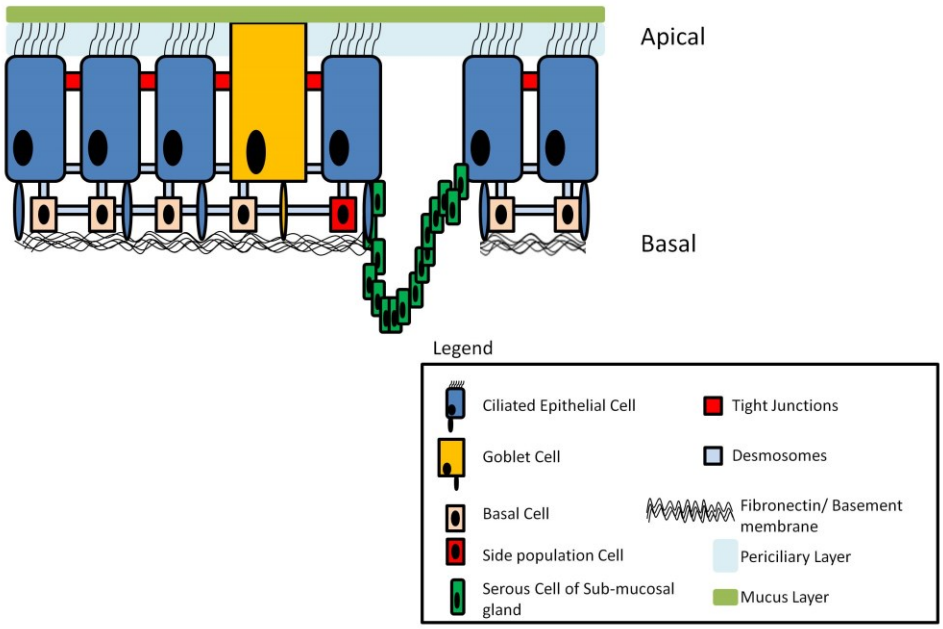


Figure 1: The Airway Epithelium as a Pseudostratified Physical Barrier.

The airway epithelium consists of basal, columnar ciliated, goblet, serous and side population cells. These cells are connected via tight junctions at the apical side of the membrane and desmosomes at the basolateral side. Each cell type also makes a connection with the underlying basement membrane. On the apical side of the epithelial cells is the low-viscosity, thin periciliary layer through which the cilia fully extend and in which they can beat easily. On top of this is a layer of more viscous mucus which traps pathogens and other debris, and that is shuttled in one direction in a process known as mucociliary clearance. Adapted from Hirota, 2012. [190]

The act of coughing on its own is enough to remove a significant amount of mucus. However, in patients with defects to their mucocillary clearance mechanisms, including patients with primary ciliary dyskinesia and CF, have obstructive airway disease and are more prone to bacterial infections.[241, 401, 470]

Airway Epithelial Cells: Chemical Barrier

Aside from mucin, AEC produce a number of different molecules that are present in the mucus that are able to inhibit microbial proliferation, effectively protecting the airway epithelium from harmful agents.[239, 382, 471] The production and secretion of different types of mediators, including anti-microbial peptides, reactive oxygen species and a variety of cytokines/chemokines, allows AEC to control infection through direct inhibition of the growth of the pathogen, or through increased recruitment of cells involved in phagocytosis of the microorganism or cells of the adaptive immunity.[26, 113, 114, 267] Table 1 lists several types of antimicrobial peptides and immune modulators that are secreted by airway epithelial cells. While an exhaustive review of the production and functions of the different molecules is beyond the scope of this thesis, their mention is important for two concepts that will be discussed later on.

Table 1: Immune modulators produced by airway epithelial cells

Compound/class of compound(s)	Function
Mucins	Trapping of pathogens, noxious particles
Antimicrobial Compounds (Lysozyme, SP-A, Lactoferrin)	Directly kill or limit bacterial/pathogen growth.
Reactive Oxygen Species (NO, H_2O_2)	Increase inflammation and tissue damage
Antioxidants (Glutathione, Catalase)	Reduce hyperoxide, lipid oxides etc. Reduce inflammation
Lipid Mediators (15-lipoxygenase pathway, COX pathway)	Immune modulation, stimulate inflammatory responses
Peptide Mediator (Endothelian, β-defensin-1 etc.)	Vasoconstriction, Mucus production, antibiotic properties
Anti-protease/ Catabolic enzymes (Elafin, CC10)	Breakdown proteases, prevent tissue damage
GM-CSF,G-CSF,M-CSF,CSF-1	Colony Stimulating Factors Cytokines
IL-6, IL-11,IL-1,IL10,TNF-α	Pleiotropic Cytokines
TGF-β,TGF-α,SCF,bFGF	Growth Factor Cytokines
IL-16	Lymphocyte Chemokine
IL-8, GRO-α, GRO-γ	C-X-C Chemokines, recruitment of inflammatory cells (e.g. neutrophils)
RANTES, MCP-1, MCP-4,Eotaxin	C-C Chemokines

Firstly, the roles of ion transport and the airway epithelium's surface liquid composition in allowing for the AECs to maintain a sterile environment, are largely due to the mechanism of mucociliary clearance and the production of immune modulators.[17, 158, 401, 445] Secondly, while many of the peptides are constitutively expressed, many can also be induced via specific interactions with pathogens. [187, 267]Both of these concepts will be discussed further on in the introduction.

The physical barrier to inhaled pathogens and toxins, the mechanism of mucociliary clearance and the presences of antimicrobial peptides together produce the physiochemical barrier functions of the airway epithelial cells of the proximal bronchi and large airways.

Pathogen Recognition by the Airway Epithelium.

Microbes express a number of different molecules or repeating motifs that can be recognized by the host innate immune system, these molecules are termed pathogen associated molecular patterns (PAMPs). In the late 1980's Charles Janeway proposed that the host had evolved a number of different pathogen recognition receptors (PRR) that could recognize and bind these PAMPs to elicit an innate immune response.[211] Over the last two decades, much research has been done not only to confirm this hypothesis, but also to elucidate the signaling cascades and

effector responses produced by the diverse array of PRRs that are currently known to exist.

Toll-like Receptors

A Background to Pathogen Recognition via Toll-Like Receptors

Prior to the identification of PRRs, the innate immune system was deemed “primitive” or “crude” and serving only to prime the adaptive immune response in humans.[346] The characterization and study of the first group of PRRs, the toll-like receptor (TLR) family, revolutionized the field of immunology. In fact, Nobel prizes have been awarded for the discovery of important mechanisms in this field, including the 2011 Nobel Prize in Physiology awarded to Jules Hoffmann and Bruce Beutler, for their descriptions of TLR roles in the innate immunities of drosophila and humans, respectively.[346]

The Toll gene was first discovered in Drosophila, and found to be important in dorsoventral polarity of the embryo.[15, 16] Significantly, the Toll-gene in Drosophila was shown to activate a homolog of NFκB transcription factor in humans, the latter having been shown to be critical in the pro-inflammatory signal transduction response of human B-cells to bacterial products.[431, 454] It had previously been established that interleukin (IL)-1 and its receptor, IL-1R, were responsible for various

functions of the immune system.[346] Therefore, when the IL-1R was cloned and subsequently shown to possess a high degree homology to the Toll receptor that was found in drosophila, a new class of receptors was born.[152, 440]

The properties described for the IL-1R were very much similar to the TLRs, which suggested that they were integral to the innate immune system. However, it was not until the seminal paper from Jules Hoffman's lab in 1996 was able to link the Toll gene to an immune function. This paper showed that the activation of Toll in Drosophila responded to fungal infection in order to up-regulate gene expression of an antimicrobial peptide. [278] [278] Hoffman's paper definitively described receptors of the innate immune system (albeit in Drosophila) that could recognize pathogens and induce gene expression to produce an antimicrobial peptide. Hoffman's experiments confirmed Janeway's initial hypothesis that receptors existed to link the host's innate immune response to microbial products, and the search for mammalian PRRs began in earnest soon after.

The first human homolog of Toll in humans was described in 1997 by Janeway's lab.[307] Subsequently, 5 human receptors were cloned with striking homology to Drosophila's Toll receptors, resulting in the

description of these 'Toll-like Receptors' or TLRs.[404] To that point, there was no conclusive function that had been discovered of these new receptors, but the previous experiments by Hoffman's group and others strongly implied a role in innate immunity. A component of the gram-negative cell wall, known as lipopolysaccharide (LPS), had already been known to cause sepsis and was being carefully studied for identification of its receptor.[276, 516] LPS had been identified as an activator of NFκB, the homolog of which was important for immune activation in Drosophila via the Toll pathway. [431] NFκB's homolog had also been demonstrated to activate p38 mitogen activated protein kinase (MAPK), a protein found to be induced via IL-1, suggesting that the receptor for LPS would likely be a member of the IL-1R family. [173]

Finally, mice housing a single mutation within the chromosome associated with their TLR4 gene have been shown to be resistant to LPS induced sepsis, indicating that a single gene product or receptor was involved in induction of inflammatory responses to LPS.[346] In 1998, Bruce Beutler's group was able to show that the gene product controlling LPS responsiveness was in fact a TLR (termed TLR4).[376] Furthermore, later work by Shizuo Akiria and others confirmed and clarified how LPS binds to and initiates signaling through TLR4.[200, 228] Taken together, these

findings represented the identification of a new branch of innate immunity. Our understanding of the innate immune system and how different components contribute to host immunity has come a long way over the past 20 years, since Janeway's initial hypothesis; and yet there is still much to discover.

General Structure of Toll-Like Receptors

To date, 10 TLRs have been discovered in humans, and have been named accordingly (TLRs 1-10).[8] They all share the same fundamental structure (that of a type 1 transmembrane glycoprotein receptor). In contrast to the 3 immunoglobulin (Ig) domains on the N-terminal of the IL-1R, TLRs have 3 leucine-rich repeats (LRRs) on their N-terminal ends to mediate ligand binding.[152] The basic LRR motif comprises 24 amino acids which forms a β-strand and an α-helix joined by a loop, resulting in a general protein recognition motif common to several different receptor families.[41, 243]

TLRs contain a unique consensus sequence within their LRR that appears repeatedly throughout the extracellular domain of TLRs; each of these sequences in TLRs 1–10 have been thoroughly characterized in terms of regions that confer ligand specificity.[41] Insertions in LRRs after 10 amino acids are predicted to provide ligand binding sites at the

concave surface of TLRs, whereas insertions at position 15 have been predicted to create binding sites at the convex surface. The binding site for flagellin has been shown to be located within the LRR consensus sequence, and mutations within this region have been found to abolish flagellin binding, providing basis for an accepted model. [316] .[316] Studies have suggested that it is the specific variations in the LRR of TLRs that contribute to the diversity of ligand binding by different TLRs.[345]

The majority of TLR form either homodimers or heterodimers with other TLR to form a 'horse-shoe like' binding domain.[226] Ligand binding has been proposed to occur at either the convex (outer face) of the TLR dimers or on the inner concave portion of the receptor.[40, 87] Crystal structures of TLR3 homodimers interacting with its prototypical PAMP (double stranded RNA (dsRNA)) revealed ligand binding sites at both the convex (outer face) of the TLR dimers and on the inner concave portion of the receptor.[287] These receptor complexes are formed via sequential and co-operative binding of the ligand to its co-receptors and the TLR elements of the complex.[345, 505] The cytoplasmic domain is highly conserved and termed Toll-IL-1R (TIR) domain due to the high degree of homology it shares with both the Drosophila Toll and the mammalian IL-1R protein. [152]

The TIR domain functions as binding domain for adaptor molecules used to propagate signaling for TLRs (discussed later in this chapter). TLR are expressed in a wide variety of both immune and non-immune cells, as described in Table 2.

The presence of TLRs on airway epithelium was first described through the identification of beta-defensin induction in tracheal epithelial cells in response to LPS.[37] Since then it has been established that airway epithelial cells have been shown to express all known TLR (1-10) to varying degrees.[163, 206, 325] TLRs are found in different compartments within the cells; TLRs 1,2,4,5,6 and 10 are anchored at the cell surface while TLRs 3,7,8 and 9 are located in endosomal compartments.[226]

Toll-like Receptor Ligand Binding and Expression in Airway Epithelial.

Toll-like receptors are expressed in a number of different cell types, including immune cells such as leukocytes, as well as non-immune cells, particularly epithelial cells. The extent of expression and localization of the TLRs in the airways is distinct to each member of TLR family. As the airway epithelium serves as a protective interface between the external environment and the host system, it employs PRRs (i.e., TLRs) as a means of surveillance. Pathogen binding to TLRs initiates signaling, eventually triggering an inflammatory response. Below is a description of

the ligands (i.e., PAMPs) recognized by each TLR and a brief description of the TLR expression in the airway epithelium.

Toll-like Receptor 1

TLR1 was one of the first TLRs to be cloned and identified in humans and since it displayed the highest degree of homology to the Toll signaling domain, it was named TLR1.[404] The TLRs are grouped into 5 different subfamilies based on phylogeny; for example, TLRs 1,2,6 and 10 make up one family.[465] TLR1 signaling occurs in the plasma membrane where it typically forms a heterodimer complex with TLR2, enabling recognition of tri-acetylated lipopeptides found in the peptidoglycan layer of bacteria.[11, 466, 518] The ectodomain of the TLR1/2 complex features a hydrophobic pocket, in which the third acyl chain of a given lipopeptide fits; this confers selectivity of the TLR1/2 complex for tri-acylated lipopeptides over di-acylated lipopeptides.[226] While many TLRs have been found to signal as homodimers, thus far there has been no evidence that would suggest TLR1's ability to do so. TLR1 is expressed on both the apical and basal surface of the airway epithelium as observed in both tissue and cell culture, and is the most abundantly expressed TLR in immortalized human airway cell cultures (i.e., BEAS-2B).[206] While TLR1/2 heterodimers are capable of being formed in the absence of

ligands, protein expression and mobilization of the complex to the apical surface is facilitated in the presence of bacterial ligands.[176, 477] The inability to properly traffic this complex to the membrane abrogates the response to tri-acylated lipopeptides.[176] Efficient signalling through the TLR1/2 complexes requires binding of the lipopeptides to CD14 which acts as a co-receptor for these complexes.[486, 507] It has also been found that the porins of outer membranes of *Neisseria* bacteria require the presence of the TLR1/2 complex in order to be recognized.[298] Interestingly, TLR10 has also been found to form heterodimers with TLR1, however a ligand for this complex has yet to be found.[177]

Toll-like Receptor 2

Toll-like receptor 2 was also amongst the first group of TLRs identified. Toll-like receptor 2 signals at the plasma membrane where they have been shown to form either homo- or heterodimers with TLRs 1, 6 and 10, and respond to a variety of different ligands.[177] TLR2 expression in airway tissue cultures and sections was found to be predominantly higher at the basolateral side of the membrane, with overall lower levels of expressions observed in culture.[206] Up regulation of TLR2 expression has also been noted in the instance of its migration to the apical side of the membrane where it can localize into lipid rafts in the membrane and

interact with a variety of receptors in response to pathogen stimulation.[2, 451] As previously mentioned, the TLR1/2 complex recognizes tri-acetylated lipopeptides, while TLR2/6 complexes recognize di-acetylated lipopeptides.[354] .[354] Much like TLR1/2, TLR2/6 requires a co-receptor for efficient signaling; CD36 modulates the TLR2/6-dependent response to di-acetylated lipopeptides.[191] TLR2 can also form homodimers at the plasma membrane, allowing for recognition of lipoteichoic acid and peptidoglycan of the bacterial cell wall.[428] Importantly, TLR2 homodimers have been shown to respond to several different lipopeptides even in the absence of TLR1 or TLR6.[74, 76] It has also been shown to interact with Dectin receptor and C-Type lectins and respond to fungal wall components and beta-glucans.[70] Of all the TLRs, TLR2 has displayed the broadest range of ligand recognition due to the wide variety of unique receptors implicated in TLR2 responses[164] Bacterial cell wall constituents including lipoteichoic acid and peptidoglycans, bacterial flagellin, mycobacterial cell wall components such as lipomannans, and the yeast cell wall component zymosan, have all been reported to induce TLR2 activation.[2, 348, 354, 428, 482] Interestingly, the 'versatility' of TLR2 has caused speculation regarding the receptor's ability to specifically interact with all these ligands; it is quite possible that preparations may contain

lipopeptide contamination which would induce TLR2 activation.[352, 532] In fact, TLR2 was initially (and incorrectly) declared the receptor for LPS ligand due to contaminations of lipoprotein in sample preparations. Further studies are required to determine the extent to which TLR2s are truly able to recognize different pathogen-associated molecular patterns.

Toll-like Receptor 3

TLR3 is the sole member in its subfamily of TLR phylogeny and is found in both the cytoplasm and at the cell surface at relatively high levels (in comparison to other TLRs) throughout the airway epithelium.[206] In the airway epithelium it is strongly expressed in cells at both the apical and basolateral sides of the epithelium.[404, 465] Toll-like receptor 3 forms signaling complexes in the endosomal compartments of both immune and non-immune cells where it recognizes dsRNA, a typical viral PAMP, to induce a type-1 interferon response. [68, 474] Double stranded RNA is often released from viral particles, however TLR3 can also recognize endogenous dsRNA that is released from necrotic cells.[10, 68] Activation of TLR3 is mediated by the binding of the dsRNA to CD14, which increases its uptake into cells where it can then interact with TLR3.[271] This complex is then associated with lysosomes. While TLR3 has been shown to interact with viral RNA, its protective effect has been called into question.

TLR3 deficient mice are actually more capable of surviving viral influenza infections compared to their wildtype counterparts.[268] The current belief is that TLR3 plays a pivotal role in the balance between tolerance and immune activation in the host.[352]

Toll-like Receptor 4

As mentioned above, TLR4 was the first identified and described of the TLRs and indeed of the PRRs. Toll-like receptor 4 is the third sub-family of TLR phylogeny and has been shown to signal via formation of homodimers and responds to lipid A of LPS [200, 322] It is expressed at low levels in the cytoplasm of airway epithelial cells, where upon stimulation is translocated to the apical side of the cell surface.[206] The lipid A moiety of LPS binds to LPS binding protein (LBP, also known as CD14) which then interacts with TLR4 homodimers to initiate signaling and a pro-inflammatory response (discussed later).[200, 516] Interestingly, it has also been reported that TLR4 can be activated as a monomer to drive NFκB expression, although at a lower efficiency than it would as a homodimer.[533] Additionally, several endogenous ligands have been shown to activate TLR4, although since these proteins tend to be overexpressed in *E. coli*, contamination with bacterial products has been shown to be a factor impacting results in several studies.[352] For instance,

several groups have shown the endogenous protein Hsp70 as being able to activate TLR4 signaling.[20, 483] However, later studies using LPS-free preparations were unable to confirm the other groups' initial findings.[151, 496] Toll-like receptor 4 has also been shown to be endocytosed and drive NFkβ and interferon expression in response to respiratory syntical virus (RSV) F protein.[260]

Toll-like Receptor 5

Toll-like receptor 5 is expressed mainly at the cell surface where it forms homodimers or heterodimers with TLR4, and is moderately expressed in the airway epithelium where it is almost exclusively found on the apical side of the membrane.[8, 315] Flagellin, a prominent component of the many bacteria that is responsible for motility, is currently the only known ligand of TLR5.[180] Flagellin has been shown that TLR5 recognizes monomeric flagellin, as recognition via TLR5 is lost in the presence of filamentous form of the bacterial flagellum.[316, 446] The binding of flagellin to TLR5 homodimers results in the activation of NFκB whereas binding through the TLR4/5 heterodimer leads to type 1 interferon and nitric oxide production.[315]

Toll-like Receptor 6

Similar to the previously discussed TLRs, TLR6 forms hetrodimers with TLR2 and binds to di-acylated lipopeptides produced by various types of bacteria. While TLR1/2 and TLR2/6 heterodimers share similar structures, TLR6 has specific differences in its amino acid sequence that preclude tri-acyl lipopeptide and favor di-acyl lipopeptide recognition. In TLR6, the side chains of two phenylalanine amino acids (F343 and F365) block the lipid residue binding pocket of the TLR2/6 dimer, resulting in a pocket about half the size of the TLR1/2 lipid binding pocket.[221, 294] This leads for an inability of the larger tri-acyl lipopeptides to fit into the TLR2/6 binding pocket.

Toll-like receptor 6 is moderately expressed in airway epithelial cells, where it is predominantly found on the apical surface of the membrane. While no specific function has been determined for TLR1/6 heterodimers or TLR6/6 homodimers, it is interesting to note that both TLRs 1 and 6 are highly expressed on B-cells, whereas TLR2 is expressed at very low levels, suggesting a possible role for interactions between TLR 1 and TLR 6 to occur.[198]

Toll-like Receptor 7

Toll-like receptors 7,8 and 9 comprise the final group of TLR receptor family. TLR7 is mainly expressed in B-cells and dendritic cells,

with a low level of expression in the airway epithelium.[206, 352] It is expressed in endosomal compartments and recognizes single stranded RNA (ssRNA), a component often found in viral pathogens.[183] The exact recognition sequence between TLR7 and ssRNA is unknown, however TLR7 has been reported to identify nucleic acids containing large repeats of guanine (G) and uracil (U). TLR7 is also able to bind small RNA sequences, including small interfering RNA (siRNA), regardless of the G or U content.[199] Signaling through TLR7 predominately results in the production of type I interferons.

Toll-like Receptor 8

Toll-like receptor 8 is found in endosomal compartments and are expressed and low levels in the airway epithelium; it shares a strong homology to TLR7 and similarly, recognizes ssRNA to drive tumor necrosis factor (TNF) expression.[8, 206] Despite this, TLR7 and TLR8 recognize different sequence structures and promote different cellular/molecular pathways. Toll-like receptor 8 is also able to recognize GU rich sequences, as well as oligonucleotides rich in adenosine (A) and U (AU-rich sequences) for promotion of TNF expression.[140] In addition to recognizing viral ssRNA sequences, TLRs 7 and 8 have been shown to respond to synthetic nucleotide sequences.[216] Importantly, mammalian

RNA is significantly less stimulatory via TLRs 7 and 8 compared to bacterial RNA; mammalian RNA contains modified nucleosides as a means to distinguish between endogenous and pathogen-derived RNA.[222]

Toll-like Receptor 9

Toll-like receptor 9 is also expressed in the endosome and is found in low levels in the airway epithelium. In unstimulated conditions, TLR9 is located in the endoplasmic reticulum; upon stimulation, it is translocated to the lysosome.[266] Toll-like receptors 3,7,8 and 9 make up the TLRs that are located in the endosome and respond to various oligonucleotide sequences. The ligand for TLR9 was found to be deoxyribonucleic acid (DNA) that contains unmethylated CpG motifs.[185] This pattern of DNA is typically found in bacteria and viruses, while mammals normally have DNA with low levels of CpG motifs (and when these motifs do occur in mammalian cells, they are most often methylated) Despite this, TLR9 is still capable of recognizing mammalian DNA complexes, implicating the receptors involvement in a number of autoimmune diseases.[269, 491] Table 2 and Figure 2 each provide a summary of the key features for the different TLRs.

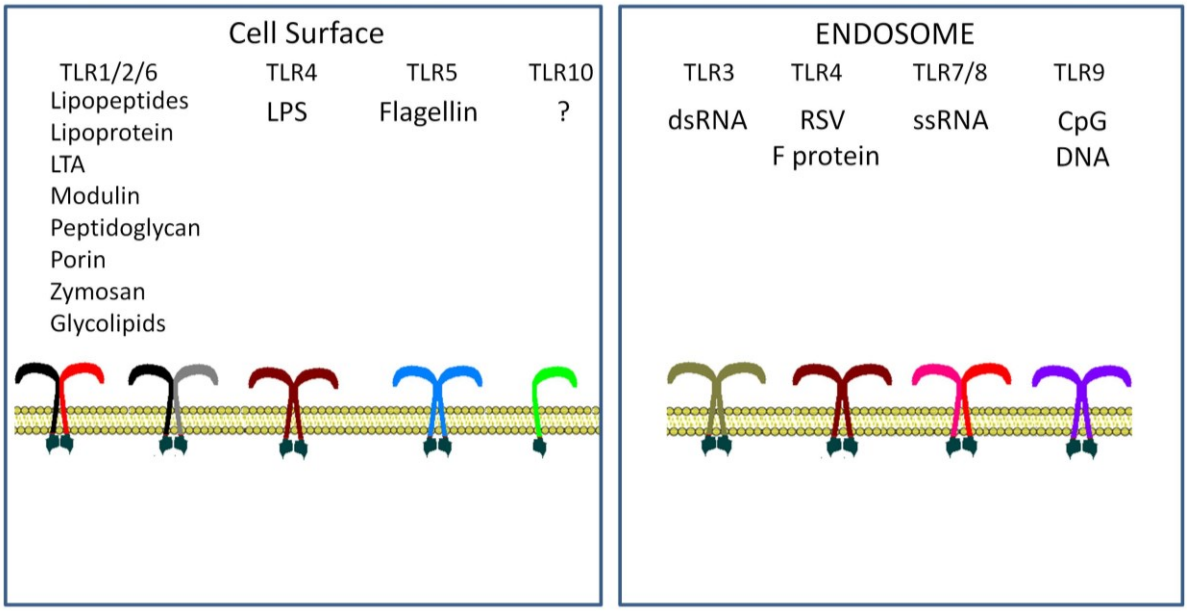


Figure 2: Toll-like receptor Ligands

As described in the text, the various Toll-Like Receptors found in humans recognize a variety of different ligands and are located in specific areas of the cell. TLR2 has been shown to heterodimerize with TLR 1 or TLR 6 on the cell surface and binds to a diverse array of PAMPS. TLR4 forms homodimers on the cell surface and recognizes LPS. TLR5 also forms homodimers and recognizes flagellin from bacteria. The endosomal TLRs main recognize intracellular pathogens or viruses as described. Table 2 further summarizes these interactions.

Table 2: Toll-like Receptor Ligands, Expression and Co-receptors

Toll-like Receptor	Ligand(s)	Expression in AEC	TLR pair/ co-receptors
TLR1	Tri-acyl lipopeptides[466, 518]	Surfaces and in the cytoplasm of AEC.	TLR2, CD14
TLR2	Peptidoglycan hemagglutinin[48, 428], glycol lipids, flagellin zymosan	Weakly expressed, on the cell surface, luminal side of AEC	TLR1, TLR6, Dectin receptor, Asialo GM1
TLR3	ssRNA virus and dsRNA virus	Surfaces and in the cytoplasm of AEC. Endosomal upon stimulation	
TLR4	Lipopolysaccride,mannan, oxidized phospholipids.	Weakly expressed on the luminal surface. Some endosomal.	MD2, CD14, LBP
TLR5	Bacterial Flagellin	Weakly expressed on the luminal surface.	
TLR6	Di-acyl lipopeptides, LTA, Zymosan, glycolipids	Weakly expressed on the luminal surface.	TLR2, CD36
TLR7	ssRNA viruses, purine analog compounds.	Endolysome and cell surface of AEC	
TLR8	ssRNA viruses, purine analog compounds.	Endolysome and cell surface of AEC	
TLR9	dsDNA from virus, CpG DNA from bacteria, hemozin (parasite)	Endolysome and cell surface of AEC	
TLR10	Unknown	Not expressed	TLR1/2(?)

Toll-like receptor 10

Toll-like receptor 10 was the last mammalian TLR discovered and has no homology to any Tolls described. It is a surface expressed TLR that has not been found to be expressed in airway epithelium. There are currently no known ligands for TLR10.

Signalling through Toll-Like Receptors

As previously mentioned, TLRs have the ability to sense a diverse array of molecular structures derived from a variety of pathogens, as well as either endogenous or synthetic sources. Since TLRs recognize conserved molecules shared among members of a particular class of microbes and generate diversity by pairing with co-receptors or other TLRs (i.e. TLR1/2 and. TLR 2/6 heterodimers) a large portion of the pathogenic repertoire can be recognized by only a few receptors. [306] The recognition of PAMPs, however, by the extracellular domain of the TLR is merely the beginning of a more complex process. Once ligand binds the TLR, a dynamic process of molecular recruitment and signal transduction takes place. Ligand binding is believed to initiate TLR dimerization and induction of conformational and structural changes that trigger adaptor proteins which in turn results in signaling.[7] Ultimately, recognition of the

microbial PAMP results in production of pro-inflammatory mediators that induce an inflammatory response for removal of the invading pathogen.

MYD88 Dependent Signaling via Toll-like Receptors

The elucidation of signaling pathways important in signal transduction began with the discovery of a key cytoplasmic adaptor protein that was initially discovered in plants. The N protein of plants is required to initiate defense against a common plant pathogen, the tobacco mosaic virus. It was discovered that the amino terminal of N protein displayed significant homology to the Toll and IL-1R cytoplasmic domains.[510] This amino terminal domain became known as the Toll-IL-1R domain, or the TIR domain. The importance of TIR in TLR signaling events was first described in C3H/HeJ mice, whereby a point mutation in the cytoplasmic domain of TIR led to a hypo-responsive reaction toward LPS as well as the inability of the TLR4 TIR domain to recruit downstream effectors.[376] In 1997, myeloid differentiation primary-response protein 88 (MYD88), a protein whose role was defined in myeloid cell differentiation, was shown to bind IL-1R1 for promotion of signalling via the NF-κB pathway.[289, 290, 327, 504]

MYD88 was found to have a C-terminal TIR domain, which would allow the protein to interact with the TLR cytoplasmic TIR domain as well

as an N-terminal death domain. This N-terminal death domain allows Myd88 to interact with IL-1R-associated kinase (IRAK) family members in order to propagate downstream signalling events. While there are four members of the IRAK family, each with distinct roles in signal transduction via TLR, IRAK4 is considered the key protein involved in downstream signaling events.[138]

Deletion of IRAK1 or 2 lead to only a partial response to LPS and, in the case of IRAK2, impaired TLR2 signaling.[224, 462, 469] In contrast, the deletion of IRAK4 in mice completely abolishes LPS induced activation of NFκB, abrogates Myd88 TLR signaling and reduces p38 activation via IL-1R.[461, 528] Upon binding Myd88 and IRAK4, IRAK1 is phosphorylated which in turn activates the kinase function of IRAK-1. IRAK-1 will then auto phosphorylate itself, recruiting tumor necrosis factor receptor–associated factor-6 (TRAF6) to the MyD88/IRAK-4/IRAK-1 complex.[81, 327, 504] TRAF6 acts as an E3 ligase that facilitates the production of lysine63 (K63)-polyubqiuitin (pUb) (K63-pUb) chains.[107] Interaction of the K63-pUb chain with TAK1 binding protein (TAB) 2 and 3 induces a conformational change that drives autophosphorylation of transforming growth factor β activated kinase 1 (TAK1).[497, 520] In addition, the Linear Ubiquitin Assembly Complex (LUBAC) produces linear-pUb chains that act in a similar manner

to bind NfκB essential modifier (NEMO) and allow canonical IκB Kinase (IKK) signaling through interaction with TAK1.[220, 244] Together, the K63-pUb and linear-pUb chains are essential for activation of TAK1 and IKK respectively.[459] The activation of TAK1 results in the activation/phosphorylation of p38 mitogen activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and inhibitor of κB kinase (IKK) activation.[108] These proteins can then induce AP-1 (activator protein-1) and NF-κB activation, ultimately leading to the transcription of genes encoding pro-inflammatory cytokines and chemokines such as TNFα, IL-6 and IL-8.[7, 181] Additional proteins can be recruited to and interact with the cytosolic TIR domain of TLRs to induce signaling. The Myd88-adaptor like protein (MAL, also known as TIR-adaptor protein or TIRAP) was shown to be a bridge between the TIR domain and the Myd88 adaptor protein for signaling through TLR4 and TLR2.[136, 197, 230] Figure 3 (pg. 30) summarizes this pathway.

Key studies in Myd88 deficient mice and Myd88 deficient cells allowed for determination of the ligands and receptors that require Myd88 dependent signaling events. All TLRs, with the exception of TLR3, have been shown to initiate signaling to some extent, in a Myd88 dependent manner. Myd88 deficient mice are unresponsive to LPS while MyD88-

deficient cells do not show any response to peptidoglycans, flagellin, CpG DNA or ssRNA, suggesting that TLRs 2, 4, 5, 7/8 and 9 all function through a Myd88-dependent signaling pathway.[1, 46, 225]

MYD88 Independent Signaling through Toll-like Receptors

The study of Myd88 deficient mice provided the first clue of a potential Myd88-independent signaling pathway in a TLR context. Myd88 knockout mice did not display any type of response linked to cytokine production, MAPK activation, or NFκB in response to TLRs 2, 5, 7 or 9. However, macrophages from these mice demonstrated increased MAPK and NFκB activation in response to LPS, implying an alternate signaling pathway was indeed possible via TLR4.[225] Furthermore, interferon regulator factors (IRF) have been shown to be activated by TLR4 and TLR3 ligands in the absence of Myd88.[228] These studies led to the characterization of the TIR domain–containing adaptor inducing IFN-β (TRIF) (also known as the TIR-containing adaptor molecule-1 or TICAM-1) as critical transducer of MyD88-independent signaling.[351, 524] The N-terminal region of the TRIF protein has been shown to activate IRF3 as well as TRAF6 to induce both the IFN response as well as the NFκB response, while the C-terminal region can only activate the TRAF6 pathway, leading to NFκB activation. Therefore, mutations in the N-

terminus can abolish IFN responses without any disruption to the NFκB response.[227, 368] It has also been shown that TRIF can activate RIP-1 to induce signaling. TLR3 is able to directly recruit the TRIF adaptor protein, while TLR4 requires an adaptor protein for this interaction to occur, known as TRIF-related adaptor molecule (TRAM; also known as TICAM2).[137, 523] Activation of TRAM occurs at a specific serine residue and results in the internalization of the TLR signaling complex into endosomes, where TRIF-related signaling, which is associated with TLR4, takes place.[217, 415] This concept reiterates the importance of TLR localization in response to ligand. Figure 3 summarizes the Myd88-dependent and Myd88-independent pathways initiated by TLR signaling.

Non Toll-like Receptor Pathogen Recognition Receptors

In addition to TLRs, the airway epithelium expresses a number of different PRRs that respond to pathogen derived materials. Since an in-depth review of these processes is beyond the scope of this thesis, a brief description is summarized below.

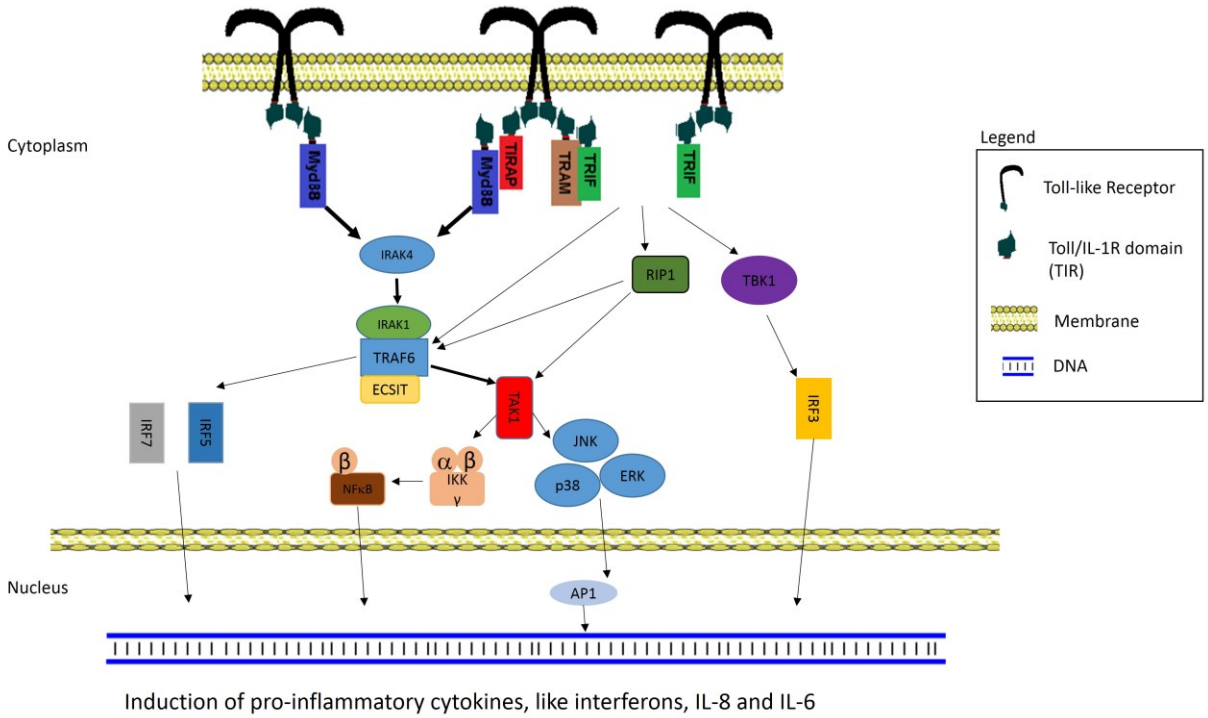


Figure 3: Toll-like Receptor Signaling.

Signaling through TLR is initiated via ligand induced dimerization of the receptors. Following this, the TIR domains of the TLR engage the TIR domains of adaptor proteins, either Myd88 or MAL (Myd88 Dependent signaling) or TRIF/TRAM (Myd88-Independent signaling). This activates downstream signalling pathways involving interactions with IRAK and TRAF molecules, leading to the activation of MAPK such as p38, ERK and JUN, leading to AP-1 activation. Activation of transcription factors NFκB and IRFs also occurs. The major consequence is the induction of pro-inflammatory genes by these transcription factors.

Abbreviations: Myd88, myeloid differentiation primary-response protein 88; **TIRAP**, ; **TRAM**, ; **TRIF**, ;TRAF,TIR domain-containing adaptor protein inducing IFNβ; IRAK, IL-1R-associated kinases; **ECSIT**, ;RIP1,receptor-interacting protein 1;TAK, TGFβ-activated kinase; TBK1, TANK Binding Kinase 1; IKK, inhibitor of NF-κB kinase; NFκB, nuclear factor-κB; JNK, JUN N-terminal kinase ; **ERK**, : AP-1 activator protein 1 IRF: interferon-regulatory factors.

NOD and NOD-like Receptors

The nucleotide-binding oligomerization domain (NOD) and associated receptors (NOD-like receptors or NLRs) belong to a family of cytosolic proteins that recognize different types of microbial and endogenous proteins.[256] These proteins are comprised of three domains: the C-terminal domain, which consists of several LRRs and which is responsible for ligand binding; the N-terminal domain, consisting of a death effector domain (DED) and a caspase recruitment domain (CARD), which are responsible for interactions with downstream effector molecules; the intermediate domain which is sandwiched between the C and N terminal domains, and consists of the nucleotide-binding and oligomerization (NACHT) domains, which are required for the formation of active receptor complexes for activation of downstream signaling.[256] Upon recognition of PAMPs, these sensors activate either NFκB or MAPK to induce the inflammatory cytokine production. The NLRs (particularly NLRP3, NLRP1 and NLRC4) can also activate the "inflammasome" via their CARD domains, which is thought to either initiate the maturation of various caspases leading to the inflammatory cytokine production or the activation cell death pathways. NLRs are activated by a number of different host pathogens, most commonly by enteropathogenic bacterial strains, intracellular pathogens, and pollutants. For further review, please refer to Kumar et al. 2011.

RIG-1 like Receptors

The retinoic acid inducible gene 1 (RIG-I) like receptor family consists of three members; RIG-1, melanoma differentiation associate protein 5 (MDA5) and the laboratory of genetics and physiology gene 2 (LGP2) receptors. RIG-1 and MDA5 each consist of N-terminal CARDs whereas LGP2 lacks this domain. All three of these proteins contain the DExD/H-box RNA helicase domain, which is required for ligand recognition and binding. These receptors mainly interact with viral oligonucleotides, primarily from the paramyxoviridae family, to induce the production of type 1 IFNs via activation of IRF proteins.

C-Type Lectin Receptors

C-type lectin (CTL) receptors play a critical role in cell-to-cell adhesions but are also able to recognize different carbohydrate motifs of microbial and fungal origins for initiation of the innate immune response. CTL receptors contain a unique protein fold that acts as the carbohydrate recognition domain permitting them to bind β-glucans on the surface of fungal cell walls. Dectin-1 has also been implicated in the detection of peptidoglycans produced from bacteria, in conjunction with TLR2.[48] The role of CTLs in AECs has been a controversial issue, as different studies have indicated that CTLs may or may not be expressed in the airway

epithelium.[362] Signaling through CTLs has been shown to activate both the MAPK and NFκB pathways.

Nuclear Factor κ B and Mitogen Activated Protein Kinase in Innate Immune Signaling

There are indeed differences in the methods of activation and proximal signaling via the above mentioned receptors, however they all initiate a pro-inflammatory response through the activation of MAPK cascades and NFκB pathways. Below is a brief review of these signaling pathways and the corresponding gene transcription regulation with regards to the innate immune system.[19, 119]

Nuclear Factor κ B Signaling

Nuclear factor κ B (NFκB) was first described as a transcriptional activator present in B cells in response to LPS stimulation.[27,432] At first, NFκB was believed to be restricted to the nucleus and Ig-producing B cells, however further studies revealed its expression in all cell types as well as its location in the cytoplasm where it exists in an inactivated state.[22, 23] It has been demonstrated that NFκB is critical in the immune response and its activation leads to the expression of numerous genes including enzymes, such as COX-2, 5-LOX, and iNOS, cytokines TNF, IL-1, IL-6, IL-8, and chemokines.[5] Five different mammalian NFκB family members have thus far been identified; NFκB1 (p50/p105), NFκB2

(p52/p100), RelA (p65), RelB, and c-Rel.[60] NFkB1 and NFkB2 are synthesized as large precursors, p105 and p100 respectively, that are processed to their DNA-binding subunits p50 and p52.[60] Both p50 and p52 proteins contains a conserved Rel homology domain (RHD) which contains a nuclear localization signal (NLS), and acts as a DNA binding domain that also has the ability to interact with IκBs, the intracellular inhibitor for NFκB.[155] The canonical pathway of NFκB signaling is driven through NFκB1 (p50). In resting cells, NFκB is found as a complex of p50 and RelA, which is sequestered in the cytoplasm in its inactive form through association with one of several inhibitory molecules, including IκB α, IκBβ, IκBγ, p105, and p100.[5] In response to environmental stimuli (i.e., cytokines and/or chemokines, viral and bacterial pathogens, and stress-inducing agents), the IKK complex becomes activated. This complex consists of IKKα and IKKβ as catalytic subunits and IKKγ (also known as NFκB essential modulator or NEMO).[60] Upon cellular activation, the IKK complex phosphorylates the inactive NFκB/IκB at two conserved serine residues within the IκB proteins. This results in the immediate polyubiquitination, disassociation and degradation of IκB proteins via the 26S proteasome.[5] Activation of the NFκB signaling cascade results in complete degradation of IκB, allowing the translocation of NFκB to the

nucleus, where it induces transcription. Activated NFκB binds to specific DNA sequences in target genes, designated as κB-elements, and regulates transcription of over 400 genes involved in immunoregulation, growth regulation, inflammation, carcinogenesis, and apoptosis.[5] Figure 4 summarizes the key steps in canonical NFκB signaling.

Non-canonical signaling has been shown to be driven through NFκB2. Briefly, IκBα phosphorylates p100/RelB which leads to partial degradation of p100 into its active form p52 and exposure of the p52 RHD. The p52/RelB dimers are then free to enter the nucleus and start transcription. The non-canonical pathway is activated by endogenous cytokines and other factors, and will not be discussed further. A more detailed review of both NFκB signal transduction pathways can be found elsewhere.[5, 60, 155] Because NFκB is an inducible protein found in the cytoplasm that can directly be shuttled to the nucleus to initiate gene transcription, it can respond rapidly to numerous stimuli without the need for further protein synthesis.[155]

The elegance of NFκB signaling is emphasized by the conserved nature of NFκB/Rel proteins throughout many different systems in nature. The discovery of the Rel homolog dorsal in Drosophila initiated the search for a Toll-like system in mammals, as described earlier. While there are

many different families for Rel proteins, each playing distinct roles, knockout mice experiments have shown the importance of p50/relA in induction of the acute immune response.[155, 433] The role of NFκB in inducing pro-inflammatory cytokine production and inducing inflammation in airway epithelial cells has been studied in a number of different disease models.[71, 166, 184, 326, 340, 464, 479, 492] For instance, NFκB was shown to be critical for the production of IL-8 and RANTES from airway epithelial cells in response to different TLR ligands. It has also been shown to be important in the response to environmental toxins, such as cigarette smoke or ozone.[61, 184] Finally, patients with cystic fibrosis have deregulated NFκB signaling leading to increased IL-8 production and neutrophil recruitment.[464, 479, 492]

Mitogen Activated Protein Kinase

Cells activated via their PRRs use signal transduction pathways to induce the expression of a number of different genes involved in the inflammatory response. The MAPK cascades are evolutionarily ancient, appearing universally in eukaryotic organisms and being implicated in a diverse array of cellular processes.[56, 282]

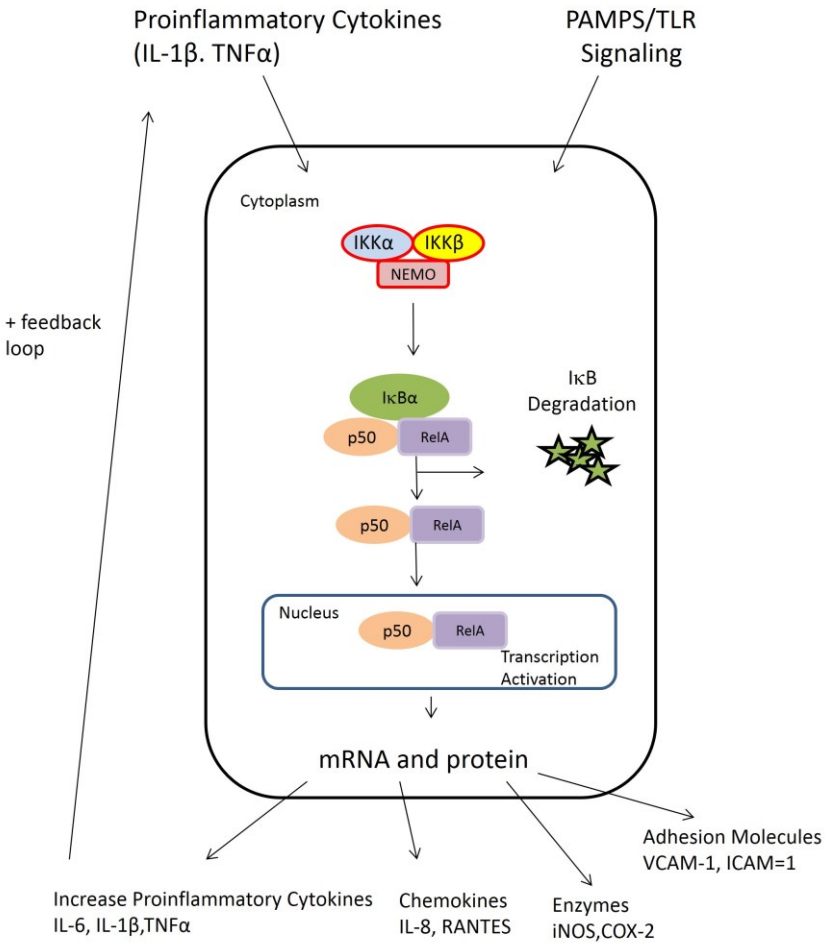


Figure 4: NFκB Signaling

In its inactive state, NFκB is present in the cytosol (represented by its p50-RelA subunits here) bound it the inhibitory protein IκB. Upon activation by relevant stimuli, the IκB kinase complex (IKK) phosphorylates IκB, which is subsequently released from NFκB and degraded. NFκB is then free to translocate to the nucleus and initiate gene transcription.[60]

It has been shown that while JNK and p38 are both activated by pathogens in insect cells, p38 appears to drive antimicrobial peptide gene expression following exposure to LPS.[174, 175, 443] In mammalian cells, 14 different members of the MAPK have thus far been described, three of those having been extensively studied for their role in activating immune responses; the extracellular signal-regulated protein kinases (ERK), c-Jun NH_2-terminal kinases (JNK) and the p38 MAPKs.[19] Each of these MAPK members are induced by different factors (although there are many commonalities amongst the groups, discussed below) and activated by different upstream kinases, however the canonical activation of all three remains conserved.

Signal transduction via MAPKs involves a primary cascade that is made up of a core tier of three kinases in which those upstream are responsible for phosphorylation and activation of those kinases located downstream.[19, 119] Signal transduction commences by the interaction between upstream signalling adaptors and small GTPases, leading to the activation of MAPK kinase kinases (MAP3Ks). In general, the ERK pathway can be activated by Ras GTPases via the Raf group of MAP3Ks while the p38 and JNK pathways are activated by the Rho family of GTPases.[4, 144, 189, 313, 506, 525, 534] The activation of MAP3Ks, in contrast, is

complex and different, such that MAP3Ks activate their targets in different manners. For instance, TGFβ-activated kinase 1 (TAK1) functions as a direct MAP3K for the p38 and JNK pathways, but is also able to indirectly activate tumour progression locus 2 (TPL2), a MAP3K for the ERK1/2 pathways.[39, 403]

Although over expression experiments have often shown that an individual MAP3K has the potential to regulate all of the major MAPK pathways, experiments using cells deficient in MAP3K have shown that MAP3Ks actually show specificity under physiological conditions.[463] In any event, MAP3Ks activate MAPK kinases (MAP2K) via serine- threonine (Thr) phosphorylation. Phosphorylated MAP2K is then able to activate the MAPK via dual phosphorylation of a Thr–X–Tyrosine (Tyr) motif (where X represents any amino acid, and is different for the ERK, JNK and p38 MAPK) in the activation loop by the MAP2K. The phosphorylation of Thr and Tyr residues is performed by a specific protein kinase cascade for each of the three pathways. The ERK pathway is activated by the MAP kinase kinases (MKK)1 and MKK2; the JNK pathway is activated by MKK4 and MKK7; and the p38 pathway is activated by MKK3 and MKK6. Specificity of MAPK phosphorylation by the appropriate MAP2Ks is

achieved through interaction of the MAPK-docking domain in the MAP2K with the domain in the appropriate downstream MAPK.[29]

The ERK1/2 and p38 pathways have been shown to activate several downstream effectors important for the activation an effective immune response, which will be described later on. Figure 5 summarizes the signaling cascade for the ERK, JNK and p38 pathways. A brief overview of each of the three MAPKs (ERK1/2, JNK and p38) and the relative contributions of each pathway to the induction of pro-inflammatory cytokines in the innate immune response of AECs is also provided below. Note that the role of each MAPK has been extensively studied for their effects in myeloid cells, in the activation of adaptive immune response, maintenance of immune regulation and in the anti-inflammatory response. How pathogens are able to hijack and utilize these pathways is also an interesting area of research. These fields show the diverse range of MAPK in signal transduction and its overall importance in cellular functions, but will not be discussed in this present work.

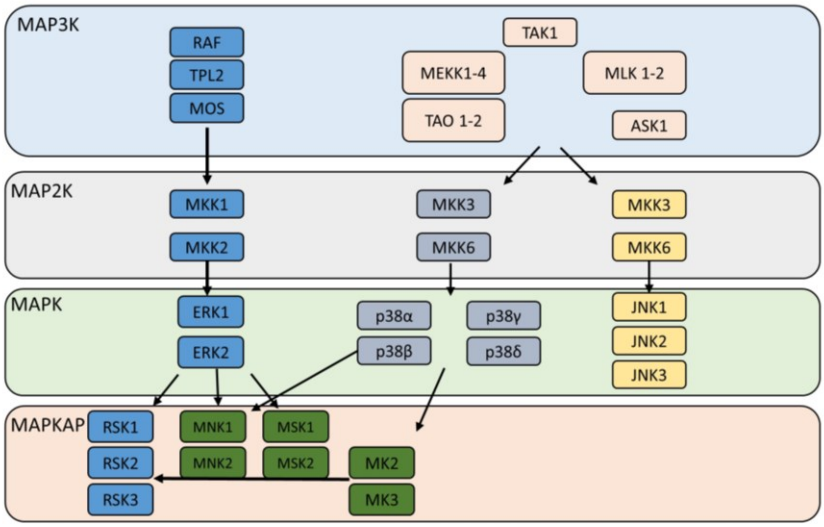


Figure 5: Mitogen Activated Protein Kinase signal transduction cascade.

Mitogen-activated protein kinase (MAPK) pathways consist of a series of at least three kinases: a MAPK kinase kinase (MAP3K) that activates a MAPK kinase (MAP2K), which in turn activates the MAPK.

ERK1/2: RAF and MOS are MAP3K that activate ERK1/2 specific MAP2K in various contexts. In innate immune responses, the tumour progression locus 2 (TPL2) MAP3K has a dominant role and is used by all Toll-like receptors (TLRs), tumour necrosis factor receptor 1 (TNFR1) and interleukin-1 receptor (IL-1R).

p38 MAPK: This family comprises four isoforms (p38α, p38β, p38γ and p38δ), which are activated by the MAP2Ks MKK3 and MKK6.

JNK MAPK: This family has three isoforms (JNK1, JNK2 and JNK3), which are activated by MKK4 and MKK7.

Many MAP3Ks can potentially activate the p38α and JNK cascades, and the specific MAP3K that is required is dependent on the stimuli and the cell type.

The activation of ERK, JNK or p38 MAPK can led to the activation of several downstream effector protein (MAPK activated proteins or MAPKAPs)

Canonical Activation of MAP3K following TLR activation

The sequences of events involved in the activation of MAPKs via TLR signaling have been described in the previous section of this thesis. The activation of the MAP3K TAK1, following stimulation by TLR and tumour necrosis factor (TNF), can subsequently activate MAP2Ks in both the p38α and JNK pathways.[498] It has been shown that TAK1 associates with TAK1-binding protein 1 (TAB1) and the ubiquitin-binding proteins TAB2 and TAB3. The recruitment of TAK1 to TRAF6 for ubiquitylation (which occurs via TAB2 and TAB3) results in the activation of TAK1 kinase activity.[308, 422, 437] Initial studies revealed that TAK1 was a major factor in the activation of JNK and p38 MAPKs, however its role has now been deemed more complex and other MAP3Ks may additionally play prominent roles.[6, 19] The MAP3K apoptosis signal-regulating kinase 1 (ASK1) can induce activation of p38α and is required for optimal LPS induction of the pro-inflammatory cytokines TNF, IL-1 and IL-6 in response to LPS.[317] Under normal physiological conditions, ASK1 forms an inactive complex with thioredoxin. Upon stimulation via TLR4, thioredoxin dissociates from the complex, promoting ASK1 auto-phosphorylation and activation.[336] An ASK1/TRAF2/TRAF6 complex then forms, which allowing for ASK1 oligomerization and subsequent activation.[145, 337] While

the role of the MAP3K MAPK/ERK kinase kinase 3 (MEKK3) is still unclear, it is known that it somehow gets recruited to TRAF6 for p38 and JNK activation[202, 330] Activation of ERK1 and ERK2 by all TLRs is mediated by the MAP3K TPL2 in macrophages and its importance in AECs has only recently been described.[150, 296] In unstimulated cells, TPL2 exists as part of a complex with the NFκB subunit precursor protein p105 (NF-κB1), which inhibits the kinase activity of TPL2.[38, 502] Macrophage stimulation via TLRs activates the IKK complex, which then phosphorylates p105 resulting in its degradation.[39] After TPL2 has been released it can phosphorylate MKK1 and MKK2 upstream of ERK1 and ERK2.

Extracellular Signal-Regulated Protein Kinases

The ERK1/2 module was the first of the mammalian MAPKs to be sequenced and identified as serine/threonine kinase. [64, 65] It was termed a mitogen stimulated kinase since this MAPK was found to be important in promoting cells to enter phases of the cell cycle.[407] ERKs 1 and 2 display a homology of 85% to one another and are ubiquitously expressed in a vast array of tissues, although certain tissue types will express each of these kinases in different amounts.[364] The characterization of ERK1/2 was mostly done in fibroblasts, which showed that phosphorylation

occurred to many different mitogen factors, including serum, growth factors, cytokines, certain stresses and transforming agents.[364, 407]

Regarding immune function, the use of specific MKK1 and MKK2 inhibitors has demonstrated that ERK1/2 activation has several consequences on inflammatory responses including induction of TNF, IL-1 β and IL-10 production following TLR stimulation and simultaneous negative regulation of IL-12, IFNβ and iNOS production.[92, 125, 218, 311, 527] Numerous studies have shown that the level of ERK1/2 phosphorylation in the airway epithelium is increased in the presence of microbial pathogens leading to increased pro-inflammatory cytokine production and gene expression.[21, 44, 94, 236, 296, 393, 521, 522, 531] For instance, the peptidoglycan from *Staphylococcus aureus* has been shown to induce increased MUC5A expression in AECs, leading to increased mucus secretion, increased cell permeability and lung damage, as well as induced cytokine production.[236, 521] Airway epithelium stimulated by *Pseudomonas aeruginosa* bacteria or flagellin have also been shown to activate ERK1/2 to induce pro-inflammatory cytokines.[21, 44, 296, 393, 522, 531]

c-Jun NH_2-terminal kinases/Stress activated protein kinases

The first form of the JNK/SAPK family of MAPK was isolated as a microtubule associated protein (MBP) from the livers of rats treated with

cyclohexamide.[261] Shortly thereafter, 2 similar proteins were identified as binders to the down-stream c-Jun protein, and termed JNK2 and JNK3.[188] In total there are three different genes that encode for ten differently spliced variants of the JNK/SAPK family of kinases.[169] The expression of JNK1 and JNK2 is ubiquitous, while JNK3 is mostly restricted to neuronal tissues, testes and cardiac myocytes.[19] Many different stimuli, including cytokines, certain ligands for GPCRs, agents that interfere with DNA and protein synthesis, as well as other stresses and microbial products have been shown to stimulate the activation of the JNK pathway.[364] The lack of a specific JNK inhibitor and embryonic lethality seen in knockout mice has made elucidating the function of JNK more challenging. However, recent a recent study with JNK1/2 being selectively deleted in cells of myeloid lineage have shown a pro-inflammatory role for JNK in macrophages in response to LPS stimulation. The role of JNK in response to microbial pathogens remains unclear, however it has been proposed suggest that JNK acts in an immunomodulatory manner to decrease the production of pro-inflammatory cytokines by blocking NFκB activation.[28]

p38 Mitogen Activated Protein Kinase

The discovery of p38 was independently described for three different functions: first, it was considered to act as a tyrosine phosphoprotein secreted from cells treated with inflammatory cytokines or LPS; second, it was thought to be the target of a drug that blocked production of TNFα; and third, it was proposed to act as an activating kinase for MAPK activated protein kinase-2 (MAPKAP-K2).[173, 273] There are four subfamilies of the p38 MAPK family termed α, β, γ, and δ, which are activated by a number of stimuli and potentially elicit different effects.[347, 379] While the α and β isoforms are ubiquitously expressed in tissues, the γ and δ isoforms are known to be differentially expressed in tissues, with the γ isoform predominant in skeletal tissues and the δ form primarily in the lung, testis, kidney, pancreas and small intestine.[213, 257, 270, 283]

The discovery of the role of these family members has been aided with the use of several specific inhibitors and various knockout mice.[19, 364] For instance, p38α and β isoforms are sensitive to pyridinyl imidazole inhibitors while γ and δ-isoforms are resistant to these drugs.[364] The compound BIRB 0796 is specific for p38 and inhibits all isoforms at differing concentrations, so it can be used to study the physiological roles of different p38 isoforms or compare the role of p38 and JNK in activating

pathways.[97, 255] An elegant study in mice by O'Keefe et al was able to show that the effects of p38 inhibitors on TLR-induced TNF production is due to the inhibition of p38α, and not to the inhibition of p38β.[342]

p38 homologs have been found in a diverse number of eukaryotic systems including yeast, Drosophila and the nematode *C. elegans.*[69, 174, 409, 478] It has been shown that p38 is activated by a number of different growth factors including granulocyte macrophage colony stimulating factor (GM-CSF), insulin growth factor (IGF), vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) amongst others. [85, 139, 384, 412] The wide array of effectors that activate p38 implicate this family in a number of different roles including cellular differentiation and cell migration, which are described in many different reviews.[97, 347, 414] The p38 MAPK system has also been extensively studied for its role in eliciting responses to various external stresses, particularly in response to microbial products and host cytokines, to induce inflammation. For instance, p38α has been shown to be activated in response to LPS, TNF, IL-17 and IL-8.[172, 373, 417, 435] The activation of p38 has been shown to drive the production of both IL-8, in response to osmotic shock, and IL-6, in response to TNFα.[47, 434] Importantly, p38 homologs have been shown to be critical for host survival in both Drosophila and *C. elegans.* In flies,

p38 has been shown to mediate and control infection, while in *C. elegans* absence of p38 leads to increased death in the presence of the bacteria *Pseudomonas aeruginosa*, indicating a critical role for p38 in the control of microbial infection.[84, 478] The role of p38 in the airway epithelium in response to microbial infection and TLR signaling has been studied numerous times and will be discussed in the context of the current work later in this chapter.

Downstream Targets of Mitogen Activated Protein Kinases

The MAPK family of proteins can activate a number of different mitogen activated protein kinase (MAPKAP or MK) as seen in Figure 5, as well as a number of different transcription factors. A brief overview will be provided here, with detailed reviews found elsewhere.[97, 347, 414] There are four main subfamilies of MKs. The p90 ribosomal S6 kinases (RSK) family was the first identified MK and are activated in the presence of mitogens via ERK2.[98, 129] Mitogen and stress activated kinases (MSK)1/2 are activated by both mitogens and stress factors, as well as by ERK1/2 and p38, but not by JNKs.[103, 372] Similarity between RSK and MSK substrate specificity has led to speculation that they may have similar physiological roles, possibly in histone phosphorylation and a nucleosomal response.[425, 450, 472] The MAPK-interacting kinase 1 and 2 (MNK1/2)

family of MKs were identified as a substrate for ERK1/2.[146] It is interesting to note that whereas MNK2 can only be activated by ERK1/2, activation of MNK1 can occur via ERK1/2 or p38.[500] MNK functions as a kinase for proteins involved in the eukaryotic initiation factor 4F (eIF4F), an important complex involved in cell growth and proliferation.[501] The MK subfamily has MK2 and MK3 members, which can be activated by both p38 and ERK1/2 in vitro, but only p38 activation has physiological roles. [42, 455] MK2/3 share similar roles in p38 driven cytokine synthesis, such as IL-6, TNFα in response to LPS.[172, 247] MK2/3 also play a role in cell migration. [182, 374] Finally, MK5 appears to signal through an atypical MAPK response and it has been suggested that MK5 may be involved in pro and anti-oncogenic signaling [450]

In addition to activation of MKs, there are also a number of different transcription factors (TFs) that become phosphorylated and subsequently activated by various MAPKs. For example, p38 has been shown to regulate numerous TFs including activating TF-2 (ATF-2), ATF-1, SRF accessory protein 1 (Sap1), p53, and more.[347] Activation of these TFs results in the expression of numerous cytokines and cell surface receptors via MAPKs. In addition to controlling activation of TFs and associated proteins, MAPKs, particularly p38 and the ERKs, are able to negatively

regulate the expression of MKs or activate proteins that modulate MAPK activation.[19]

Particularly important in the context of TLR signaling is the dual-specificity protein phosphatases (DUSPs). These proteins are able to dephosphorylate the MAPKs, leading to their deactivation. Following activation by Toll-like receptors (TLRs), and the subsequent activation of MAPKs, ERK1/2 and p38α promote the transcription of the DUSP1, DUSP2 and DUSP4 genes. This initiates a negative feedback loop to limit MAPK activation. DUSP1 targets p38α and Jun N-terminal kinases (JNKs), DUSP2 targets JNKs, and DUSP4 targets ERK1/2.[19] Of note, DUSP1 is a major target of glucocorticoids and is partly responsible for their anti-inflammatory affects through regulation of MAPK.[467] For a more detailed review, see Caunt et al.[82]

Chemokines and Cytokines in Airway Inflammation

In response to microbial pathogens and induction of NFκB and MAPK signal transduction pathways, the airway epithelium can produce and secrete a number of different cytokines and chemokines to modulate the immune response.[3, 279, 375] Many of these epithelial-derived substances can act locally, affecting both neighboring cells and tissues, so that structures and functions of the epithelial cells themselves become

influenced/altered. For instance, inflammatory mediators and cytokines may induce mucin secretion, affect ion transport, or increase ciliary beating, thus modulating local inflammatory responses and pulmonary defenses against infection.[279] In addition, the secretion of various chemokines can result in increased neutrophil recruitment to the site of inflammation in an effort to control the infection.[233, 249, 358, 494]

The airway epithelium is able to produce a number of different types of cytokines including colony-stimulating factors (CSFs), pleiotropic cytokines, chemokines, and growth factors (see Table 1). Granulocyte/macrophage colony stimulating factor (GM-CSF) was the first of the CSFs detected as a product of epithelial cells and can function as a potential mediator of inflammation due to its broad spectrum of biological activity, including prolongation of eosinophil survival, mediator release, and the enhancement of phagocytic activity for neutrophils, eosinophils, and macrophages.[233, 249, 280, 295, 358, 448] Interleukin-6 is able to elicit varying effects, however in the context of asthma and inflammation due to microbial activation of AECs, IL-6 appears to elicit a pro-inflammatory response in AECs.[18, 279, 335] Chemokines, specifically IL-8 and to a lesser extent RANTES, are secreted in relatively large amounts by cultured AEC and is prominent in the bronchial alveolar lavage (BAL) of patients with

chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), where they act as chemoattractants for neutrophils. Therefore, the presence of IL-8 and RANTES could explain the presence of excess neutrophils in the aforementioned diseases/illnesses [93, 391, 481]

In conjunction with chemokines and cytokines, lipid mediators, peptide mediators, reactive oxygen species, enzymes, and inhibitors have all been shown to be secreted by the airway epithelium in response to pathogens as a way of modulating protective features of the AECs. Taken together, the physical barrier of AECs, mucociliary clearance, and induction of cytokines/chemokines all contribute to the maintenance of a relatively sterile environment in the lungs so that foreign pathogens may be successfully detected and inflammatory responses may be induced.[114, 159, 207, 223]

Cystic Fibrosis

A Brief Perspective of Cystic Fibrosis

Cystic fibrosis is an autosomal-recessive disorder that is classified as the most common genetic disease in the western world, with an incidence of approximately 1/2500 live births among the Caucasian population.[54, 55] As early as the start of the 17th century, reports of children with “salty sweat and emaciation leading to an early death” was

described in the medical annals.[12] While many prior reported cases closely resembled CF cases and were classified as such, the first true pathological and epidemiological report of the disease was conducted by Dorothy Andersen in 1938, who described the observed cases as “fibrotic cysts in the pancreases of diseased children”.[14] During the early 1940s, a pediatric doctor (Dr. Farber) summarized that the disease was not only restricted to the pancreas, but that it was in fact a systemic disease of mucosal layers and buildup.[130] At approximately the same time, the first study linking CF to an inherited disease was published.[201] It was also firmly established that the pathology of the pancreas and the inflammation in the lung were from the same disease.[513] With better understanding came better treatment options and the first recommendations for specific diets came out for patients diagnosed with CF.[13] Importantly, patients with CF and associated lung disease showed improvements with aerosolized penicillin treatment, suggesting that bacterial infections played a role in the morbidity of patients. In the early 1950s, it was established that CF patients were displaying abnormal chloride transport in their sweat glands, paving the way for new theories on the etiology of the disease and novel, less invasive diagnostic procedures (i.e., the sweat test).[112, 314] By the end of the decade, new diagnostic tests and treatment therapies had

led to a remarkable increase in CF patient survival. A disease once thought to lead to death within the first couple years of life, patients were now seeing survival, on average, into teenage years and several patients surviving into early adulthood.[111, 439] Optimism spurred clinicians to find a definite treatment for CF patients, and by the end of the 1960's a plan was in place that is still generally in practice today. The 'treatment plan' incorporated many of the components now regarded as essential for modern CF centre care including early correct diagnosis, a comprehensive physical therapy program and antibiotic regimen to deal with all the aspects of the disease and data collection to validate the impact of the treatment on morbidity and survival.[117, 118, 302] While all the evidence was pointing to a disruption in ion transport and genetic disease, there was still little known about basis of the disease. In order to develop new treatments and increase survival further, an understanding of the genetic basis for the disease was required.

The Genetic Basis for Cystic Fibrosis

Evidence mounted of a primary defect in ion transport of epithelial cells throughout the 1960s and 70s. However, it was not until 1980, during a conference held by the Canadian CF Foundation, that the first evidence of ion transport in epithelial cells was recognized as the primary

defect in patients with CF.[240] 1981 was the year during which chloride (Cl^- ion transport malfunction followed by rapid absorption of sodium in epithelial cells of CF patients was observed and published for the first time, marking a milestone achievement in the field of CF.[240] This was a landmark paper in the field of CF. By measuring a single parameter, the transepithelial potential difference (TPD), Knowles and colleagues were able to demonstrate that ion transport *in vivo*, an event that occurs within hours of birth, was greatly elevated in CF patients compared to patients without CF, thus suggesting a primary genetic epithelial disorder in CF patients. Eventually researchers uncovered a defect of epithelial cells in CF patients such that there was a significantly reduced ability of chloride (Cl) ions to move across CF cells (impermeability of Cl^- ions) resulting in a very rapid reabsorption of sodium, thus creating an imbalance of salt and water properties in the epithelium.[386] This led to the theory that CF airway epithelium has increased NaCl and water absorption into the epithelial cells leading to dehydration of the epithelial secretions, reduced clearance of mucus which leads to an increase in chronic airway infection.[54, 385] Armed with this new information about the basic defect in CF and improved techniques in reverse genetics, the search for the causative gene mutation leading to CF began.

In 1985, three papers where published in the same issue of Nature that identified the gene locus, located on chromosome seven, thought to be responsible for CF.[242, 495, 508] The gene responsible was soon found to be a chloride ion channel, as a report was published showing that chloride channels were differential regulated in airway epithelial cells of patients with CF.[143] Finally, in September of 1989, three papers published in Science identified and described the gene ultimately responsible for CF.[231, 400, 405] The gene responsible for CF was termed the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), spanning 189 kilobases and containing 27 exons and 28 introns.[480] The authors of these papers had also cloned the functional gene into primary cells obtained from the pancreatic duct or airways of CF patients, which resulted in restored transepithelial potential difference, as had been expected.[86, 123, 398]

The Cystic Fibrosis Transmembrane Conductance Regulator: Function and Mutations Leading to Cystic Fibrosis

The Cystic Fibrosis Transmembrane Regulator (CFTR) protein is a phosphorylation dependent, low conductance Cl^- ion channel that is predominately expressed on the apical side of epithelial cells.[399, 436] The CFTR plays a central role in overall salt transport, fluid flow and ion concentrations across the epithelial. The CFTR is a rather large glycoprotein, consisting of 1480 amino acids and is composed of two

motifs, each of these motifs containing a membrane spanning domains (MSD) containing 6 transmembrane segments and a nucleotide binding domain (NBD) that is capable of interacting with adenosine tri-phosphate (ATP).[204] In CFTR, the MSD and NBD are linked by a unique domain known as the regulatory (R) domain, which contains multiple consensus phosphorylation sites.[436] The MSDs of CFTR are responsible for the selectivity of Cl^- ions through the pore, while the NBD hydrolyzes ATP to gate the channel and the R domain is phosphorylated to control activity of the channel.[319, 436] For further review of the biophysical properties of CFTR and the structure/function relationship of the pore, refer to Riordan et al. and Sheppard et al.[399, 436]

Mutations in the CFTR that lead to a CF phenotype can affect the protein in a number of different ways, and thus there are six classes on CFTR mutations that result in CF.[55] The mutations are divided into how they affect the CFTR protein, and a summary of these classes can be found in Table 3 below.[55, 503, 537] The most common mutant allele is a three-nucleotide deletion at the 508th codon in CFTR causing the deletion of a phenylalanine residue, or the ΔF508 mutation, resulting in defective intracellular processing of the protein.[55] While over a thousand mutations have been identified within CFTR, almost 70% of all CF patients have the

ΔF508 mutation. Amongst the North American population, approximately 1 in 26 people is a carrier for this mutation, and about 1 in 3500 live births will result in CF.[246] The phenotype of this mutation can range from relatively mild, with sufficient pancreatic function, and a more extended lifespan, to more severe, with lowered pancreatic functioning resulting in fat malabsorption as well as more pronounced deterioration of lung function and higher incidence of malnutrition and liver disease.[537]

The expression of CFTR can be found in the epithelial cells of a variety of tissues and organs, including the lung and trachea, pancreas, liver, intestines, and sweat glands.[165, 476, 499] While the disease affects each of these systems, and is thus a systemic disease, the majority of morbidity and mortality is attributed the pulmonary manifestations that lead to hyper-susceptibility to chronic lung infections and increased inflammation. The focus in this thesis is on the pulmonary manifestations of defective CFTR in the lungs; a description of CF's other aspects can be found in the review published by Quinton.[385]

Cystic Fibrosis Phenotype in the Lung

Cystic fibrosis was traditionally seen as a mucoviscidosis disease, arising from abnormal mucus secretion, in terms of airway pathology.[383] The first notable sign of a CF phenotype in the lungs young patients, even

before any anatomical manifestations, is the appearance of hyper-secretion of mucus in the bronchi. [538] While the submucosal glands seem to be anatomically intact, they contain excessive number of goblet cells and they are filled with a dense mucus in patients with CF.[397] The presence of bacterial products as a result of chronic infection, in addition to a dehydrated mucus, led to the presence of this thick mucus within the lungs of CF patients. [378, 397] While the mucins themselves maybe of a different composition, it is clear that the presence of bacterial DNA contributes to the viscous mucus, and recombinant DNase has been shown to decrease mucus viscosity of sputum from CF patients.[83, 378, 519] While it remains unclear of how the differences in the viscous mucus is ultimately produced in the CF lung compared to the non-diseased lung; it is clear that bacterial infections in the bronchioles precede any detectable loss in lung function.[349]

One would imagine that having thick, viscous mucus in the lungs prior to or in the absence of infection would lead to impaired lung functioning; therefore, hyper secretion of mucus could very well play a role in susceptibility of CF patients to infection, but this is certainly not the only cause. The airway epithelium plays an important role in innate immune defense, as previously described in this section.

Table 3: Classes and Function of CFTR Mutations

Class	Description (Disease state, severe or mild)	Mutation Example (Prevalence in Canada)
I	Failure to synthesize full length CFTR. Little or no CFTR. (Severe)	G542X (2.2%) W1282X (0.9%)
II	Improper protein folding and defective delivery to membrane. Little or no CFTR. (Severe)	ΔF508 (71.4%) Δ1507 (1.9%)
III	Defect in opening of CFTR channel at membrane. Function impaired. (Severe)	G551D (3.1%)
IV	Structural defect in CFTR that reduces ion passage through channel. (Mild)	R117H (0.9%)
V	RNA splicing errors lead to reduced quantity of functional CFTR. (Mild)	A455E (1.4%)
VI	Causes increased cell surface turnover and degradation of CFTR. (Mild-Severe)	Q1412X (ND)

The defective ion transport in airways of patients with CF results in a dehydrated PCL, inhibiting proper ciliary function and mucociliary clearance. It has been demonstrated that bacterial killing is impaired due to this dehydrated PCL.[63, 301, 445] The use of hypertonic saline solution to restore proper ciliary clearance and rehydrate the airways has been proposed for a treatment to CF.[80, 395, 402] In addition, it has been reported that secreted antibacterial compounds produced by the AEC do not

function properly in CF patients due to the altered salt concentrations in the ASL, however subsequent experimentation has not confirmed these findings.[445] The most recent evidence generated from the CF pig hints towards defective bacterial killing in the acidic environment of the CF lung.[370]

Taken together, the innate immune defenses of the airway epithelial cells in CF do not work properly, leading to a niche for bacteria to grow and establish chronic airway infections. A four part detailed review on the current state of CF, involving epidemiology, genetics, microbiology and other factors from worldwide clinics was recently released and provides a more detailed review on the global perspective of CF.[78, 418-420]

Microbiology of the CF airways

The altered physiological composition of the airways in CF patients offers the ideal environment for bacterial colonization. As a result, CF patients suffer from chronic infections that result in a progressive decline in lung function, often associated with an increase in morbidity and mortality. The central dogma of CF microbiology is that only a restricted number of bacterial and fungal infections set up the chronic infections in the lungs of CF patients. However, recent studies have highlighted the actual diversity of bacterial species that are able to colonize the lungs of

CF patients, revealing a great amount of diversity amongst bacterial species, effectively creating a “microbiome” in the lungs of CF patients.[292, 458] In addition to the species diversity found in the adult CF lung, recent studies have stressed that there is a diversity amongst the strains of bacteria infecting the lungs.[324] Despite these studies, it has become clear that certain bacterial species dominate the CF airways in an age-dependent manner. While the majority of CF patients are colonized with *Staphylococcus aureus* during their infant/early childhood years, they tend to become chronically infested with *Pseudomonas aeruginosa* during their teenage years and from young adulthood onwards. Table 4 summarizes the predominant bacterial species of the CF lung.[286, 457] As treatments continue to improve and life expectancies become greater in CF patients, *P. aeruginosa* can clearly be considered the most prevalent and important bacterium colonizing the CF lung.

Table 4: Bacterial Microbiology of the CF Lung

Bacteria	Overall Prevalence	Prevalence (Over age 18)
Pseudomonas aeruginosa	52.5%	79.8%
Staphylococcus aureus	50.9%	21.2%
Haemophilus influenzae	16.3%	8.3%
Burkholderia Species	5%	5%

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram negative, unipolar flagellated bacterium that is ubiquitous in nature and is a major opportunistic pathogen in hospital-acquired infections.[105] As *P. aeruginosa* displays a high amount of genetic diversity and numerous virulence factors that allow it to colonize a variety of different niches, it is a particularly widespread pathogen in hospital-acquired infections.[195, 232] The versatility in *P. aeruginosa* can be attributed in part to the large number of genes encoded by the bacteria; the complete genome sequence contains 6.3 million base pairs with approximately 5,570 open reading frames, making it the most complex bacteria genome sequenced to date. [456] In a medical setting, *P. aeruginosa* is especially important in the context of urinary tract infections,

ear infections, ventilator associated pneumonia, burn patients and chronic infection in CF patients.[232, 238, 286, 416, 490]

Pseudomonas aeruginosa in Cystic Fibrosis

Pseudomonas aeruginosa is the most prevalent bacteria in the lungs of CF patients with an incidence of 10 to 30% between the ages of 0 –5 years and increases to a staggering prevalence of 80% in the CF population over the age of 18.[286] Furthermore, the early acquisition of *P. aeruginosa* is a strong predictor of a declining prognosis.[127] The inflammatory response elicited by chronic infection is associated with a significant deterioration in lung function, and an overall increase in the morbidity and ultimate mortality in CF patients, with chronic infection increasing the risk of death 2.6-fold.[284, 392]

Pseudomonas aeruginosa infection is restricted to the proximal and small airways, while being absent from the alveolar compartments.[161] Multiple studies have also revealed that *P. aeruginosa* is present in the thick mucosal layer directly above the airway epithelium, distal from the AECs, in a nutrient poor, hypoxic environment.[27, 160, 179, 321] The genetic versatility of *P. aeruginosa* in part explains its ability to successfully exploit this niche in the CF lung, however it does not completely explain the absence of other bacterial species within the CF lung. Several hypotheses

have attempted to find a reason for this; one such theory describes the ability of *P. aeruginosa* to thrive in the salt deficient mucus of the CF lung, another describes the potential lack of CFTR leading to a dysfunction in the ability of AECs to bind and clear *P. aeruginosa*, both which are reviewed elsewhere.[79] In addition, *Pseudomonas* undergoes two critical phenotypic changes that impact its ability to form chronic infections, namely the formation of biofilms and the switch to a mucoid phenotype, both which will be discussed below.

Pseudomonas aeruginosa Biofilms in Cystic Fibrosis

Bacteria are able to grow in two distinct manners; as free swimming, motile bacteria unattached to a surface, known as planktonic growth, or as bacterial aggregates attached to a surface or encased in exopolymeric substance, known as biofilm growth. Biofilm formation occurs after a motile bacterium has attached to a surface or aggregate in sufficient amounts to induce a switch in gene expression and induce the production of exopolymeric substances (EPS), composed of various secreted carbohydrates, bacterial proteins, DNA and RNA, which encase the bacterial community.[120, 171] This genetic switch to a biofilm state also results in a loss of motility/loss of flagellum expression and differential expression pili.[120, 209] Within the mature biofilm, nutrient channels are

formed to allow efficient oxygen and nutrient dispersion throughout the colony. The encased bacteria represent a heterogeneous population of bacteria, with various regions of bacteria within the community expressing different genes, and facing different local oxygen and nutrient environments.[120, 171, 178] The encased bacterial communities are able to release bacteria, which can then revert back to a planktonic state, to colonize different areas. Biofilms provide many advantages to bacteria involved in chronic infections, including increased antibiotic resistance, decreased phagocytosis and an efficient strategy for sequestering limited nutrients.[34, 193, 293]

Several different studies have shown that *Pseudomonas aeruginosa* likely forms aggregated biofilms within the mucus of CF airways *in vivo*.[100] Clusters of cells representing microcolonies of Pseudomonas have been visualized within the CF airways and have been excised from post-mortem samples of patients with CF.[27, 264] Further, biofilm like colonies were observed in freshly excised lung sections, lung abscess and the sputum of CF patients, respectively.[194, 441, 515] While planktonic ligands, such as flagellin or LPS, have been well studied for their ability to induce inflammation in epithelial cells, little work has been

done on the ability of biofilm materials to stimulate inflammatory responses in these cells.

Quorum Sensing in *Pseudomonas aeruginosa*

An important aspect in the formation and maintenance of bacterial biofilms is the expression of secreted genes that allow the bacteria to sense their neighbors. This type of bacterial sensing is known as quorum sensing. Pseudomonas is able to sense and respond to their population density via self-produced small diffusible molecules termed N-acylated homoserine lactones (acyl-HSL).[157, 215] Quorum sensing (QS) is a complex system that will not be reviewed in detail here. However, it is important to note that *P. aeruginosa* has three important QS pathways, of which two will be briefly described. The first system is the las system, consisting of the signal synthase LasI, which produces N-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL), and the signal receptor LasR, which binds 3OC12-HSL and activates transcription of certain target genes.[148, 363, 365] The second QS system is the Rhl system, consisting of the signal synthase RhlI, which generates N-butanoyl-homoserine lactone (C4-HSL), and its signal receptor RhlR.[338, 339, 366] LasR and RhlR also induce the transcription of their own genes, creating a positive feedback loop, or autoinduction and are arranged in a hierarchical fashion as the

LasR–LasI system activates the RhlR–RhlI system.[265, 369, 427] These molecules have been detected at nanomolar to micromolar concentrations in lung tissues and sputum samples from CF patients experiencing *P. aeruginosa* infections.[132, 310, 441] At such concentrations, HSLs have displayed immunomodulatory effects including induction of IL-8 secretion from AECs and activation of p38 MAPK phosphorylation.[196, 252, 303] In addition to quinolone, which is involved in the third QS pathway (also termed *P. aeruginosa* Quinolone Signaling or PQS quorum sensing), *P. aeruginosa* possesses an intricate system for controlling the regulation of many genes, including those crucial to virulence and biofilm maintenance.[100, 237, 427] Novel strategies in therapeutic development have targeted bacterial QS systems for the potential treatment of CF patients.[51]

Mucoid *Pseudomonas aeruginosa* in Cystic Fibrosis

The nature of the environment to which *P. aeruginosa* is exposed in the CF lung, specifically high levels of reactive oxygen/nitrogen species, results in various mutations that are selected for and that confer an adaptive advantage to the bacteria.[66] In fact, clinical isolates of *P. aeruginosa* from patients with CF tend to show a hypermutability phenotype, leading to increased mutation rates and selections so they can

better adapt to the environmental cues.[31, 122, 343, 344] While mutations can have an impact on any of the bacterial genes, the lungs of most CF patients are initially infected with a “non-mucoid” isolate of CF which, over time, mutates to become a mucoid phenotype.[73, 88] ‘Mucoidy’ is a descriptive term, referring to the overproduction of the exopolysaccharide alginate by the bacteria.[389] The production of alginate is a complex process, requiring many genes in its synthesis that is reviewed in the Ramsey text.[389] While any gene in the process could be mutated to derive a mucoid phenotype, 80% of isolated mucoid CF strains have a mutation in the *mucA* gene or genes that lead to *mucA* degradation, suggesting a functional hotspot for mutation.[62] MucA is a gene that sequesters an activator of alginate production (AlgT), and thus its mutation leads to free cytosolic AlgT and constitutive expression of alginate genes.[300]

The switch to a mucoid phenotype also control a number of virulence factors in *P. aeruginosa* while also conferring several advantages to the bacteria that make eradication of the chronic infection more difficult. [299] Mucoid strains tend to be more resistant to antibiotic treatments, resist phagocytosis by immune cells of the host and prevent binding of the complement opsonization.[162, 371, 429] In addition, mucoid

strains of Pseudomonas seem to have enhanced ability to form aggregate biofilms and increased adhesion molecules expression.[333, 381, 388] Mucoid strains of Pseudomonas have also been shown to decrease pulmonary function and contribute to increase inflammatory profile of the CF airways.[367] Interestingly, mucoid *P. aeruginosa* has been shown to upregulate several different types of lipoproteins, which may have an effect on induction of inflammation in airway epithelial cells. [135] The role of mucoid lipoproteins or alginate on the induction of inflammation has not yet been elucidated. Figure 6 summarizes the interaction between *P. aeruginosa* and the airway epithelium.

Airway Inflammation in Cystic Fibrosis

Inflammation in CF is defined by a persistent and overwhelming presence of neutrophils within the lung. [121, 245] Much evidence has been generated to support the fact that chronic infection leads to a substantial portion of this increased inflammation.[25, 380]

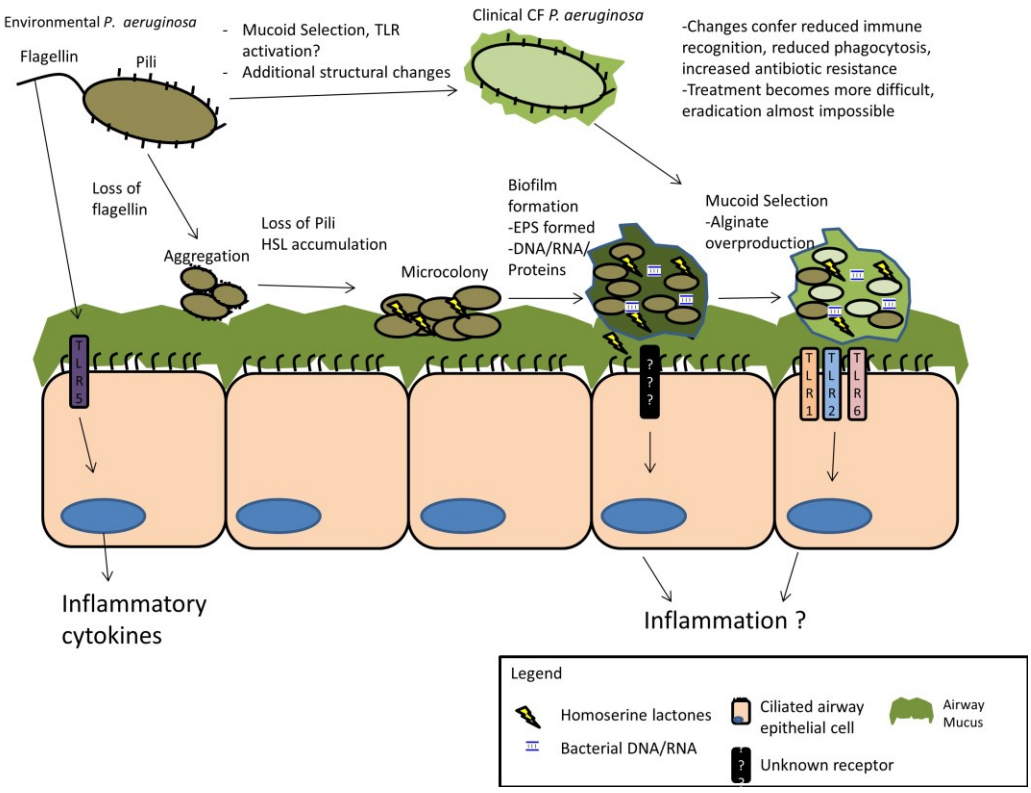


Figure 6: *Pseudomonas aeruginosa* infection in the CF lung.

Environmental strains of *P. aeruginosa* set up infections in the mucosal layer of the CF lung. Planktonic *P. aeruginosa* is mostly detected via TLR5 to induce a pro-inflammatory response that is insufficient in clearing the infection. *P. aeruginosa* aggregates into bacterial communities within the mucus layer, altering gene expression and producing bacterial biofilms. These biofilms create QS molecules and other factors, and it is currently unknown how they affect AEC. In addition to biofilms, environmental strains undergo mutations leading to 'clinical isolates' of the bacteria, often exhibiting a mucoid phenotype and antibiotic resistance. This leads to chronic bacterial infections that cannot be eradicated and can lead to increased lung inflammation.

Many studies have also linked various components of *P. aeruginosa* to the induction of pro-inflammatory cytokines, such as IL-8, in airway epithelial cells in the context of cystic fibrosis. For instance, *P. aeruginosa* has been shown to activate TLR5 in AEC to induce p38 activation and lead to IL-8 production.[163, 535] Cells lacking CFTR also show an increased inflammatory response to these products.[44] These studies, outlined in Table 5 below, exhibit the ability of *P. aeruginosa* to induce inflammation, and contribute to the CF phenotype in the lung.

In addition, it has been observed that in CFTR-deficient cells there is an increased pro-inflammatory signaling in the absence of infection.[93] Analysis of fetal tracheal explants showed an increased level of endogenous NFκB signaling and IL-8 production in the absence of bacterial infection.[245, 473, 488] Furthermore, elevated levels of IL-8 and TNF (in comparison to the perceived bacterial load) have been found in the bronchial airway lavage (BAL) fluid of CF patients despite the absence of any recognized pathogens.[234, 406] While it may be difficult to obtain samples that are truly not in the presence of bacterial infection and epigenetic/other host factors may play a role in this increased signaling, it is still worth mentioning that CF patients seem to have an intrinsic increase in pro-inflammatory signaling or gene transcription.

Table 5: Induction of Inflammation by *P. aeruginosa* in CF lung

Study	Summary
Schiotz et al. 1983[424]	Inflammation levels in lungs of CF patients chronically infected with *P. aeruginosa* (PA) and the effects of steroids.
Pedersen et al. 1990[367]	Ability of alginate from PA to modulate inflammation.
Elborn et al. 1993[126]	Inflammatory markers before and after first PA culture from lung of CF patients.
DiMango et al. 1995[116]	Diverse PA gene products, including HSL, stimulate respiratory epithelial cells to produce interleukin-8.
Yu et al. 1996[530]	Virulance properties of mucoid PA.
Kammouni et al. 1997[219]	Induction of cytokine secretion from AEC by PA LPS.
Feldman et al. 1998[133]	Role of PA flagella in pathogenesis of lung infection
DiMango et al. 1998[115]	Activation of NFκB by PA in normal and CF AEC
Denning et al.[110]	PA Pyocyanin increases IL-8 in human AEC
Epelman et al. 2000[128]	PA exoenzyme S induces cytokine production in AEC
Koyama et al. 1999 &2000[250, 251]	PA-LPS is less inflammatory but still induces cytokine secretion in AEC
Kube et al. 2001[253]	Proinflammatory cytokine responses to PA in AEC
Liedal et al.[277]	Small molecular weight secreted materials from PA increase IL-8 in AEC
Wieland et al. 2002[512]	Inflammatory response in AEC stimulated by PA LPS, exotoxin A and phospholipase
Lizweski et al. 2002[288]	Mucoidity is essential for PA pathogensis
Smith et al. 2002[447]	PA autoinducers (HSLs) induce inflammation in AEC
Aldallal et al. 2002[9]	Inflammatory responses to PA in primary CF AEC
Denning et al. 2003[109]	Secreted metabolites of PA effect immune-modulatory proteins in AEC
Firoved et al.2004[135]	Lipoproteins in mucoid PA and activation of MAPK in AEC

Cobb et al. 2004[91]	Flagellin and alginate elicit distinct inflammatory responses in AEC
Kowalski et al. 2004[248]	Localization of CFTR into lipid rafts is required for PA induced activation
Greene et al. 2005[163]	TLR induced inflammation in CF and non CF cells
Zhang et al. 2005[535]	PA flagellin acts through TLR5 to induce inflammatory response
Raia et al. 2005[387]	Inhibition of p38 MAPK modulates inflammatory response
Wu et al. 2005[517]	Airway epithelial response (via MAPK) and tolerance to PA
Bjarnsholt et al 2005[50]	Blocking QS leads to increased clearance of PA in lungs
Dalgado et al. 2006[106]	PA DNA induction of IL-8 in AEC depends on p38 but not NFκB
Pan et al. 2006[356]	Steroids block PA produced pyocyanin IL-8 induction in AEC
Bauman et al. 2006[30]	Vesicles secreted from PA induce inflammatory response
Bragonzi et al. 2009[66]	Microevolution of PA leads to adapted inflammatory induction
Raoust et al. 2009[390]	LPS or flagellin are sufficient to activate TLR induction of inflammatory cytokines in AEC
Becker et al. 2010[36]	Lipid rafts are important in mediating inflammatory signals in CF
Ciornei et al. 2010[89]	Biofilm PA has structural changes to LPS and induce greater inflammatory responses
Berube et al. 2010[44]	Loss of functional CFTR increases IL-6 production in response to PA in AEC
Roussel et al. 2011[413]	PA drives IL-8 through redundant TLR and NADPH oxidase in CFTR deficient AEC
Cosgrove et al. 2011[95]	PA proteases contribute to IL-8 production in AEC
Mayer et al. 2011[303]	PA HSL induces a hyperinflammatory response in AEC
Gellatly et al. 2012[153]	PA QS regulation determines virulence of bacteria
Martel et al. 2013[296]	TPL2 is essential for the activation of inflammatory cytokine production via ERK MAPK in AEC
Parker et al. 2013[361]	Uptake of PA flagella in AEC induces inflammation
Park et al. 2013[360]	Bacteria free secreted vesicles induce inflammatory responses in AEC
Valentine et al. 2013[484]	Inhibiting HSL molecules of PA reduces inflammation in AEC

If this is the case, not only would inflammation need to be controlled by targeting the bacterial infection, but also by correcting the basic defect.

Taken together, this intrinsic pro-inflammatory state, in addition to chronic bacterial infection that cannot be cleared, leads to a vicious progression of increasing inflammation in the CF lung. This leads to a dramatic increase in pro-inflammatory cytokines, particularly IL-8, and an increased number of neutrophils and their products. A marked increase in reactive oxygen species is observed within the AEC, the presence of which can lead to damage of the epithelial cells and fibrosis.[93, 147] It has also been proposed that CFTR is a channel for antioxidants, such as glutathione, and this only adds to the effect of increased ROS.[408] The end result for the patient is often bronchiectasis, reduced pulmonary function and ultimately respiratory failure leading to death.[323, 396]

Animal Models in Cystic Fibrosis

It is currently a goal in CF research to determine a suitable and tractable animal model that would allow examination of how CFTR mutants achieve increased bacterial infection and inflammation, and to demonstrate how modifications to CFTR mutants could possibly generate more favourable effects for the CF patient. While CFTR-deficient mice have been successfully generated, they still are not suitable

representatives of the human form, due to lack of spontaneous bacterial infection, differential leukocyte recruitment and absence of increased mucus production.[90, 93, 449] New models in pigs and ferrets have undoubtedly contributed to the elucidation of certain aspects regarding physiology of the disease, however the microbiology of the CF lung has yet to be fully replicated in these models.[229] Importantly, the aforementioned animal models do not spontaneously generate *P. aeruginosa* infections, providing an obstacle to the study of chronic or biofilm infections. In fact, to date there have been no animal models developed that would adequately describe the microbiology of the CF lung. Table 6 summarizes the current models.

Table 6: Animal Models used in CF Research

		Mouse			
Phenotype	Human	CFTR -/-	ENaC OE	Ferret	Pig
Chronic Infection	*P. aeruginosa;* *S.aureus* streptococci	Absent	Absent	Streptococci Staph. infection	Streptococci Staph. infection
Airway Inflammation	Neutrophils; NFκB/MAPK induction; IL-8 and TNF-α	Macro-phage recruited	Neutrophil NFκB	ND	Neutrophils
Mucus Production	Mucus dehydration; Excessive secretion	Not Present	Mucus Plugs	Lesions in newborns lungs	Hydrated Mucus; Excessive secretion

Rationale for Current Study

Airway epithelial cells contain a plethora of receptors that are able to monitor the host environment and respond to bacterial infections by inducing inflammation in order to maintain a sterile surface. In normal physiological conditions, inflammation plays a critical role in host defense; however, in the case of CF, chronic infections coupled with excessive, prolonged inflammation ultimately leads to lung destruction and respiratory failure and possible death. Patients with CF experience chronic bacterial infections that induce inflammation in a variety of ways. As table 5 summarizes, different individual ligands have been shown to activate AECs. These ligands, however, are typical of planktonic bacteria and therefore knowledge is limited regarding the mechanisms employed by bacterial biofilms (predominant in chronic CF infections) to induce airway inflammation. Additionally, the host inflammatory pathways triggered by mucoid *P. aeruginosa,* the most common type of bacterium cultured from chronically infected CF patients, initiate this type of response. Further studies of how changes in *P. aeruginosa* effect host response will certainly clarify the host-pathogen interaction occurring in the CF lung. These new tools would undoubtedly reveal new findings concerning the dynamic activation of inflammatory pathways in AECs. This will lead to new assays

for the identification of inhibitors to bacterial components that cause inflammatory induction.

Objectives

The main objectives of this thesis are to

1) Identify how planktonic and biofilm materials are recognized by the airway epithelium to induce pro-inflammatory mediators.
2) Identify the pathways involved in detecting mucoid and non-mucoid bacteria by airway epithelial cells.
3) Develop tools to study host-pathogen interaction in order to determine key virulence mediators produced by bacteria in a changing environmental state and over time.

CHAPTER 2: Differential activation of Airway Epithelial Cells by Planktonic and Biofilm *Pseudomonas aeruginosa* and the onset of innate immune activation.

Adapted from[33]:

Beaudoin T., Lafayette S., Roussel L., Bérubé J. Nguyen D and Rousseau S. **The level of p38α MAPK activation in airway epithelial cells determines the onset of innate immune responses to planktonic and biofilm Pseudomonas aeruginosa.** (2013). J. Infectious Disease. 207(10):1544-55.

Preface:

Airway epithelial cells (AECs) have a variety of pathogen recognition receptors (PRR) to sense environmental pathogens and help prevent infection. Activation of these PRR induce a number of proinflammatory signaling pathways, including NFκB and MAPK pathways as described above. Traditional purified ligands, such as flagellin and LPS, have been well characterized for their ability to activate these pathways. However, in chronic infection, bacteria often grow in matrix-encased colonies known as biofilms. With this new mode of growth comes a different gene expression pattern and upregulation of different virulence factors along with the repression of others. In addition, *Pseudomonas aeruginosa* is speculated to form bacterial biofilms in the airway epithelium of patients with Cystic Fibrosis (CF). How airway epithelial cells respond to these bacterial biofilms has not been characterized previously. Furthermore, since it has been shown that CF AECs are hyperresponsive to bacterial stimuli, how biofilm material activates CF AECs is of particular interest. This is the rational for the study detailed below.

Statement of Contribution

The main text of the article was prepared by Dr. Simon Rousseau. Materials and Methods and Figure legends were prepared by Trevor Beaudoin. Editing prior to submission was performed by all authors, but significant revisions were contributed by Trevor Beaudoin and Dao Nguyen. Correction of Proofs was done by Dr. Simon Rousseau and Trevor Beaudoin.

All Figures and data analyses were performed by Trevor Beaudoin. Contribution to individual experiments are listed below:

Table 8 and Figure 10: Shantelle LaFayette aided in performing microbiological characterization of PACF508, PAO1 and mutant strains.

Figure 14 B, 26: Guy Martel and Julie Berube made the NFkB and ATF2 reporter plasmids and the stably transfected, polyclonal BEAS-2B cell lines used in this experiment. Experiments were performed by Trevor Beaudoin

Figure 22: Shantelle LaFayette performed the reconstitution assay in the las mutants to confirm exogenous HSL can stimulate lasR gene transcripts.

Figure 25: Julie Berube performed the p38 MAPK activity assay.

Neutrophils were collected by Razieh Rabini, nasal polyps were provided by Dr. Emmanuelle Brochiero and Dr. Martin Desrosiers

Abstract:

Biofilm microcolonies of *Pseudomonas aeruginosa* chronically infect the airways of patients with cystic fibrosis and fuel ongoing destructive inflammation, yet the impact of the switch from planktonic to biofilm growth on host responses is poorly understood. We report that in airway epithelial cells a threshold of p38α mitogen-activated protein kinase (MAPK) activation was required to trigger neutrophil recruitment, which is influenced by extrinsic and intrinsic factors. Planktonic *P. aeruginosa* diffusible material (PsaDM) induced stronger p38α MAPK activation as compared to biofilm PsaDM. Biofilm PsaDM activated p38α MAPK in a Toll-like receptor–independent fashion via the lasI/lasR quorum-sensing system, but this activation was insufficient to recruit neutrophils. However, in airway epithelial cells from patients with cystic fibrosis with hypersensitivity to injurious stimuli, biofilm PsaDM activated p38α MAPK strongly enough to recruit neutrophils, which can contribute to lung injury.

Background/Introduction

Bacteria can exist as free-swimming, planktonic bacteria or as matrix-encased colonies known as biofilms. This allows for the bacteria to exploit different niches, with planktonic cells having increased motility and growth rates, while biofilms are able to sequester nutrients and have several advantages within the host, including increased resistance to antibiotics and phagocytosis.[171, 293] However, much less is known about how these modes of growth influence the host innate immune response and thus determine the outcome of the host-pathogen interaction. This likely has important implications for patients with cystic fibrosis (CF) who are chronically infected with biofilm microcolonies of *Pseudomonas aeruginosa*. *P. aeruginosa* is the most significant pathogen in CF, with up to 80% of patients eventually being chronically infected by their teenage years.[286] In essence, lungs of CF patients form a specialized ecological niche exploited by *P. aeruginosa.* Multiple findings suggest that the chronic *P. aeruginosa* infections in the CF patients involve biofilms, with the formation of large aggregates of bacteria within the airways.[49, 441] This chronic infection leads to neutrophilic inflammation that causes progressive lung tissue destruction. Detection of pathogens by the innate immune system relies on pattern-recognition receptor families that activate

host defense mechanisms. Many of these pattern-recognition receptors converge on common intracellular signaling pathways to drive inflammation.[43, 332] An essential element of host defense against bacterial pathogens activated by many pattern-recognition receptors is the p38α mitogen-activated protein kinase (MAPK).[410] Interestingly, *Caenorhabditis elegans* nematodes deficient for pmk-1, a p38 MAPK ortholog, are susceptible to bacterial killing by *P. aeruginosa*.[235] This highlights the ancient role played by p38α MAPK in regulating host defense against *P. aeruginosa.* Furthermore, destructive inflammation in intestinal epithelial cells does not occur with constitutive activation of NFκB, but requires additional activation of MAPKs.[168] We have investigated p38α MAPK activation, cytokine synthesis, and neutrophil recruitment in airway epithelial cells (AECs) exposed to planktonic or biofilm *P. aeruginosa* diffusible material (PsaDM). We hypothesized that the level of p38α MAPK activation by *P. aeruginosa* correlates with the capacity of AECs to mount a host defense response.[168]

Materials and Methods

Materials

BIRB 0796 was kindly provided by Professor Sir Philip Cohen (MRC PPU, University of Dundee, UK). All chemicals were bought from Fisher

Scientific (Fair Lawn, NJ, USA). FSL-1, LPS from *Pseudomonas aeruginosa* and *S. typhimurium* flagellin were bought from Invivogen (CA, USA).

Pseudomonas aeruginosa strains

Two strains of *P. aeruginosa* were investigated: PAO1, a common laboratory strain and PACF508, a stable mucoid clinical isolate from the sputum of a patient with CF (CFTRΔF508 homozygous; Hôpital Sainte-Justine, Montréal) (PACF508).[43] The *lasR* mutant is a transposon mutant obtained from the PAO1 transposon library.[208] The *lasI* mutant was constructed by allelic replacement using pSC301 to create an unmarked deletion in *lasI* from +90 to 572 bp.

Bacterial Media Preparation

Bacteria were grown in either 4% peptone (Fisher Chemical, Fair Lawn, NJ, USA) or in Synthetic Cystic Fibrosis Media (SCFM), to replicate nutritional cues that *Pseudomonas aeruginosa* will find in the CF lung. SCFM was described as in Palmer et al.[355] Briefly, amino acids (Purchased from Acros, New Jersey, USA) were added from 100-mM stocks to a buffered base (6.5 ml 0.2 M NaH_2PO_4, 6.25 ml 0.2 M Na_2HPO_4, 0.348 ml 1 M KNO_3, 0.122 g NH_4Cl, 1.114 g KCl, 3.03 g NaCl, 10 mM MOPS, 779.6 ml deionized water) in the following volumes: L-

aspartate, 8.27 ml; L-threonine, 10.72 ml; L-serine, 14.46 ml; L-glutamate·HCl, 15.49 ml; L-proline, 16.61 ml; L-glycine, 12.03 ml; L-alanine, 17.8 ml; L-cysteine·HCl, 1.6 ml; L-valine, 11.17 ml; L-methionine, 6.33 ml; L-isoleucine, 11.2 ml; L-leucine, 16.09 ml; L-tyrosine, 8.02 ml; L-phenylalanine, 5.3 ml; L-ornithine·HCl, 6.76 ml; L-lysine·HCl, 21.28 ml; L-histidine·HCl, 5.19 ml; L-tryptophan, 0.13 ml; and L-arginine·HCl, 3.06 ml.) SCFM was adjusted to pH 6.8 and filter sterilized through a 0.2-μm-pore-size filter. After sterilization, the following sterile components were added per liter: 1.754 ml 1 M $CaCl_2$, 0.606 ml 1 M $MgCl_2$, and 1 ml 3.6 mM $FeSO_4 \cdot 7H_2O$. Amino acids were maintained as 100-mM stocks in deionized water and stored in the dark at 4°C.

Crystal Violet Assay

Each of the bacterial strains studied was inoculated in 4 mL of desired media (SCFM or 4% Peptone) and left overnight with shaking at 37°C. Cultures were then diluted 1/100 into fresh media and 100 μL was plated into one well of a 96 well PVC microtiter plate (BD Falcon). Each sample was plated in triplicate Plate was incubated for 24 hours without shaking at 37^0C. After 24 hours, plate was washed by submerging two times in tap water to remove planktonic bacteria. 150 μL of 0.1% (w/v) crystal violet in water was then added to each of the inoculated wells. After twenty

minutes, the plates were washed as above and left to dry. Attached bacteria formed a ring on the plate. The stained bacteria was then solubilized using 200µL 30% (w/v) acetic acid for 15 minutes, 125 µL of this mixture was transferred to optically clear flat bottom 96 well plates (PerkinElmer) and OD was measured at 595 nm.

Phenotypic Characterization of Bacteria.

Swimming motility was described as positive if a zone of growth was visible after 24h incubation following a stab inoculation of 0.3% agar plates.[475] Twitching motility was described as positive if a visible halo was observed (twitching zone) after 24h growth following a stab inoculation of thin 1.5% agar plates. Pyocyanin production was determined qualitatively after 24-h growth.[514] Briefly, 0.6mL chloroform into 1mL of an overnight culture, mixed and spun at 1300Xg. The bottom layer was removed and transferred to a fresh tube with Add 0.5mL of 0.2M HCl, mixed and spun down at 13,000xg. The upper layer was removed and absorbance was read at 520 nm. Pyoverdine production was assayed by spinning down an overnight culture at 3000xg for 10 minutes, removing the supernatant and measuring absorbance at 405 nm. Unless otherwise specified, bacterial cultures were incubated in LB media 37^{o}C. All assays were done at least

in triplicate. The laboratory strain PAO1 was used as a control in all assays.

Transmission Electron Microscopy:

Biofilm and planktonic cultures were grown in SCFM as described above. Cells were collected and fixed with 2.5% gluteraldehyde in 0.1M sodium cacodylate. Fixed samples were washed and post-fixed with 1% osmium tetroxide in 1.5% potassium ferrocyanide and placed on ice for 2 h. Following three 10-min washes in water, tissues were dehydrated in graded acetone series (50% to 100%) and embedded in Epon. Random section were cut and mounted on grids before visualization on a Phillips Tecan transmission electron microscope (120 kV).

Bacterial Growth Curves

Bacterial growth curves were conducted for *P. aeruginosa* PAO1 and PACF508 in 4% peptone and in SCFM. 25 mL of desired media was inoculated with 20 µL an overnight culture such that the optical density at 600 nm (OD_{600}) was undetectable. Every 30 minutes, 100 µL aliquot was removed and read on a Tecan M1000 plate reader in order to determine the optical density at the given time point. This was performed for 12 hour time course and again at 16 hours and 24 hours after inoculation.

Air-Liquid Interface Biofilm Images

Static biofilms were grown in 6 well plates (corning) as followed: 2 mL of media was placed in the culture plate along with 50 µL of overnight culture of bacteria. After three hours of incubation at 37^{o}C with no shaking, the media was removed and replaced with fresh media. The plates were allowed to grow for an additional 24 hours at 37^{o}C without shaking. After this time, the media was removed from the bacteria and the wells were washed 3X with PBS to remove unattached bacteria. The wells were then imaged using brightfield of a standard inverted microscope at 400X magnification.

P. aeruginosa diffusible material preparation

P. aeruginosa diffusible material from planktonic (Plank. PsaDM) was produced in 4% peptone (Fischer scientific) or synthetic CF medium SCFM (described above). *P. aeruginosa* was grown in 5 mL of desired media in 12 mL test tubes at 37 degrees for 72 hours with shaking at 250 RPM. Following this growth, the culture was centrifuged at 2100xg for 20 minutes to pellet cells. The supernatant was collected and filtered through a 0.22 µm filter (Millepore), aliquoted and stored at -20^{o}C for use within the month or -80^{o}C for longer storage. Prior to use, the total protein content was determined via a standard Bradford assay. Static Biofilm PsaDM was

prepared in peptone or SCFM as follows: ~5x10^7 log phase cells were used to seed each 6 mm polystyrene tissue culture wells (Falcon). After three hours of initial attachment, the media was removed, replaced with fresh media, and the attached bacteria were incubated statically at 37^{o}C for an additional 24 hours. After this time, the attached cells were scraped off of the plate and were combined with the cell suspension within the well. Bacteria were centrifuged at 2100 X g for 30 minutes and the supernatant was collected and filtered through a 0.22 µm filter. Total protein of filtrates was measured by the Bradford method. Prior to use, bacterial filtrates were heat inactivated at 95^{o}C for 10 minutes (to inactivate proteases) and allowed to cool to room temperature.

Antibodies

Neutralizing antibodies against TLR2, TLR4 and TLR5 were used at 5 µg/mL and purchased from Invivogen (CA, USA). Anti-phospho-ERK1/2 (Thr202/Tyr204), Anti-ERK1/2 MAPK, Anti-phospho p38 MAPK (Thr180/Tyr182, 09-272) and Anti-p38 MAPK (Thr180/Thr182) was used at 1/1000 dilution and purchased from Millipore (Billerica, MA, USA). Anti-phospho JNK was used at a dilution of 1/100 and purchased from Cell Signaling (Danvers, MA). Anti-GAPDH was used at a dilution of 1/4000 and was purchased from Millipore. Goat anti-rabbit IgG DyLight^{TM}800

(35571; 1:15,000) and Goat anti-mouse IgG DyLight™680 (35518; 1:15,000) were bought from Thermo Scientific (Rockford, IL, USA).

Cell Culture

Immortalized human bronchial epithelial cells (BEAS-2B) were purchased from ATCC (Rockville, MD, USA). BEAS-2B were maintained at 37°C, 5% CO_2, 100% humidity in DMEM (4.5g/L D-glucose) supplemented with 10% v/v heat inactivated foetal bovine serum (FBS), 100U penicillin G and 100 μg/mL streptomycin (P/S). Cells were grown to confluence in a 6-well plate (9 cm^2, Corning (Tweksbury, MA, USA) in 1.5 mL of media. Prior to stimulation with agonists, media was removed and replaced with DMEM containing 0.5% FBS and P/S. Following stimulation cells were either lysed for protein extraction or media was collected for ELISA.

Human airway epithelial cell line NuLi was derived from a normal lung of a 36-year-old male patient and CuFi airway epithelial cell line derived from lung of a 14-year-old female patient with cystic fibrosis homozygous for the CFTRΔF508 mutation, purchased from ATCC and used until passage 17, were maintained at 37°C, 5% CO_2, 100% humidity in CnT17 with supplements (CellnTec via Cedarlane, Burlington, On.)

Cells were grown in 6 well plates (Corning), treated with human Collagen IV.

Cell lysis and Immunoblotting.

Following stimulation, cells were lysed in ice-cold buffer A (50 mM Tris-Cl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton x-100, 1 mM Sodium orthovanadate, 5 mM Sodium pyrophosphate, 0.27 M Sucrose, Complete-Mini protease inhibitor cocktail and 2 mM DTT). Proteins were quantified using the Bradford method, 20 µg of lysates were submitted to SDS-PAGE, transferred to nitrocellulose and immunoblotted with specified antibodies. The signal was detected and quantified using a Licor Odyssey imaging system.

ELISA

Human IL-6 and IL-8 were purchased from Preprotech. For IL-6, 100µL of supernatant collected after stimulation of cells was used for quantification as per manufacturer's protocol. For IL-8, samples were diluted 1:20 in reagent dilutant (0.1% BSA in PBS with 0.05% Tween-20) used for quantification as per manufacturers protocol.

NFκB Reporter Assay

The NFκB consensus response element (GGGACTTTCC) was synthesized in 4 copies and cloned at the XhoI/BglII sites of the pGL4.28

vector (Promega, Madison, WI, USA). The resulting vector was transformed into DH5α bacterial strain and purified with Invitrogens Purelink maxi prep kit. (Invitrogen, Burlington, Ontario) pGL4.28 NFκB was then stably transfected into BEAS-2B and maintained with DMEM as described above with the addition of 200μg/mL of hygromycin. These cells were plated in 12 well plates (Corning) to confluency and then stimulated with agonists for 3 hours. Following stimulation, cells were washed twice with ice-cold phosphate-buffered saline, and 40 μL of reporter lysis buffer (Promega) was added per well. After 5 minutes, cells were scraped and collected into a microcentrifuge tube and spun down at 13 000 × g for 3 minutes. A total of 30 μL of supernatant was collected in a new tube and stored at −20°C. Ten microliters of sample was used in 96-well plates for the reporter assay. A total of 25 μL of luciferase assay reagent (20 mM Tricine, 1.07 mM $(MgCO_3)\cdot 4\ Mg(OH)_2\cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM ethylenediaminetetraacetic acid, 33 mM dithiothreitol, 270 μM coenzyme A, 0.477 mM D-luciferin, and 0.533 mM adenosine triphosphate) was added to each well, using an automatic injector. Emission units were read on a Tecan Infinite M1000 plate reader.

Ex Vivo Nasal Biopsy Assay

All patients with chronic rhinosinusitis participated voluntarily and signed an informed consent form approved and supervised by the institutional review board. All subjects (age, ≥18 years) had a diagnosis of chronic rhinosinusitis, according to published American Academy of Otolaryngology–Head and Neck Surgery guidelines and biopsy specimens were obtained as previously described.[27, 309] Surgical biopsy specimens were washed 3 times with ice-cold phosphate-buffered saline before being cut into pieces of equal weight. Each biopsy piece was distributed to one of 6 experimental groups in 12-well tissue culture plates and cultured in 75% Dulbecco's Modified Eagle's medium (DMEM)/25% Hanks' buffer solution (100 U/mL penicillin G and 100 µg/mL streptomycin) without serum for 72 hours. Every 24 hours, medium was refreshed. After the 72-hour period, biopsy pieces were left untreated or were pretreated with 0.1 µM BIRB 0796 for 1 hour followed by stimulation with 1 µg/mL of planktonic or biofilm PsaDM for 24 hours.

RNA extraction and cDNA synthesis

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The RNA was quantified and 1 µg was treated with DNAse I Amp Grade (Invitrogen, Carlsbad, USA) and

reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, USA), according to the manufacturer's protocols.

Quantitative PCR

BEAS-2B were left untreated or where pre-treated for 1 hour with 100 nM of BIRB 0796 before stimulation by 1µg/mL of planktonic or biofilm PACF508 PsaDM grown in SCFM for 6 hours. After stimulation RNA was extracted using phenol-chloroform method. Semi-quantitative real-time PCR (qPCR) was performed in 96 well plate format using SYBR Green based detection on a Step-One-Plus machine (ABI) with each 20 µl reaction containing approximately 50 ng cDNA, 0.3 µM of sense and antisense primers (see below) and 1X Quantitect SYBR Green supermix (Qiagen). The plate was sealed and cycled under the following conditions: 95°C/ 10 min, 50 cycles of 95°C/ 10 s and 60°C / 45 s. Each reaction was performed in duplicate, mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for normalization and fold induction was determined from Ct values using Pfaffl method. PCR efficiencies were determined from the slope of a standard curve generated using five-fold dilution series of the DNA template. The following primers were used in the qRT-PCR:

Table 7: Primers for qPCR

Gene	Forward Primer (5'-3)
RANTES	GAAGCCTCCCAAGCTAGGAC
GM-CSF	ACTACAAGCAGCACTGCCCT
TNF-β	TATCACTGTCCTGGCTGTGC
GRO-α	AGGGAATTCACCCCAAGAAC
IL-6	GTGTGAAAGCAGCAAAGAGG
IL-8	GTGCAGTTTTGCCAAGGAGT
GAPDH	AGCAATGCCTCCTGCACCACC

Neutrophil recruitment assay:

BEAS-2B AECs were cultured as previously described and treated for 24 hours with filtrates, with or without 1 hr pre-treatment with 0.1μM BIRB 0796. After 24 hr the media was removed (termed conditioned media or CM) and used for neutrophil migration assays or ELISA. For neutrophil migration, 500 000 freshly isolated neutrophils from human blood were placed in the upper chamber and conditioned media collected from treated AECs placed in the bottom, separated by a polycarbonate membrane with 5 μm pore. After 12 hr, neutrophils crossing the membrane were counted with a haemocytometer.

Kinase Assays

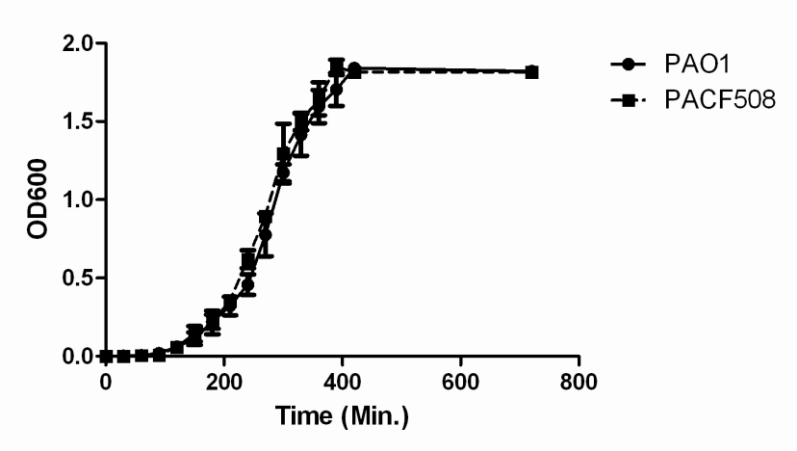
p38α MAPK activity was assayed as previously described.[411] Immunoprecipitated p38 α MAPK was assayed using ATF2-GST as substrates. Assay medium contained 50 mM Hepes, pH 7.4, 50 mM β-glycerophosphate, 50 mM $MgCl_2$, 0.2 mM Na_3VO_4, 4 mM dithiothreitol, ATF2-GST, and [γ-32P] ATP (3000 Ci/mM). The activity of the kinases was quantified by measuring the incorporation of radioactivity into the specific substrate after SDS-PAGE.

Experimental Results

Characterization of *Pseudomonas aeruginosa* PAO1 and PACF508

Two strains of *Pseudomonas aeruginosa* were used for this study; the reference laboratory strain PAO1 and a clinically isolated, stable mucoid strain PACF508. In addition, nutritional cues can affect the genotypic expression of bacteria and so a synthetic CF media (SCFM) was prepared to better mimic the conditions the bacteria would face in the CF lung.[355] In order to better compare these strains, some basic characterization of different properties were performed. Growth curves were performed on PAO1 and PACF508 both in peptone and SCFM. As can be seen in Figure 7A, there was no difference in the growth curves for PAO1 and PACF508 when grown in peptone. On the other hand, PAO1 grew slightly slower in SCFM then did PACF508, as seen in comparing Figure 7B although the total number of bacteria, reflected by the OD_{600}, was equal in the stationary phase (10-24 hours) in both conditions. Because clinical isolates can lose certain features, including pili and flagella, during their course of infection, both strains were tested for twitching motility (dependent on pili) and motility (dependent on flagella) assays. Relevant mutants were used for comparison. Table 8 provides a summary of the results.

A



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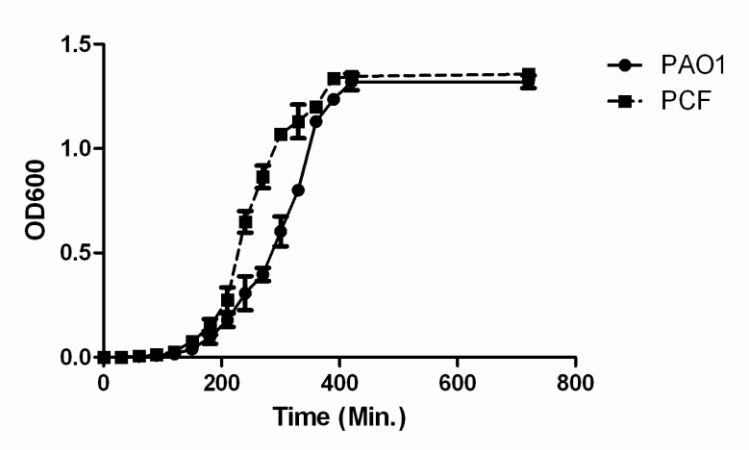


Figure 7: Growth Curves of PAO1 and PACF508

Pseudomonas aeruginosa strains PAO1 (circles, solid line) and clinical isolate PACF508 (squares, hashed lines) were grown for up to 72 hours in either 4% peptone media (A) or synthetic cystic fibrosis media (SCFM, B). Every 30 minutes, 100uL of media was removed and its optical density at 600 nm was read a plotted to generate the growth curves for the first 12 hours.

Table 8: Swimming and twitching motility assays for PAO1 and PACF508

Strain	PAO1	PACF508	*fliK*
Swimming (+ or -)	+	+	-

Strain	PA01	PACF508	*pilA*
Twitching (+ or -)	+	-	-

Both strains showed increased diameter in the swimming assay, indicating the presence of a flagella, while only PAO1 was positive for the twitching motility, possibly representing a down-regulation of pili in this strain. Because pili can be important in bacterial attachment, the ability for these strains to adhere to plastic surfaces and form biofilms was tested via a crystal violet assay and microscopy, Figures 8 and 9 respectively.[381] PAO1 and PACF508 are both able to grow biofilms well in peptone media. However, PAO1 is unable to grow significant biofilm mass in the same time period (24hrs) in SCFM when compared to the clinical isolate. Because we wish to study the response of airway epithelial cells in response to *P. aeruginosa* infections in the context of CF, I have focused my experiments on PACF508 grown in SCFM for this study. Where PAO1 mutants are used, they were grown in peptone and compared to wildtype PAO1 grown in peptone, as described.

Pyocyanin and Pyoverdine are important exoproducts that have been shown to be important in virulence of *P. aeruginosa.*

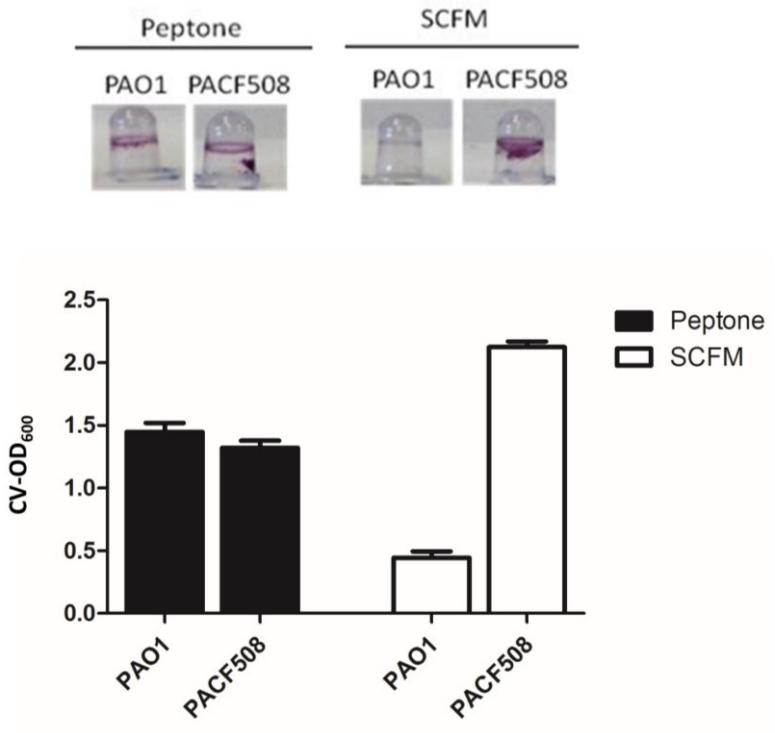


Figure 8: Crystal violet assay for biofilm formation.

Biofilm formation was assessed via crystal violet assays. An overnight culture of PAO1 or PACF508 was plated at a concentration of 1/1000 into a plastic microtiter plate and grown in either 4% peptone (black bars) or SCFM (white bars). Bacteria was allowed to attach to surface for 24 hours, after which the plate was washed 3X in water then 200 µL of 0.1% (w/v) crystal violet dye in ethanol was added to each well for 10 minutes. Following this, pictures were taken and 100% ethanol was added to each well to dissolve crystal violet and the OD was taken and plotted.

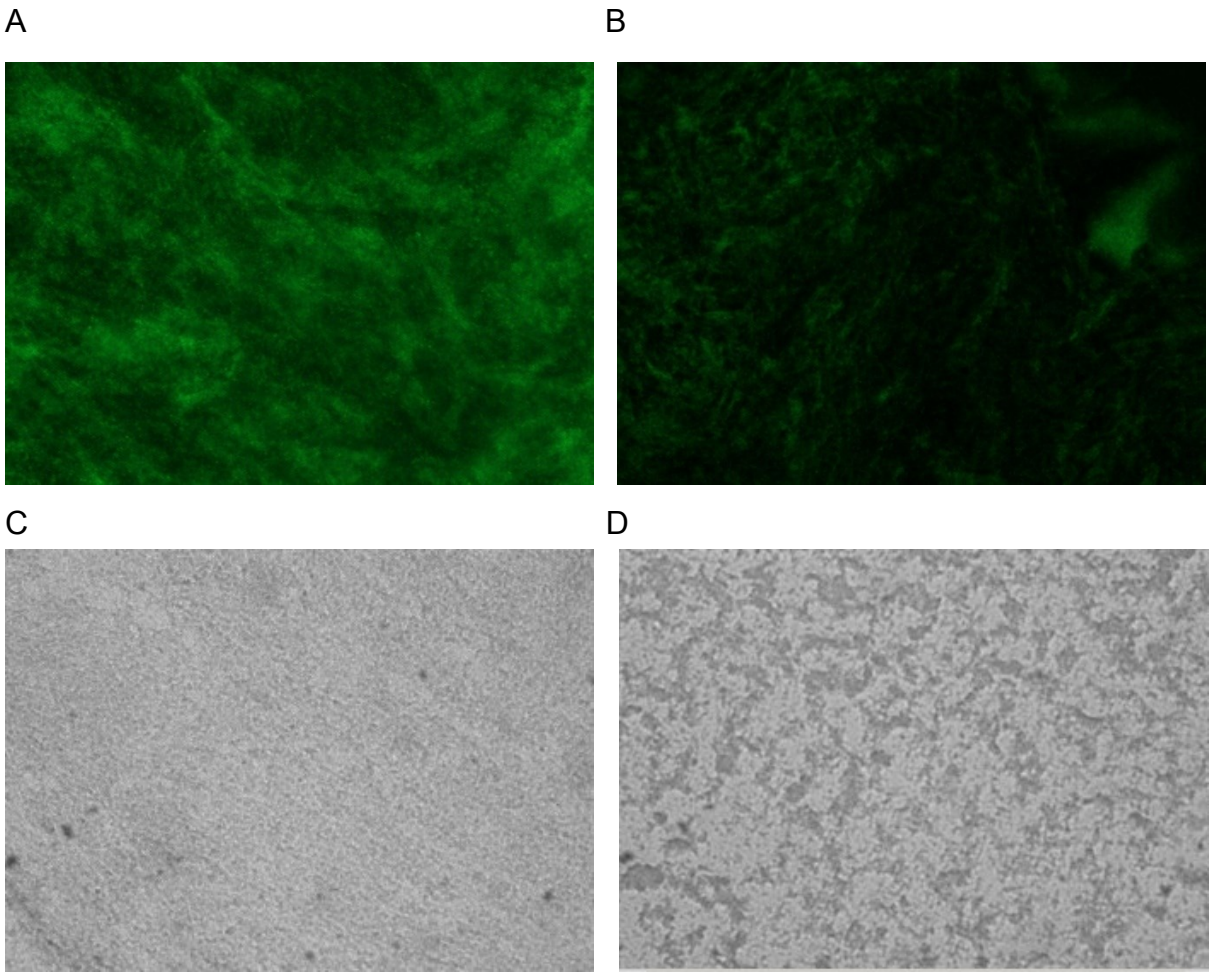


Figure 9: Biofilm formation of PAO1 (GFP) and PACF508 in Peptone and SCFM on tissue culture plates.

PAO1 expressing green fluorescent protein (GFP) was plated in 6 well plates for 24 hours without shaking in either 4%peptone (A) or SCFM (B). PACF508 was grown in 6 well plates for 24 hours without shaking in either 4% peptone (C) or SCFM (D).

As seen in Figure 10, PAO1 is able to produce both factors, while PACF508 cannot produce pyocyanin. The structure of the bacteria grown in different media and as planktonic or biofilm cultures was assessed via transmission emission microscopy (Figures 11 and 12).

Finally, since Pseudomonas infections are thought to occur in the thick mucus layer and distal to the airways, I used diffusible materials to stimulate the AEC. The *Pseudomonas aeruginosa* Diffusible Material (PsaDM) filtrates was prepared as described in the materials and methods. OD_{600} readings and colony forming unit counts were taken during preparation to confirm adequate growth between the samples. Prior to use, the PsaDM filtrates were assayed for total protein content via standard Bradford assay and quantified as in Figure 13. As can be seen from Figure 13A, the total protein content of PAO1 grown in peptone is similar, while PA01 grown in SCFM produces substantially less when grown as a biofilm compared to planktonic. This could be due to the fact that much less biomass is produced by PAO1 biofilms when grown in SCFM (refer to Figures 8 and 9). On the other hand, PACF508 total protein content is similar between planktonic and biofilm conditions for both types of media (Figure 13B).

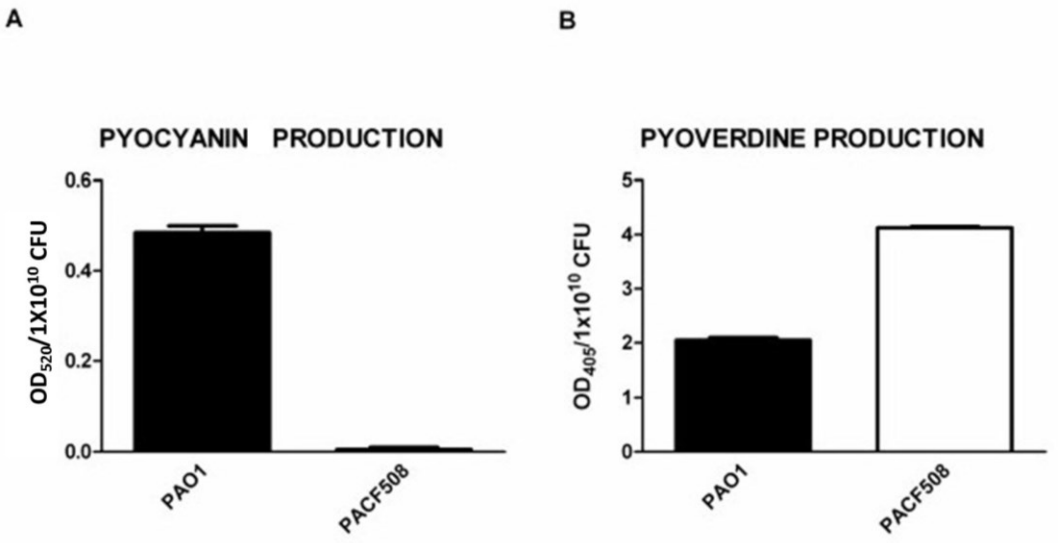


Figure 10: Pyocyanin and Pyoverdine Production by PACF508 and PAO1 *Pseudomonas aeruginosa.*

P. aeruginosa strains PAO1 (Black bars) and PACF508 (White bars) where grown overnight in 4% peptone media. Bacteria was assessed for the production of pyocyanin and pyoverdine by colometric assays as described in the materials and methods.

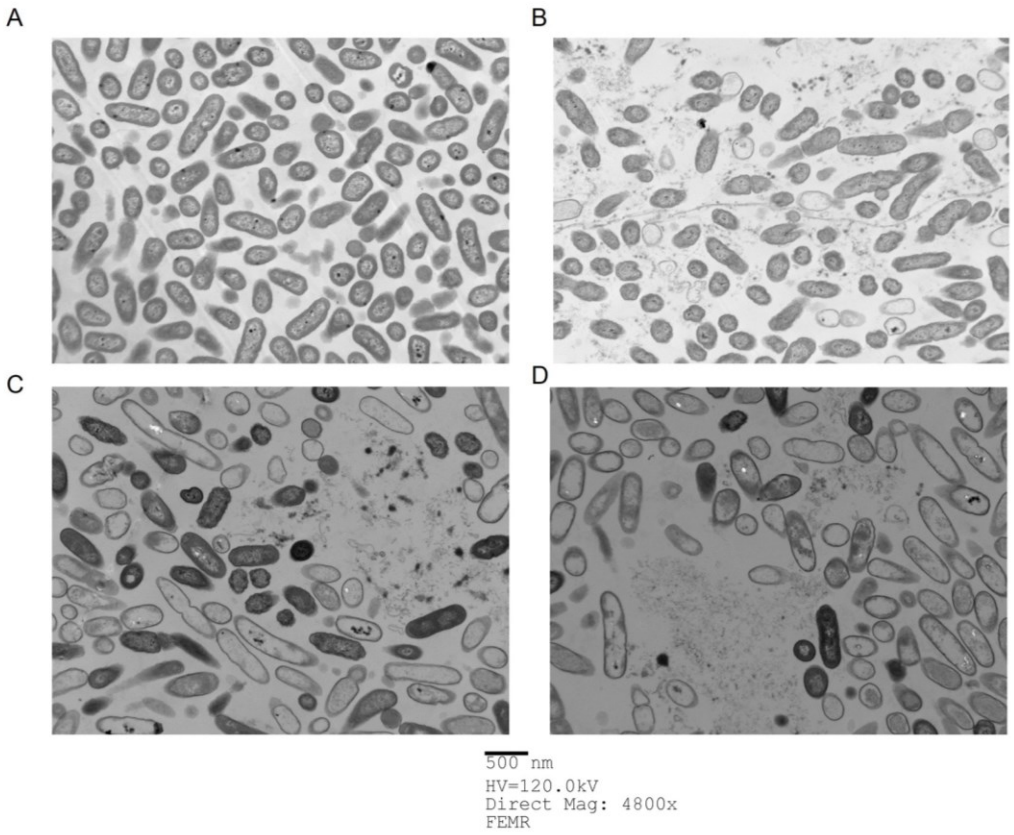


Figure 11: TEM of PAO1 and PACF508 Planktonic and Biofilm bacteria grown in SCFM.

P. aeruginosa strains PACF508 (A and B) and PAO1 (C and D), were grown in SCFM in planktonic (A and C) or biofilm (B and D) conditions for 24 hours. Transmission emission microscopy was used to visualize the bacteria.

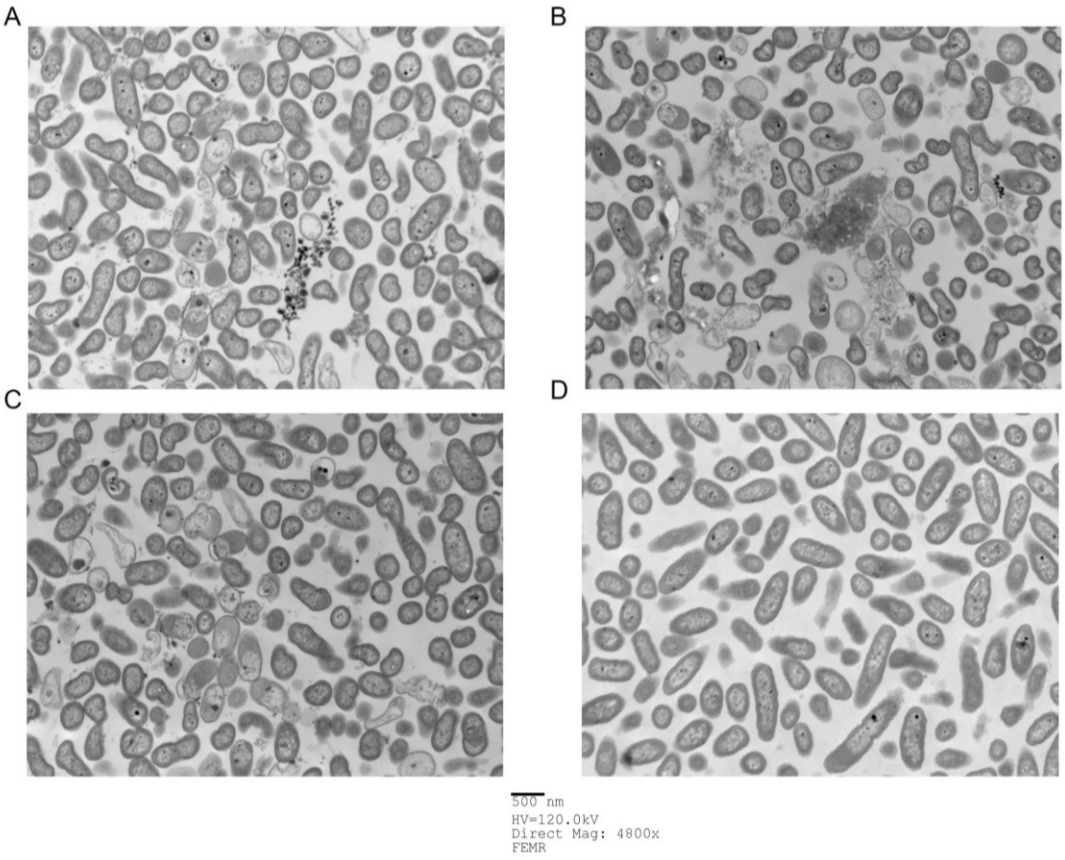
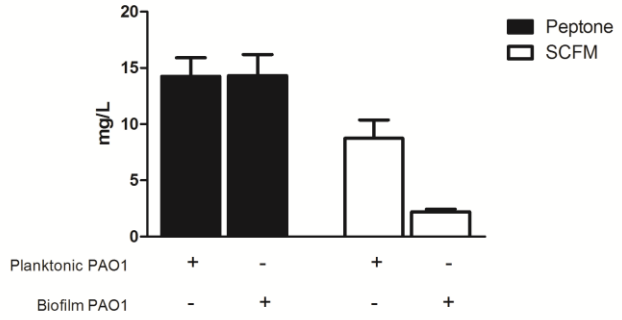


Figure 12: TEM of PAO1 and PACF508 Planktonic and Biofilm bacteria grown in Peptone.

P. aeruginosa strains PACF508 (A and B) and PAO1 (C and D), were grown in 4% peptone in planktonic (A and C) or biofilm (B and D) conditions for 24 hours. Transmission emission microscopy was used to visualize the bacteria.

A



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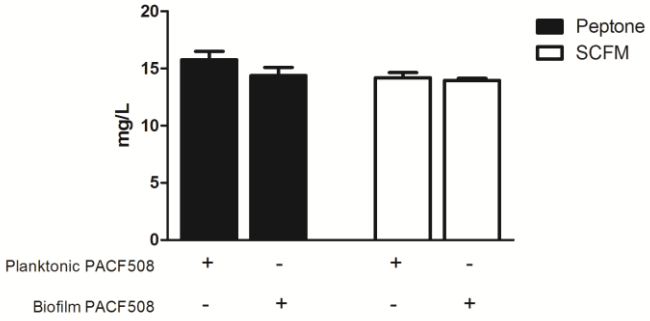


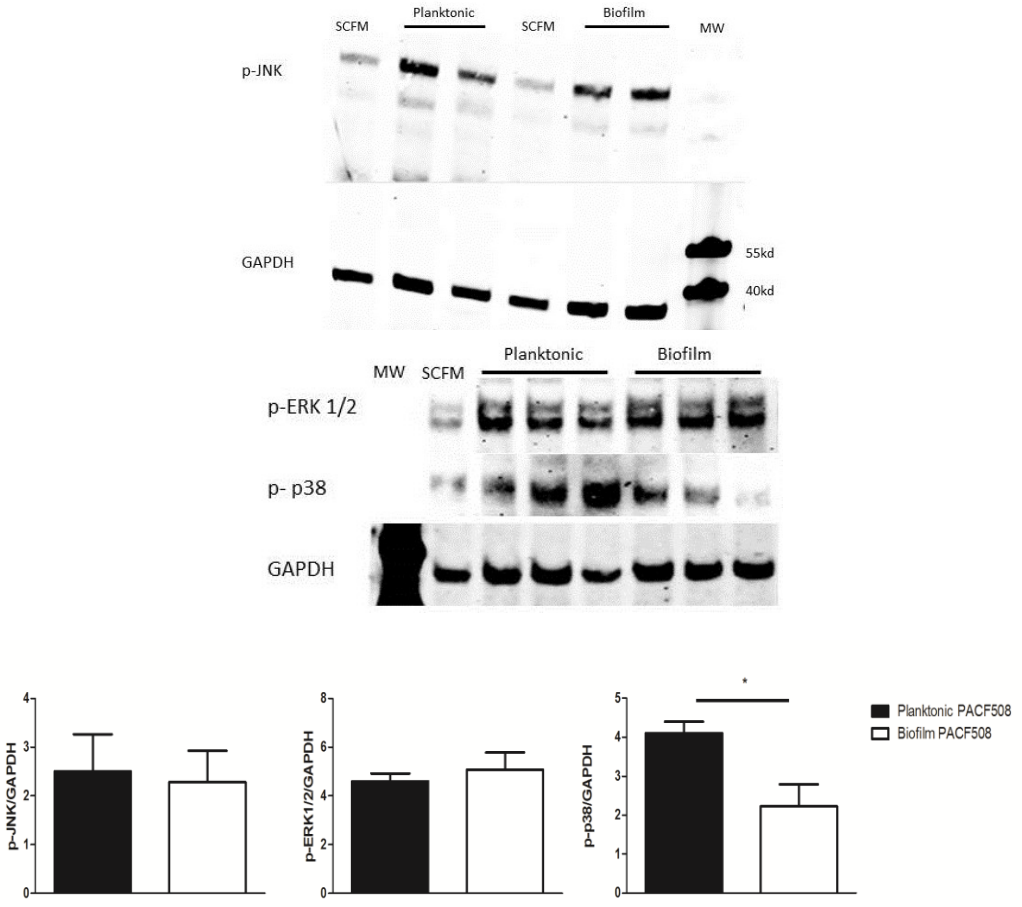
Figure 13: Total protein content of PAO1 and PACF508 planktonic and biofilm PsaDM in Peptone and SCFM

Total protein content of PAO1 (A) and PACF508 (B) grown in 4% peptone (Black bars) and SCFM (White bars) grown as either planktonic or biofilm as described in the materials section and assessed via Bradford assay.

Activation of p38α Mitogen Activated Protein Kinase by Planktonic and Biofilm *Pseudomonas aeruginosa* material.

As was stated in the introduction, MAPK and NFκB play critical roles in initiating proinflammatory cytokine production in AEC as a response to planktonic *Pseudomonas aeruginosa* and other inhaled pathogens. Therefore, it was of great interest to investigate and compare the response of planktonic and biofilm *P. aeruginosa* derived materials in an epithelial cells line. Compared to media alone, both planktonic and biofilm PACF508 material was able to phosphorylate JNK and ERK1/2 MAPK to similar extents in the immortalized bronchial epithelial cell line, BEAS-2B (Figure 14A). In addition, NFκB was activated strongly by both planktonic and biofilm PsaDM, as seen using a stably transfected luciferase reporter construct transfected into BEAS-2B (Figure 14B). Interestingly, p38α MAPK was activated much more strongly by planktonic PACF508 PsaDM then it was via biofilm PsaDM (Figure 14A). Because of p38α ancient role in defense against *P. aeruginosa* infection, as well as the increased presence of phosphorylated p38 in histological samples of CF patients, these results led us to focus on the outcome of p38α activation by planktonic and biofilm PsaDM.

A


SCFM
Planktonic
SCFM
Biofilm
MW
p-JNK
GAPDH
55kd
40kd
MW
SCFM
Planktonic
Biofilm
p-ERK 1/2
p- p38
GAPDH
p-JNK/GAPDH
p-ERK1/2/GAPDH
p-p38/GAPDH
*
Planktonic PACF508
Biofilm PACF508

B

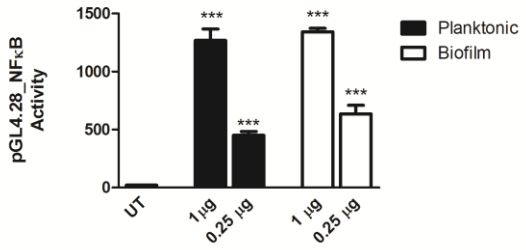

pGL4.28_NFκB Activity
1500
1000
500
0
***
***
***
***
UT
1 µg
0.25 µg
1 µg
0.25 µg
Planktonic
Biofilm

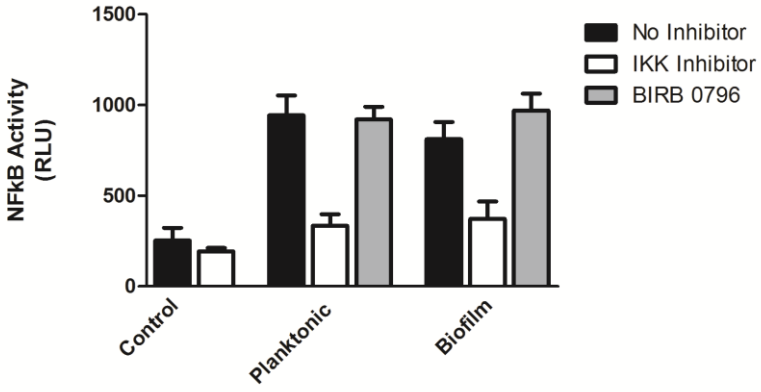
Figure 14: Activation of MAPK and NFκB via Planktonic and Biofilm PACF508

A) BEAS-2B cells were grown in 6 well plates to confluency and starved overnight with DMEM with 0.05% FBS. Cells were then stimulated with 1µg/mL of planktonic or biofilm PACF508 PsaDM filtrate for 30 minutes. Following this, cells were lysed and protein was collected and run on a 10% SDS gel. The appropriate antibodies were used and the fold change (plotted against media alone) was plotted.

B) NFκB activity was assessed using a minimal promoter of NFκB upstream of a luciferase reporter and stably transfected into BEAS-2B cells. These cells were grown to confluency as described in (A) and treated with indicated doses of planktonic (Black bars) and Biofilm (White bars) PACF508 PsaDM.

The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** $P \leq .001$, ** $P \leq .01$, and * $P \leq .05$, compared with control; #$P \leq .05$ for comparison between groups.

A



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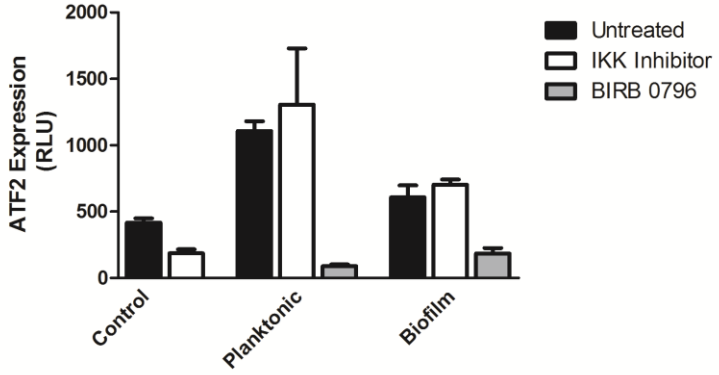


Figure 15: ATF2 induction by planktonic and biofilm PsaDM

The activation of NFκB (A) and ATF2 (B) was assessed via luciferase reporter construct using repeat elements of the transcription factors in front of a minimal promoter driving luciferase production. Cells were pretreated for 1 hour with 10μM IKK inhibitor or 0.1μM BIRB 0796 (p38) inhibitor prior to stimulation for 3 hours with 1μg of total protein content from planktonic or biofilm diffusible material (PsaDM)

The activation of NFκB by planktonic and biofilm PsaDM and could be blocked by the IKK inhibitor (10μM) but not by the p38 specific inhibitor BIRB 0796 (Figure 15A). In contrast, ATF2, a transcription factor downstream of p38, was induced to a greater extent in BEAS-2B by planktonic PsaDM in comparison to biofilm PsaDM. This was blocked completely by BIRB 0796 but not affected by the IKK inhibitor (Figure 15B).

Studying the activation of p38α MAPK in AECs in response to planktonic and biofilm *P. aeruginosa* PsaDM

We first studied the ability for PACF508 grown in SCFM or peptone, as well as PAO1 grown in those media. As seen in Figure 16A, PACF508 planktonic PsaDM led to an approximately 40% greater activation of p38α MAPK as compared to biofilm PsaDM. Similar results were observed for PAO1 when grown in peptone (Figure 16B) but not when grown in SCFM. This is likely explained by the fact that planktonic PAO1 grew poorly in SCFM and formed minimal biofilm in this medium, in contrast to PACF508 (Figures 8, 9 and 13).

To verify that the greater activation of p38α MAPK by planktonic PsaDM was not due to greater bacterial growth, we measured the total protein content of the bacterial filtrates (Figure 13A and B). Moreover, if the differences were solely quantitative, the

same maximal level of activation of p38α MAPK should be reached in dose-response curves. This was not the case, as stimulation of BEAS-2B AECs with increasing concentrations of planktonic PsaDM reached a higher plateau than stimulation with biofilm PsaDM (Figure 17). Additionally, the kinetics of p38α MAPK activation further support a greater activation by planktonic PsaDM (Figure 18). These results provide evidence of a qualitative, rather than a quantitative difference between planktonic and biofilm PsaDM in activating p38α MAPK. To understand the qualitative differences between planktonic and biofilm activation of p38α MAPK in AECs, we compared the bacterial phenotypes and morphology of PAO1 and PACF508, using functional assays and transmission electron microscopy. While both strains were motile by swimming (flagellum mediated), and produced Pyoverdine, only PAO1 was positive for twitching (type IV pilus mediated) (Table 8) and pyocyanin production (Figure 10). As both strains induced greater p38α MAPK activation when growing in planktonic mode, these exoproducts likely do not explain the differences in p38α MAPK activation by biofilms.

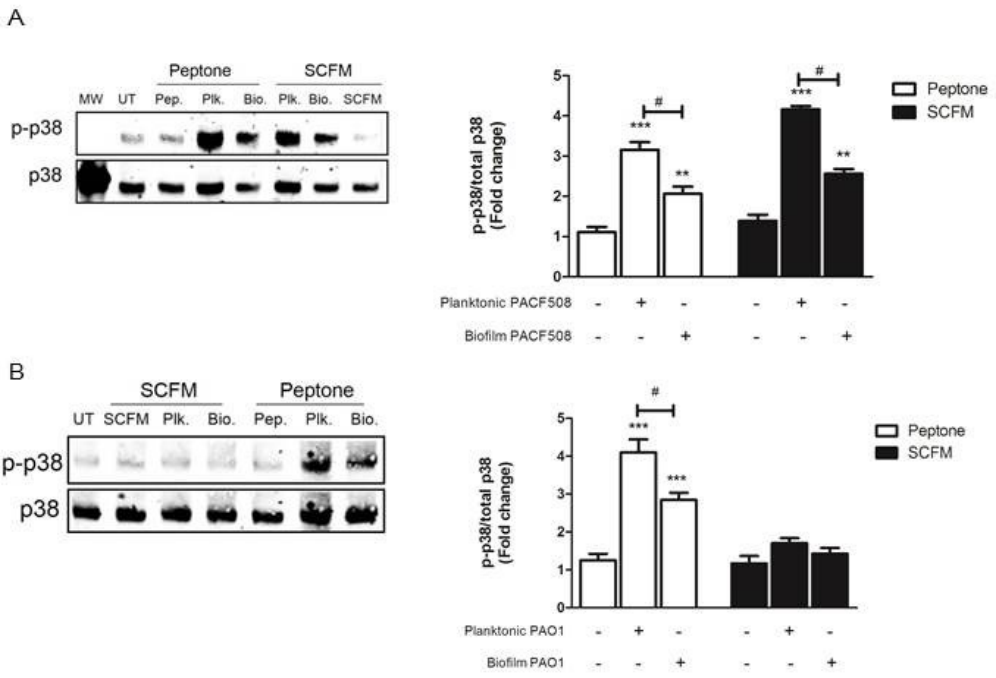


Figure 16: Activation of p38α in BEAS-2B by planktonic and biofilm PsaDM

BEAS-2B airway epithelial cells (AECs) were left untreated (−) or treated (+) with 1 μg/mL of planktonic or biofilm PACF508 PsaDM (A) or PAO1 (B) for 30 minutes. Following stimulation, cells were lysed, and 20 μg of Triton-soluble material was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. After transfer to nitrocellulose, the membranes were probed with antibodies recognizing only the phosphorylated forms of p38α MAPK or with antibodies that recognize all forms of p38 MAPK. Quantitative analysis of the signals from each antibody was performed using the Licor infrared Odyssey imaging system. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** P ≤ .001, ** P ≤ .01, and * P ≤ .05, compared with control; #P ≤ .05 for comparison between groups.

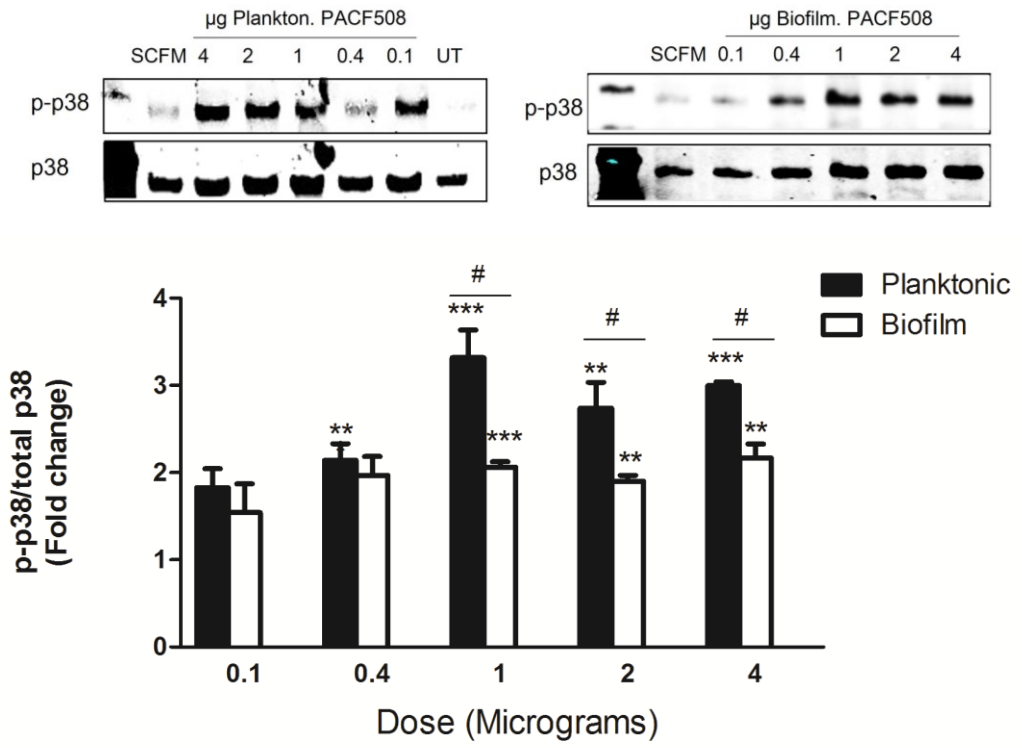


Figure 17: Dose response of Planktonic and Biofilm activation of p38α in airway epithelial cells.

BEAS-2B AECs were exposed to the indicated increasing concentrations of planktonic and biofilm PACF508 PsaDM grown in SCFM for 30 minutes. p38α MAPK activation was determined as in described in Figure 16. Black bars represent planktonic PsaDM–stimulated cells, and white bars represent biofilm PsaDM–stimulated cells. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** P ≤ .001, ** P ≤ .01, and * P ≤ .05, compared with control; #P ≤ .05 for comparison between groups.

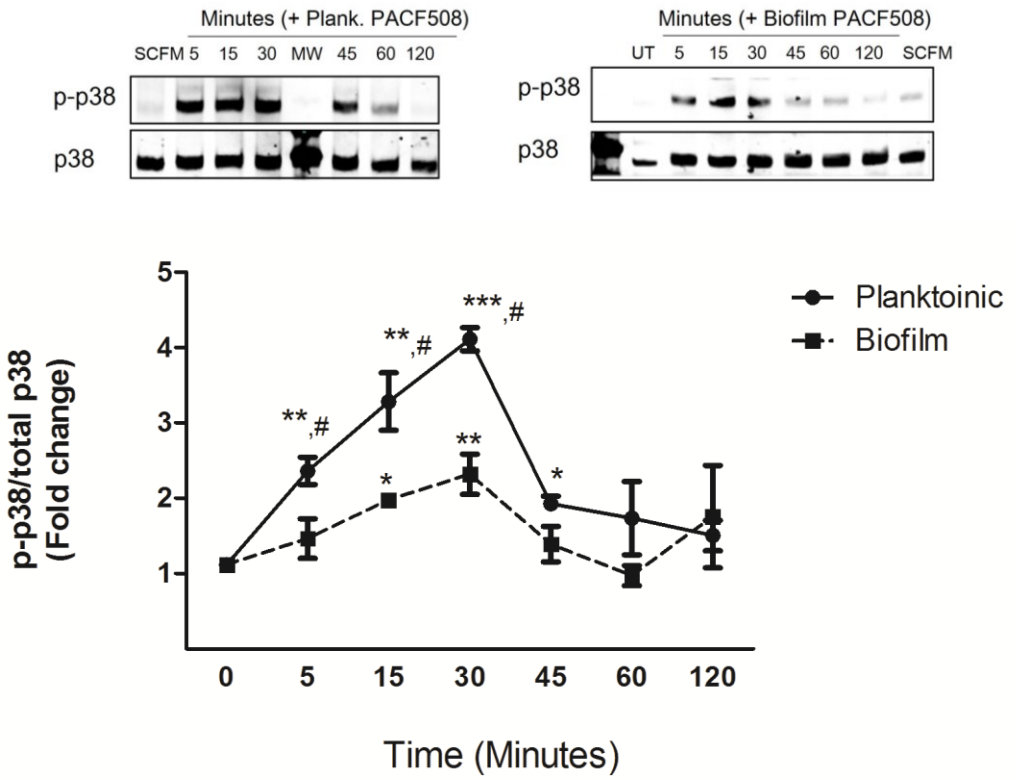


Figure 18: Time course of p38α activation by planktonic and biofilm PsaDM

BEAS-2B cells were exposed to 1 µg/mL planktonic (solid line) or biofilm (dotted line) PACF508 PsaDM grown in SCFM for the indicated times. p38α MAPK activation was determined as in panel A. Fold change is expressed in relation to untreated samples. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** P ≤ .001, ** P ≤ .01, and * P ≤ .05, compared with control; #P ≤ .05 for comparison between groups.

Lipid rafts are essential for the activation of p38α MAPK via planktonic, but not biofilm, PsaDM

The Cystic Fibrosis transmembrane conductance regulator (CFTR) localizes to lipid rafts, cholesterol-enriched plasma membrane microdomains required for TLR-mediated CXCL8 synthesis in epithelial cells.[205, 248, 451] Depletion of cholesterol by the drug methyl-β-cyclodextrin prevented p38α MAPK activation by signals emanating from planktonic but not biofilm PsaDM (Figure 19). These results provide additional evidence that planktonic and biofilm PsaDM induce innate immune signaling by distinct signaling mechanisms.

Signaling pathways in the activation of airway epithelial cells by Biofilm PsaDM

We then investigated which bacterial component(s) could account for the difference in p38α MAPK activation between the planktonic and biofilm PsaDM. Whereas planktonic PsaDM activates p38α MAPK in a TLR-dependent fashion (Refer to Chapter 3 and references for more details), TLRs are either not involved or have a minor role in biofilm PsaDM–stimulated AECs (Figure 20).[32, 413, 535] This led us to determine the molecule(s) responsible for biofilm-

mediated activation of p38α MAPK. Putative candidates are quorum-sensing signal molecules that are abundant in biofilms.[101, 102]

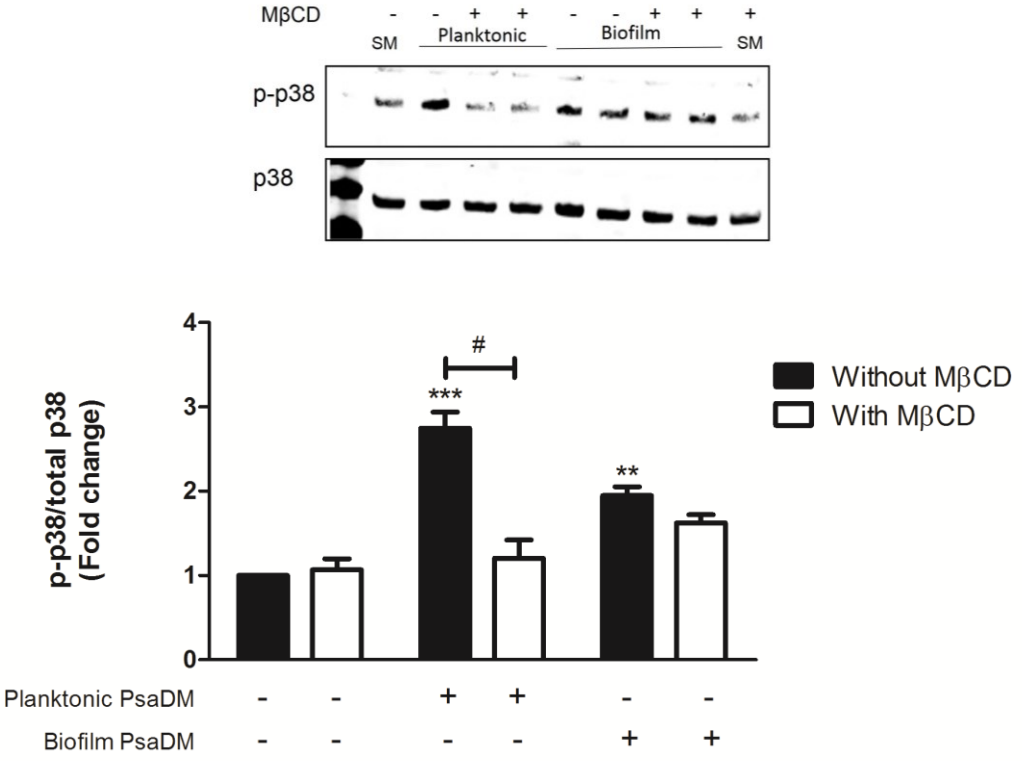


Figure 19: Lipid rafts involved in planktonic but not biofilm p38α activation

BEAS-2B AECs were left untreated (−) or pretreated for 1 hour without (black bars) or with (white bars) 100 μM methyl-beta-cyclodextrin (MβCD) and then stimulated (+) with 1 μg/mL planktonic or biofilm PACF508 PsaDM grown in SCFM for 30 minutes. p38α MAPK activation was determined as previously described. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** $P \leq .001$, ** $P \leq .01$, and * $P \leq .05$, compared with control; #$P \leq .05$ for comparison between groups.

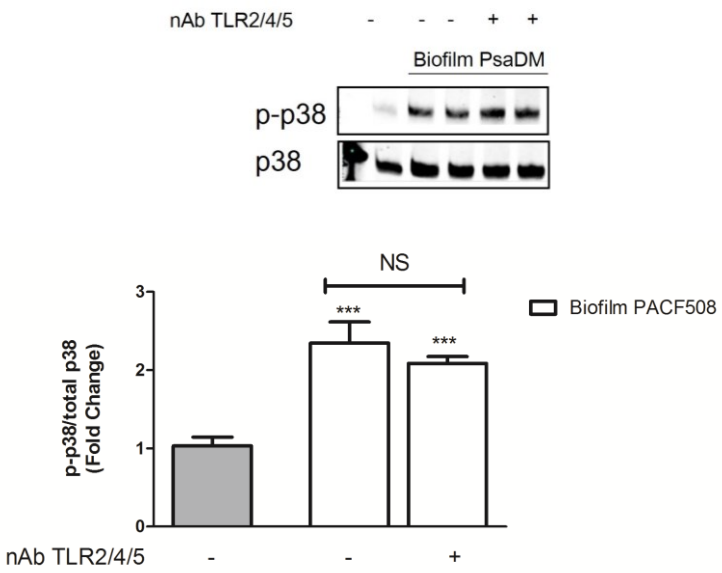


Figure 20: Activation of p38α by biofilm PsaDM is not dependent on TLR 2,4 or 5.

BEAS-2B were left untreated (−) or pretreated with a combination of 5 µg/mL Toll-like receptor 2 (TLR2), 5 µg/mL TLR4, and 5 µg/mL TLR5 (+) neutralizing antibodies for 30 minutes prior to stimulation with 1 µg/mL of biofilm (white bars) PACF508 material. Western blot analysis was performed as previously described. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** $P \leq .001$, ** $P \leq .01$, and * $P \leq .05$, compared with control; No significance was found for comparison between groups.

The *P. aeruginosa* quorum-sensing molecule N-(3-oxodo-decanoyl)-L-homoserine lactone (3OC12-HSL) activates p38α MAPK in BEAS-2B AECs in a TLR1/2/4/5/6-independent manner (Figure 21), a result consistent with previous findings in macrophages and epithelial cells.[252, 304, 493] Since PAO1 biofilms act primarily in a TLR-independent manner, we hypothesized that 3OC12-HSL mediates biofilm-induced p38α MAPK activation. In *P. aeruginosa,* 3OC12-HSL is synthesized via LasI. Thus, we tested biofilm PsaDM from a *lasI* PAO1 mutant (which is unable to produce 3OC12-HSL) and found that it could not activate p38α MAPK, in contrast to its wildtype isogenic parent (Figure 22A). Although we have shown that 3OC12-HSL can directly activate p38α MAPK in AECs (Figure 21), this quorum-sensing molecule also induces expression of other bacterial products when bound to the transcriptional activator LasR. To address whether 3OC12-HSL activates the p38α MAPK directly or indirectly, we first tested a *lasR* PAO1 mutant unable to activate the 3OC12-HSL–responsive genes even in the presence of 3OC12-HSL. As p38α MAPK phosphorylation is similarly reduced when AECs are exposed to the *lasI* and *lasR* mutant (Figure 22A), this

suggests that LasR-regulated genes are responsible for activating p38α MAPK.

Since LasR regulates *lasI* expression and 3OC12-HSL is nearly undetectable in the *lasR* mutant, we cannot rule out that the LasR-regulated gene responsible for p38α MAPK activation is *lasI*.[363] If this is the case, exogenous 3OC12-HSL should restore p38α MAPK activation in both the *lasI* and *lasR* mutants (but other LasR-regulated gene will only be rescued in the *lasI* but not the *lasR* mutant). Accordingly, exogenous 3OC12-HSL rescued expression of LasR-controlled elastase in the *lasI* but not the *lasR* mutant (Figure 23). Most importantly, exogenous 3OC12-HSL restored p38α MAPK activation in the *lasI* but not the *lasR* mutant (Figure 22A), suggesting that 3OC12-HSL alone is not sufficient for p38α MAPK activation by biofilm PsaDM. No significant differences in p38α MAPK activation were observed from planktonic PsaDM derived from both of these mutant strains (Figure 22B). Taken together, these results show that although 3OC12-HSL can directly activate p38α MAPK, in *P. aeruginosa* biofilms other genes regulated through the *P. aeruginosa* LasR transcriptional regulator are key to p38α MAPK activation.

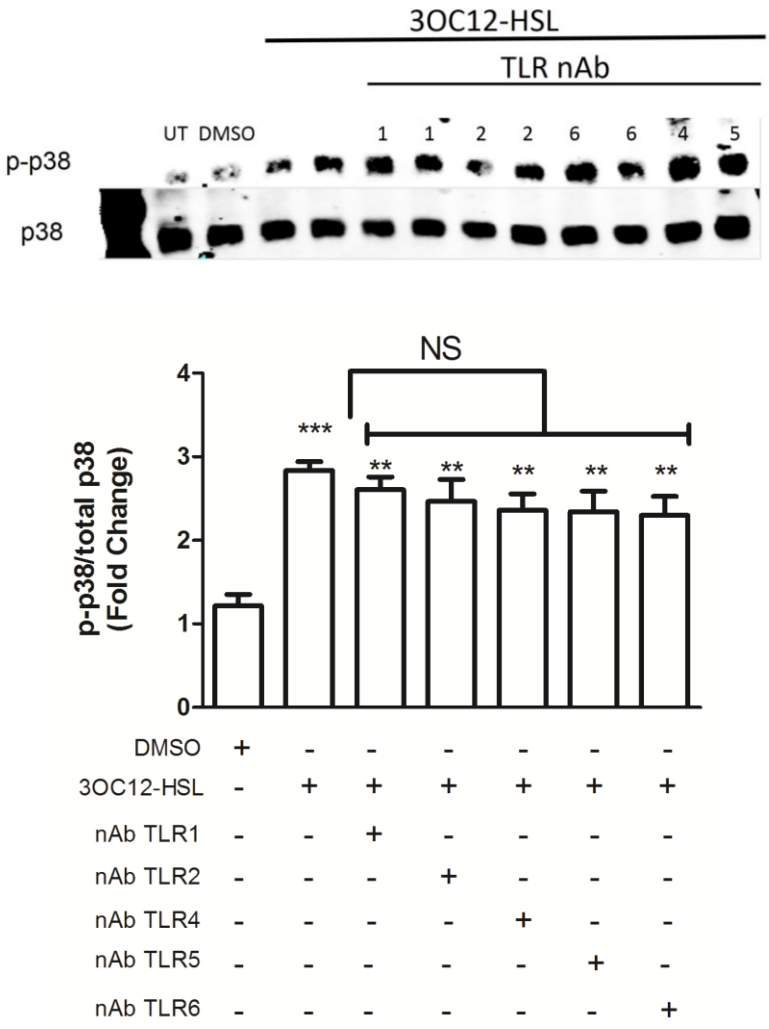


Figure 21: HSL activates p38 in AEC independent of TLR

BEAS-2B airway epithelial cells (AECs) were left untreated or pretreated without or with 5 μg/mL neutralizing TLR1, TLR2, TLR4, TLR5 or TLR6 antibodies, followed by exposure to 100 μM N-(3 oxododecanoyl)-L-homoserine lactone (3OC12-HSL) for 30 minutes. Western blot analysis was performed as previously described. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** $P \leq .001$, ** $P \leq .01$, and * $P \leq .05$, compared with control; No significance was found for comparison between groups

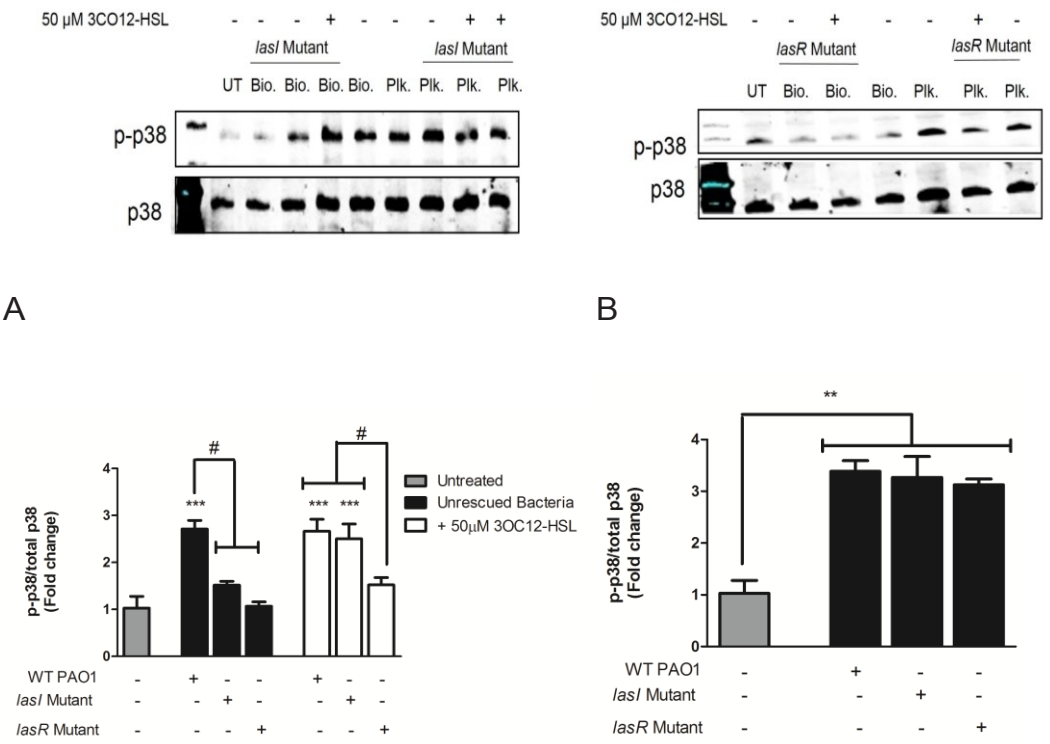


Figure 22: The LasR/LasI quorum-sensing system in *Pseudomonas aeruginosa* is responsible for p38α MAPK activation by biofilm PsaDM.

BEAS-2B AECs were treated with biofilm PsaDM from PAO1 wild type, *lasI* mutant, or *lasR* mutant that had been left untreated (black bars) or exposed to 50 µM (A). BEAS-2B AECs were treated with planktonic PsaDM from PAO1 wild type, *lasI* mutant, or *lasR* mutant all grown in peptone. 3OC12-HSL (white bars) for 6 hours prior to extraction (B). Western blot analysis was performed as previously described. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** P ≤ .001, ** P ≤ .01, and * P ≤ .05, compared with control; #P ≤ .05 for comparison between groups.

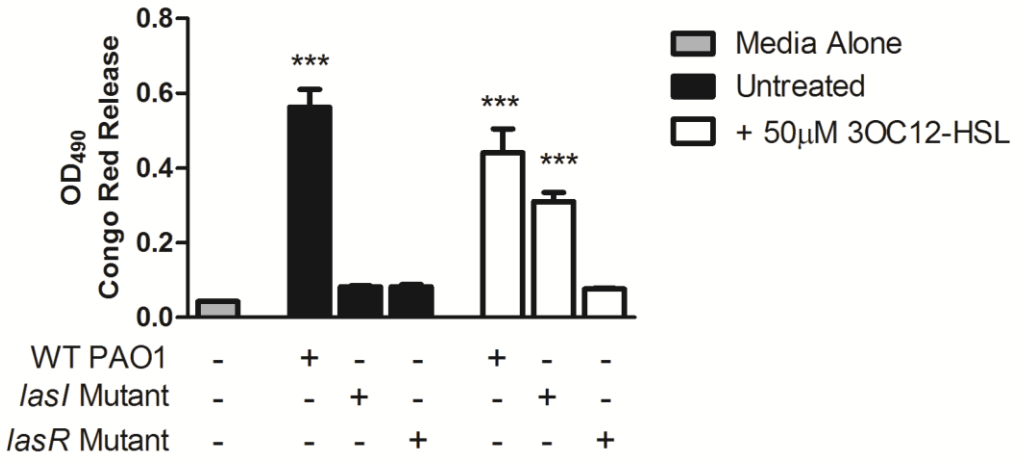


Figure 23: Rescuing wild type gene expression in LasI *Pseudomonas aeruginosa* mutant via addition of exogenous 3OC12-HSL

PAO1 wild type, *lasR* mutant, and *lasI* mutant were grown in the absence (black bars) or presence (white bars) of 50 μM 3OC12-HSL for 24 hours with shaking. Following this, elastase production was measured via an elastase Congo red procedure, with absorbance measured at 490 nm. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** $P \leq .001$, ** $P \leq .01$, and * $P \leq .05$, compared with control.

Planktonic, but not biofilm, PsaDM triggers neutrophil recruitment in a p38 dependent manner.

We next looked at the ability for planktonic and biofilm PsaDM to elicit pro-inflammatory cytokines in a p38α dependent manner. In order to do this, we used a p38 specific inhibitor, BIRB 0796, to block activation of p38α. At the concentrations used, BIRB 0796 specifically inhibits p38 without affecting the activation of ERK1/2 or JNK MAPK (Figure 24 and 25). In addition, BIRB 0796 is able to abrogate p38 MAPK activity in a dose dependent manner, as assessed via a kinase assay (Figure 26). BIRB 0796 is also able to down-regulate activation of the transcription factor ATF2 while having no effect on NFκB activation when stimulated by planktonic or biofilm PsaDM (Figure 15B). Neutrophils are characteristic of chronic CF inflamed airways and are recruited to sites of inflammation in response to various environmental cues, including the presence of CXCL8 and interleukin 6 (IL-6).[203, 423] p38α MAPK was shown to play a role in the synthesis of both cytokines at transcriptional and posttranscriptional levels in epithelial cells.[526] Stimulation of BEAS-2B cells with PACF508 planktonic PsaDM led to a greater increase of IL-6 and CXCL8 than biofilm PsaDM, while there was no significant up-regulation of other inflammatory mediators, such as granulocyte macrophage colony-stimulating factor

(GM-CSF), LTα, GROα, and RANTES, by either planktonic or biofilm PsaDM stimulation of BEAS-2B AECs (Figure 27).

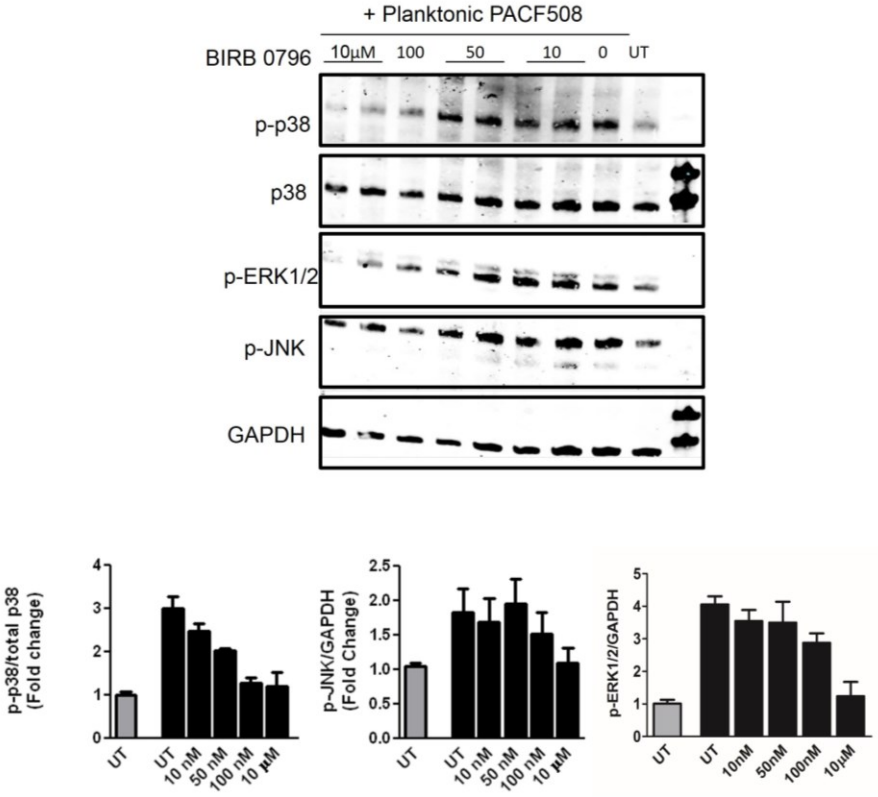


Figure 24: Effect of BIRB 0796 on MAPK pathways in AEC after treatment with planktonic PsaDM

BEAS-2B AECs were pretreated for one hour with various concentrations of BIRB 0796 (p38) inhibitor. Following this, they were treated with 1μg/mL of planktonic PsaDM for 30 minutes prior to protein extraction. Protein samples were assessed for phosphorylation of p38α, JNK and ERK1/2 in the presence of BIRB 0796. Western blot analysis was performed as previously described.

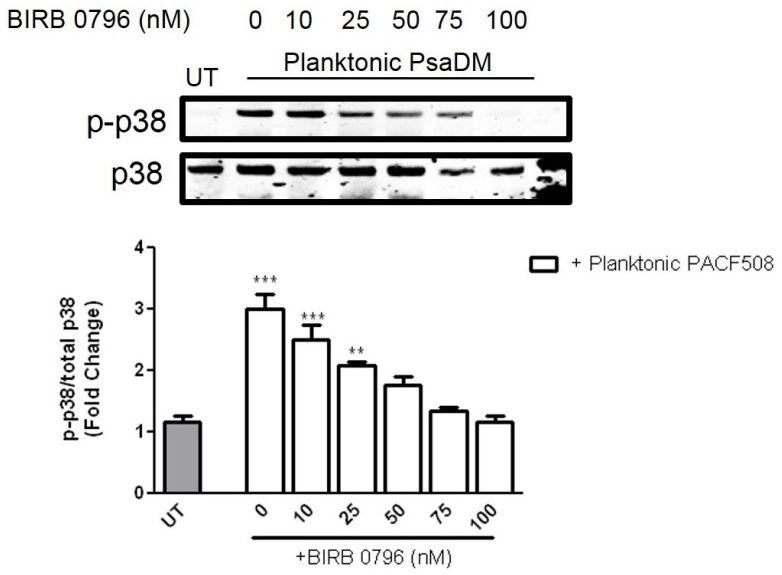


Figure 25: BIRB 0796 dose response on p38 phosphorylation by PsaDM

BEAS-2B AECs were left untreated or treated with various concentrations of BIRB 0796 for 1 hour prior to stimulation with 1 µg/mL of planktonic PACF508 PsaDM for 30 minutes. Western blot analysis was performed as previously described. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** $P \leq .001$, ** $P \leq .01$, and * $P \leq .05$, compared with control.

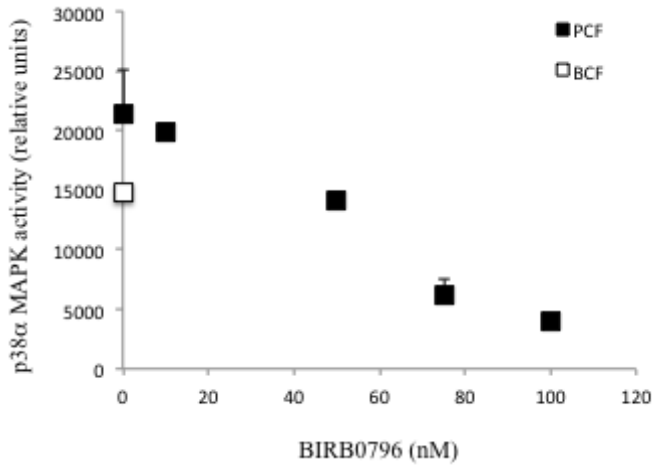


Figure 26: BIRB 0796 dose response on p38 Kinase Activity in response to PsaDM

BEAS-2B AECs were left untreated or pretreated with various concentration of BIRB 0796 for 1 hour prior to stimulation with 1µg/mL of planktonic PACF508 for 30 min. The activity of the kinases was quantified by measuring the incorporation of radioactivity into the specific substrate after SDS-PAGE.

The reported half maximal inhibitory concentration (IC_{50}) of BIRB 0796 toward p38α MAPK is 40–70 nM, which is in accordance with the values we determined (50–75 nM; Figures 24 and 25). Using suboptimal doses (<100 nM) of BIRB 0796, we found a dose-dependent inhibition of IL-6 and CXCL8, with respective IC_{50} values of 45 nM and 10 nM (Figure 28).[255]

To determine whether the stimulation of BEAS-2B AECs by planktonic and biofilm PsaDM was sufficient to provide important cues for neutrophil recruitment, cell culture medium from PsaDM exposed BEAS-2B AECs was assayed for its capacity to drive neutrophil migration. Exposure of BEAS-2B AECs to planktonic but not biofilm PsaDM led to a significant increase (>2-fold) in migrating neutrophils (Figure 29). Therefore, in BEAS-2B AECs, the level of p38α MAPK activation and cytokine synthesis in response to biofilm PsaDM is not sufficiently high to elicit an inflammatory response, in contrast to the level in response to planktonic PsaDM, which reaches a required threshold for neutrophil recruitment.

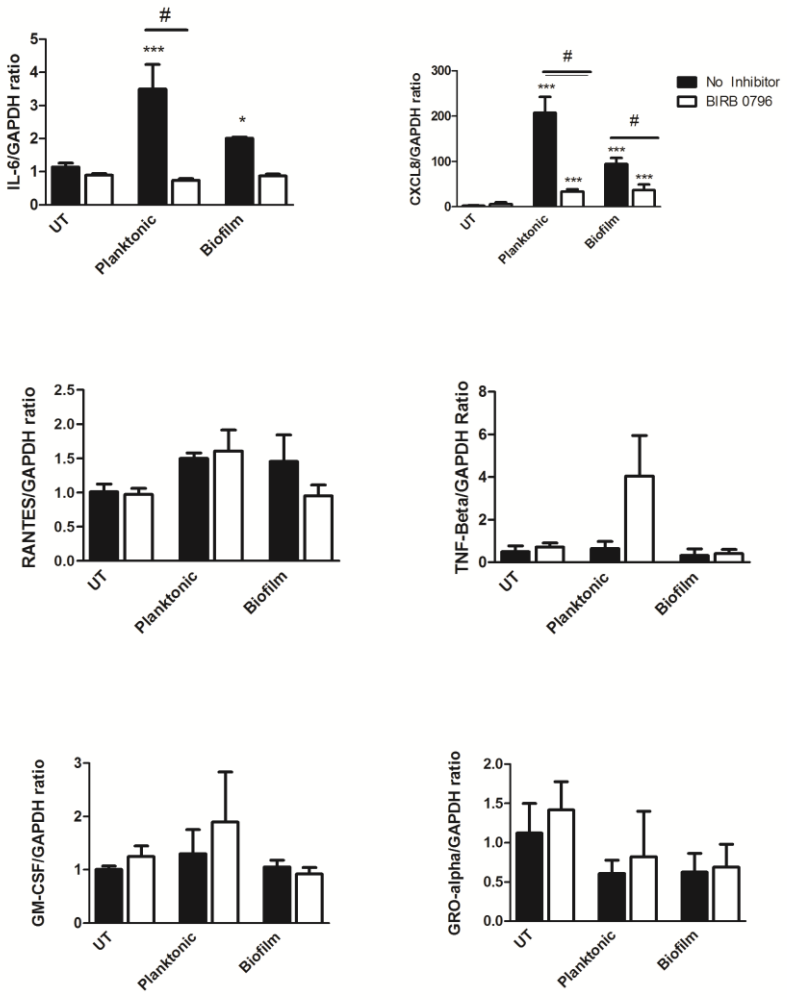


Figure 27: mRNA pro-inflammatory cytokine production in response to PsaDM.

BEAS-2B cells were left untreated (Black bars) or pretreated for 1 hour with BIRB 0796 (White bars) followed by stimulation with 1µg/mL of planktonic or biofilm material for 6 hours. RNA was extracted and qRT-PCR was performed on genes related to various cytokines. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** P ≤ .001, ** P ≤ .01, and * P ≤ .05, compared with control; #P ≤ .05 for comparison between groups.

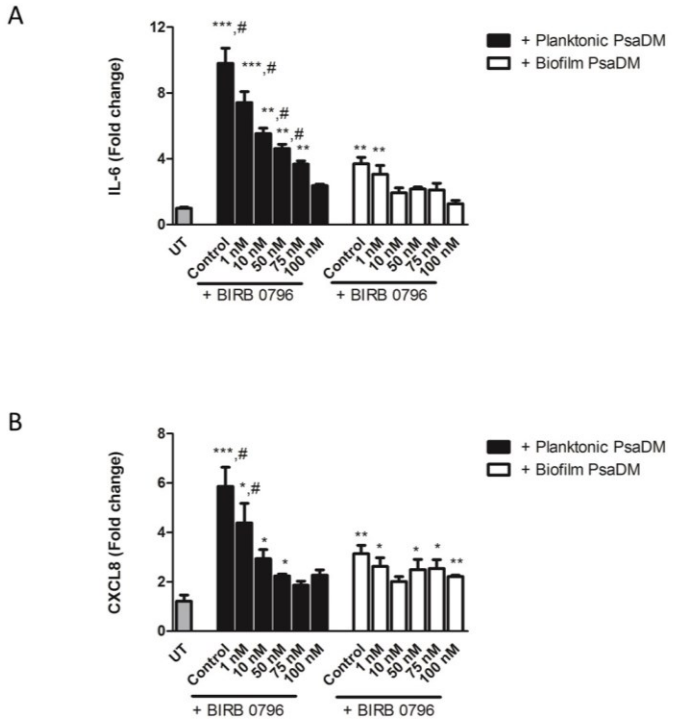


Figure 28: IL-6 and IL-8 Cytokine production in response to PsaDM is blocked via BIRB 0796

BEAS-2B airway epithelial cells (AECs) were left untreated or pretreated with increasing concentrations of the p38 MAPK selective inhibitor BIRB 0796 for 1 hour before exposure to 1 µg/mL planktonic or biofilm PACF508 PsaDM grown in synthetic cystic fibrosis medium (SCFM) for 6 hours. After stimulation, the culture medium was collected, and the abundance of interleukin 6 (IL-6; A) or CXCL8 (B) was determined by enzyme-linked immunosorbent assay. Fold change to mean of untreated control (90 pg/mL IL-6, 330 pg/mL IL-8). The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** $P \leq .001$, ** $P \leq .01$, and * $P \leq .05$, compared with control; #$P \leq .05$ for comparison between groups.

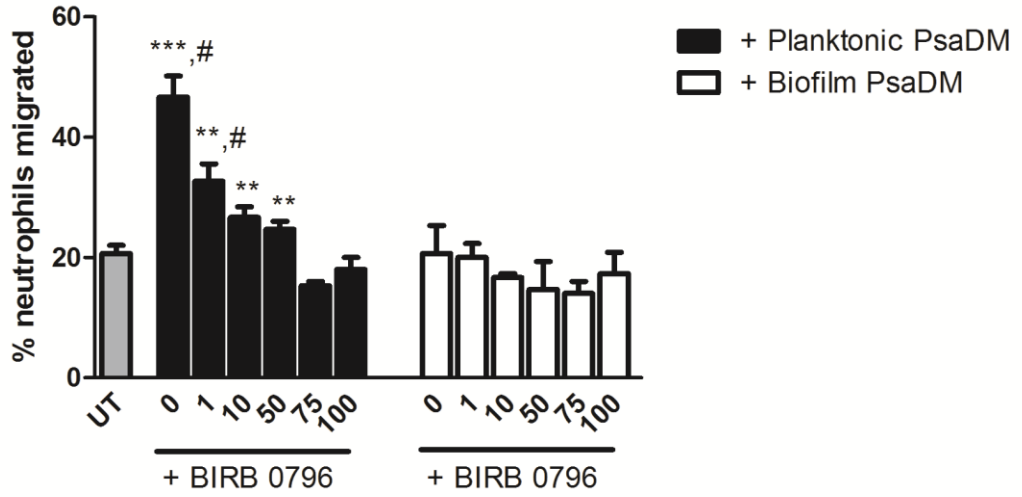


Figure 29: Planktonic but not Biofilm PsaDM induces increased neutrophil recruitment capacity of airway epithelial conditioned media in a p38α dependent manner.

BEAS-2B AECs were left untreated or pretreated with increasing concentrations of the p38 MAPK inhibitor BIRB 0796 for 1 hour before exposure to 1 μg/mL planktonic or biofilm PACF508 PsaDM grown in SCFM for 2 hours. Following this treatment, medium was removed, cells were washed, and serum-free medium was added for an additional 24 hours. This medium was then collected and used in a neutrophil recruitment assay. The results are expressed as the numbers of neutrophils recruited in response to stimulation, compared with the unstimulated condition. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** $P \leq .001$, ** $P \leq .01$, and * $P \leq .05$, compared with control; #$P \leq .05$ for comparison between groups.

Interestingly, the IC_{50} for neutrophil recruitment was 10 times lower (1 nM; Figure 29). Therefore, while a relatively linear relationship exists between p38α MAPK activation and IL-6 and CXCL8 synthesis, a certain level of p38α MAPK activation (10%–40% of maximal planktonic PsaDM stimulation, based on data presented in Figure 15A and 29) is required for neutrophil recruitment.

Planktonic PsaDM Leads to Greater Cytokine Synthesis and Neutrophil Recruitment than Biofilm PsaDM in Nasal Biopsy Explants

To support our finding that planktonic PsaDM leads to greater neutrophil recruitment than biofilm PsaDM, we used an ex vivo model of nasal polyps surgically removed from patients with chronic rhinosinusitis. Stimulation of the biopsy specimens with planktonic PACF508 PsaDM led to a greater increase of IL-6 and CXCL8 than stimulation with biofilm PsaDM (Figure 30). Accordingly, exposure of the explants to planktonic PsaDM led to greater neutrophil recruitment than exposure to biofilm PsaDM (Figure 31). Cytokine synthesis and neutrophil recruitment induced by planktonic PsaDM were sensitive to p38α MAPK inhibition by BIRB 0796 (Figures 30 and 31). Therefore, planktonic PsaDM leads to greater activation of host defense mechanisms in a p38α MAPK–dependent manner, compared with biofilm PsaDM, in *ex vivo* nasal biopsy specimens.

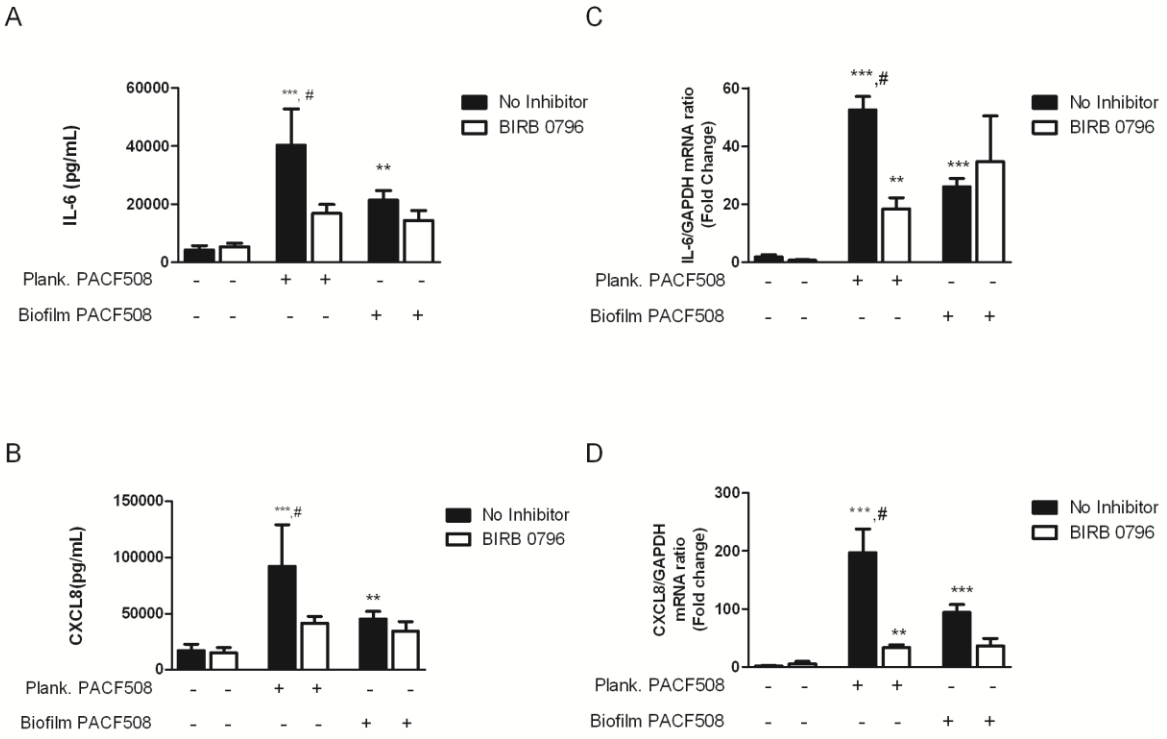


Figure 30: Planktonic PsaDM leads to greater cytokine synthesis and neutrophil recruitment than biofilm PsaDM in nasal biopsy specimens

Ex vivo nasal biopsy specimens were left untreated (black bars) or pretreated (white bars) with 0.1 μM BIRB 0796 for 1 hour prior to exposure to 1 μg/mL planktonic or biofilm PACF508 PsaDM grown in synthetic cystic fibrosis medium for 24 hours. After stimulation, the culture medium was collected to determine the abundance of interleukin 6 (IL-6; A) or CXCL8 (B) by enzyme-linked immunosorbent assay. RNA was also extracted from the tissue, and the messenger RNA (mRNA) abundance of IL-6 (C) or CXCL8 (D) was measured by quantitative reverse transcription polymerase chain reaction. For panels A and B, the mean value (±SD) of 15 samples is shown for each condition. The data were analyzed using nonparametric methods (the Friedman analysis), followed by the Dunn multiple comparisons tests. For panels C–D, the mean values (± standard error of the mean) of 3

samples for each condition are shown. Analysis was performed using 1-way analysis of variance, followed by a t test with the Bonferroni correction. *** P≤.001, ** P≤.01, and * P≤.05, compared with untreated condition; #P≤.05 for comparison of planktonic and biofilm groups.

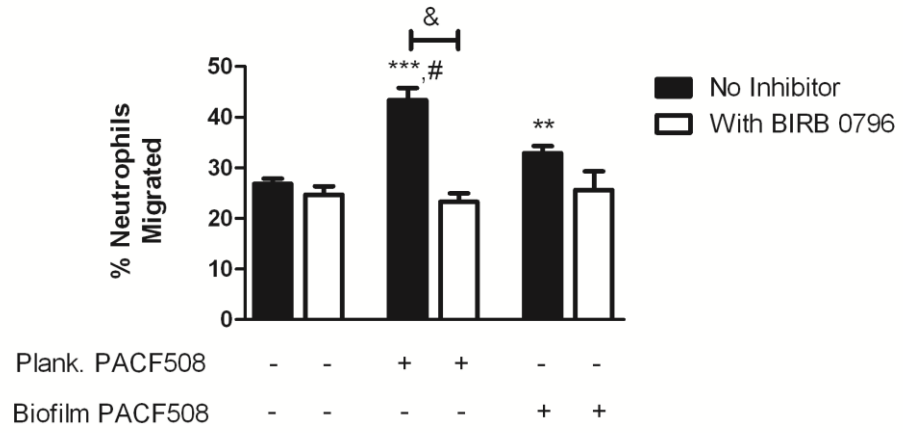


Figure 31: Neutrophil migration in response to conditioned media from Nasal Explants

Ex vivo nasal biopsy specimens were left untreated (black bars) or pretreated (white bars) with 0.1 μM BIRB 0796 for 1 hour prior to exposure to 1 μg/mL planktonic or biofilm PACF508 PsaDM grown in synthetic cystic fibrosis medium for 24 hours. After stimulation, the culture medium was collected and used for neutrophil migration assays as previously described the mean values (± standard error of the mean) of 3 samples for each condition are shown. Analysis was performed using 1-way analysis of variance, followed by a t test with the Bonferroni correction. *** P≤.001, ** P≤.01, and * P≤.05, compared with untreated condition; #P≤.05 for comparison of planktonic and biofilm groups.

Biofilm PsaDM Elicits Neutrophil Recruitment in CFTRΔF508 AECs Because of Their Hypersensitivity to Injurious Stimuli

The results presented so far suggest that although biofilm PsaDM elicits p38α MAPK activation and cytokine synthesis, the levels are too low to fully induce host defense mechanisms. However, we have recently reported that AECs expressing the most common mutation found in CF patients, CFTRΔF508, are hypersensitive to external injurious stimuli. Therefore, we hypothesized that CFTRΔF508 AECs will reach a p38α MAPK activation threshold at lower concentrations of injurious stimuli, or conversely, will have greater activation in response to the same concentration of bacterial ligands than their non-CF counterparts. p38α MAPK activation was greater in CFTRΔF508 AECs, compared with non-CF AECs, in response to both planktonic PsaDM (2.7 times higher) and biofilm PsaDM (2.1 times higher; Figure 31A). In fact, biofilm-mediated p38α MAPK activation in CFTRΔF508 AECs was greater than planktonic mediated p38α MAPK activation in non-CF AECs (Figure 32A). Similar results were obtained when PsaDM was prepared from PAO1 grown in peptone (Figure 32B). In accordance with our previous results (Figure 22), biofilm PsaDM-mediated p38α activation occurs through a *lasR*-dependent gene distinct from *lasI* (Figure 33).

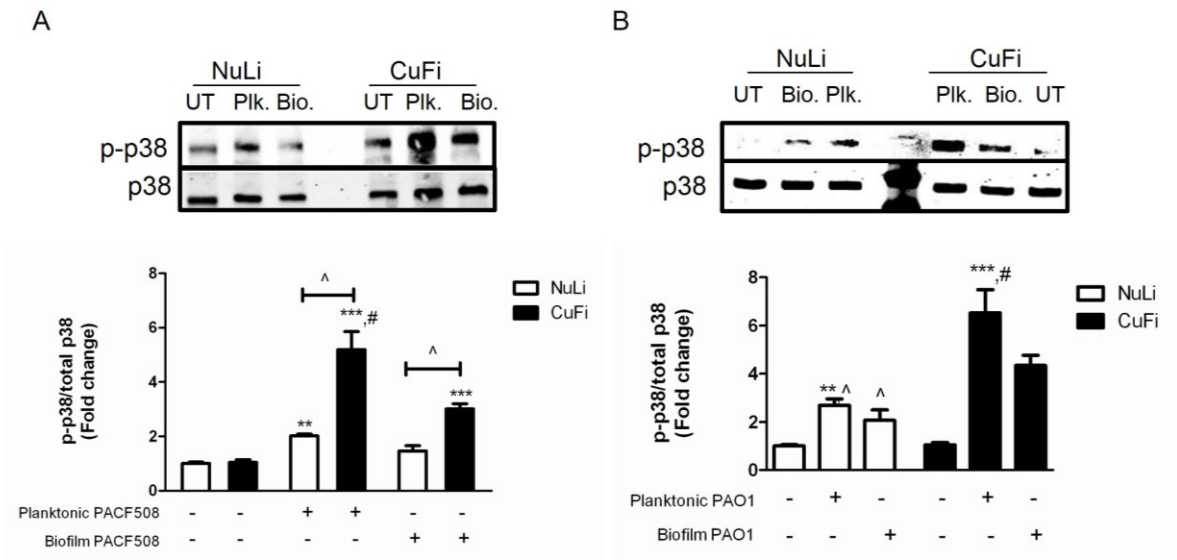


Figure 32: Planktonic and biofilm PsaDM activates p38α MAPK in CFTRΔF508 airway epithelial cells (AECs).

NuLi (non–cystic fibrosis; white bars) or CuFi (CF; black bars) AECs were left untreated (−) or treated with 1 μg/mL of planktonic or biofilm PACF508 PsaDM grown in synthetic CF medium (A) or PAO1 grown in peptone (B) for 30 minutes. Western blot analysis was determined as previously described. The mean value (± standard error of the mean) of 3 experiments is shown. Statistical analyses were performed using 1-way analysis of variance with Bonferroni post-test analysis. *** P≤.001 or ** P≤.01, compared with control; #P≤.05 for comparison of planktonic and biofilm groups; ^ P > .05 NuLi compared to CuFi; &P > .05 treated compared to untreated.

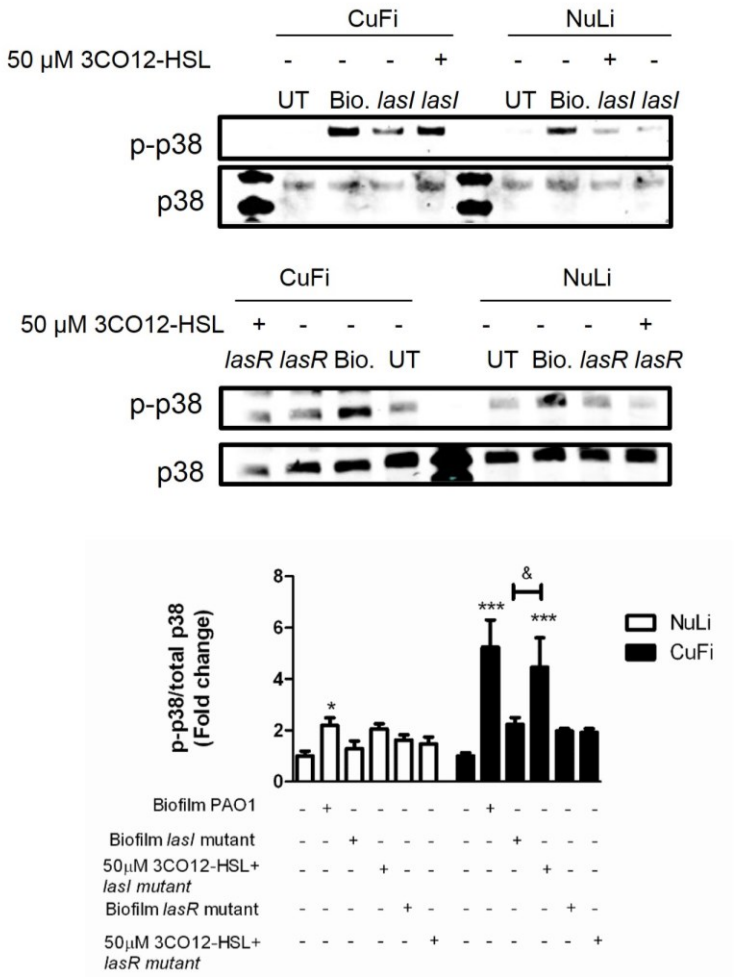
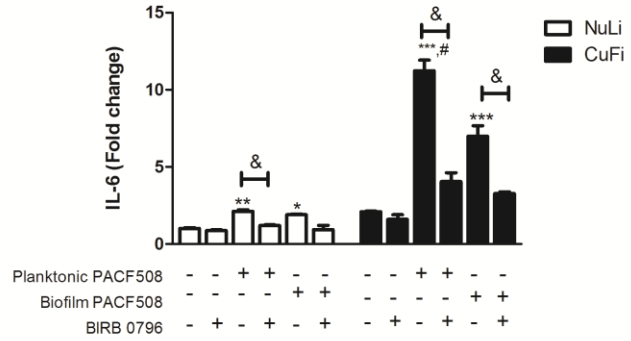


Figure 33: Biofilm PsaDM activates p38α MAPK in CFTRΔF508 airway epithelial cells (AECs) via LasR regulated products.

NuLi (non-CF; white bars) or CuFi (CF; black bars) were treated with biofilm PsaDM from PAO1 wild type, *lasI* mutant, or *lasR* mutant and assessed for p38α MAPK activation. PAO1 (wt) or mutants were all grown in peptone. The mean value (± standard error of the mean) of 3 experiments is shown. Statistical analyses were performed using 1-way analysis of variance with Bonferroni post-test analysis. *** P≤.001 or ** P≤.01, compared with control; #P≤.05 for comparison of planktonic and biofilm groups; ^ P > .05 NuLi compared to CuFi; &P > .05 treated compared to untreated.

IL-6 and CXCL8 synthesis was greater in the CFTRΔF508 AECs than in their non-CF counterparts in response to both planktonic and biofilm PsaDM (Figure 34A and 34B). In all cases, cytokine synthesis was significantly decreased when p38α MAPK activation was inhibited with BIRB 0796 (Figure 34A and 34B). Accordingly, planktonic PsaDM stimulation led to greater neutrophil recruitment by CFTRΔF508 AECs than by non-CF AECs (Figure 35). Importantly, in contrast to BEAS-2B and non-CF AECs, biofilm PsaDM stimulation was strong enough to trigger an increase in neutrophil recruitment in CFTRΔF508 AECs, which is p38α MAPK dependent (Figure 34). Activation of p38 by planktonic and biofilm PsaDM in other CF cell lines shows similar trends to that observed in NuLi and CuFi cells (Figure 36).

A



B

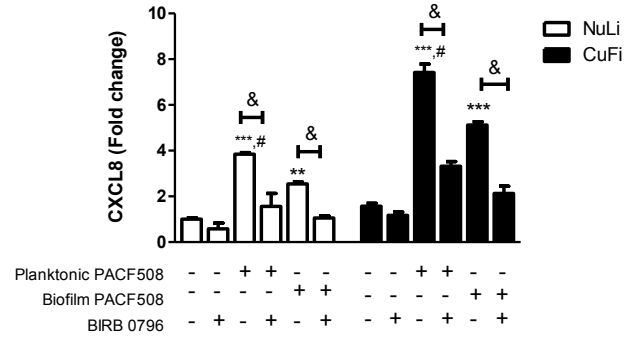
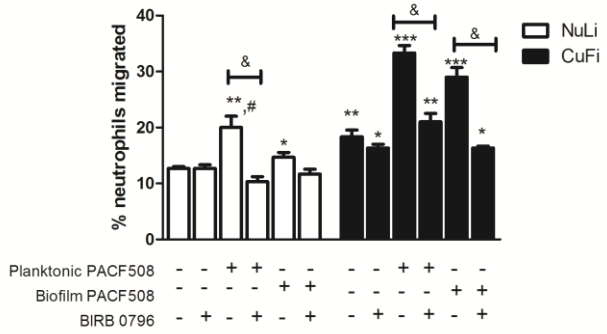


Figure 34: Biofilm PsaDM elicits a strong IL-6 and IL-8 in CuFi cell lines but not NuLi.

NuLi (non–cystic fibrosis [CF]; white bars) or CuFi (CF; black bars) AECs were left untreated or pretreated without or with 0.1 μM BIRB 0796 for 1 hour before exposure to 1 μg/mL planktonic or biofilm PACF508 PsaDM grown in synthetic CF medium (SCFM) for 6 hours. After stimulation, the culture medium was collected, and the abundance of interleukin 6 (IL-6; A) or CXCL8 (B) was determined by enzyme-linked immunosorbent assay. *** P≤.001, ** P≤.01, and * P≤.05, compared with untreated NuLi cells; &P > .05 compared to the presence of BIRB 0796; #P≤.05 for comparison between planktonic and biofilm groups, by 2-way analysis of variance, followed by a t test with the Bonferroni correction post-test. Fold-changes were calculated with respect to the untreated NuLi control.

A



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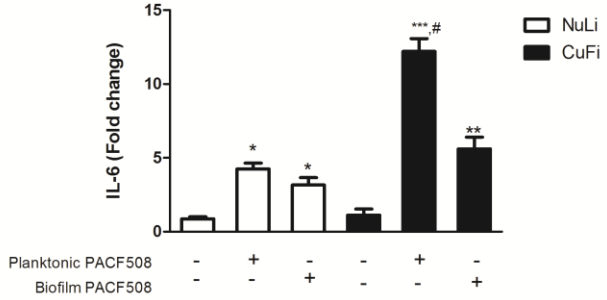

Figure 35: Biofilm PsaDM elicits neutrophil recruitment in CFTRΔF508 airway epithelial cells (AECs).

NuLi (non–cystic fibrosis [CF]; white bars) or CuFi (CF; black bars) AECs were left untreated or pretreated without or with 0.1 μM BIRB 0796 for 1 hour before exposure to 1 μg/mL planktonic or biofilm PACF508 PsaDM grown in SCFM for 2 hours. Following this treatment, medium was removed, cells were washed, and serum-free medium was added for an additional 24 hours. This medium was then collected and used in a neutrophil recruitment assay (A) or the abundance of IL-6 determined by enzyme-linked immunosorbent assay (B). *** P≤.001, ** P≤.01, and * P≤.05, compared with untreated NuLi cells; &P > .05 compared to the presence of BIRB 0796; #P≤.05 for comparison between planktonic and

biofilm groups, by 2-way analysis of variance, followed by a t-test with the Bonferroni correction post-test. Fold-changes were calculated with respect to the untreated NuLi control.

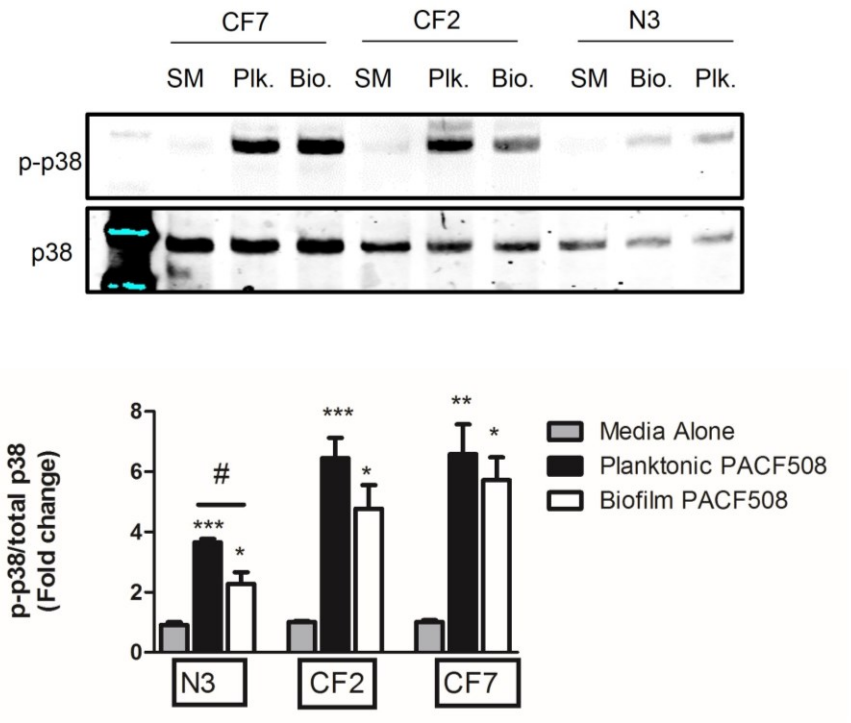

Figure 36: Planktonic and biofilm PsaDM activates p38α MAPK in additional CF AEC lines.

N3 (non–cystic fibrosis), CF2 (from patient with CF508) and CF7 (CF cells from patient with stop-codon mutation) AECs were treated with SCFM (grey bars) or treated with 1 µg/mL of planktonic (Black bars) or biofilm (White bars) PACF508 PsaDM grown in synthetic CF medium for 30 minutes. Western blot analysis was determined as previously described. The mean value (± standard error of the mean) of 3 experiments is shown. Statistical analyses were performed using 1-way analysis of variance with Bonferroni post-test analysis. *** P≤.001 or ** P≤.01, compared with control; #P≤.05 for comparison of planktonic and biofilm groups.

Discussion and Conclusions

Lungs of CF patients are a specialized ecological niche for *P. aeruginosa* biofilms. In this article, we asked if it matters to AECs whether they encounter bacteria-derived products from planktonic or biofilm microcolonies of *P. aeruginosa*, since many bacteria from biofilms to evade adverse environmental conditions. We report 4 main findings: (1) there exists a threshold of p38α MAPK activation required to engage host defense mechanisms, (2) planktonic PsaDM induces a stronger p38α MAPK activation as compared to planktonic PsaDM, (3) biofilm PsaDM activates p38α MAPK via the product of the *lasR* gene distinct from *lasI*, and (4) in AECs that do not express functional CFTR, the threshold of p38α MAPK activity required to recruit neutrophils is lower than that for their non-CF counterparts.

The relationship between p38α MAPK and host defense mechanisms against pathogens is ancient and is found in organisms ranging from nematodes to humans.[67, 235, 273] Moreover, a number of pathogens have devised immune evasion mechanisms by targeting this pathway.[57, 170, 350, 359] On the basis of our results, we propose that the switch from planktonic to biofilm growth is another strategy to decrease activation of p38α MAPK and the initiation of host defense

mechanisms. Although biofilm-derived material does activate p38α MAPK activation, functionally this is not sufficient to recruit neutrophils in our in vitro assay. Therefore, a threshold of p38α MAPK activation exists that has to be overcome in order to respond. This threshold is influenced by both extrinsic factors (i.e., signals emanating from the environment, such as planktonic or biofilm-derived material) and intrinsic factors (i.e., signals in the cellular context, such as CF AECs vs non-CF AECs) that determine the p38α MAPK pathway's sensitivity to infection.

Previous work has shown that TLR5 is a major activator of p38α MAPK in response to planktonic *P. aeruginosa*.[536] Moreover, we have recently reported that the switch to a mucoid phenotype by planktonic *P. aeruginosa* engages host responses through TLR2 in addition to TLR5.[32] Whether TLR2 act as a homodimer or a heterodimer with TLR1 or TLR6 is unknown. In contrast to planktonic PsaDM, we report here that biofilm PsaDM activates p38α MAPK in a TLR-independent fashion via the *lasI/lasR* quorum-sensing system. Previous findings have shown that the 3OC12-HSL signal can trigger inflammation directly, an observation that our data support. However, in the context of *P. aeruginosa* biofilms, genes

regulated through the LasR transcriptional activator are more important to p38α MAPK activation than 3OC12-HSL direct stimulation. This is an important finding of our study, as it means that other biofilm-derived virulence factors act on AECs to activate p38α MAPK. While biofilm PsaDM does activate p38α MAPK, the planktonic PsaDM, signaling through TLR5/TLR2 found in lipid rafts, induces greater p38α MAPK activation than is required to trigger neutrophil recruitment. Our results support the model of a switch from planktonic to biofilm growth that is accompanied by loss of the flagellum, a strong inducer of p38α MAPK activation and innate immune responses in AECs.[509]

In CF, a “hyper-inflammatory” phenotype exists, resulting in increased MAPK activation and increased synthesis of proinflammatory cytokines.[37, 58, 59, 253, 453, 487] Therefore, in CF, biofilms activate p38α MAPK earlier to trigger neutrophilic inflammation. Contrary to what is postulated to occur in non- CF cells, biofilm PsaDM is now considered “virulent” enough to induce a response by the epithelium, which would be a contributor to lung tissue destruction.

Although blocking the p38α MAPK activity can decrease neutrophil recruitment that is induced by planktonic PsaDM in in vitro

and ex vivo nasal biopsy specimens, and while it has been previously shown to control lipopolysaccharide- induced mucosal inflammation in ex vivo nasal biopsy specimens from CF patients, its complete inhibition may not be a successful strategy because of the numerous functions it regulates.[387] Moreover, since innate immune mechanisms do have protective functions against pathogens, particularly in the setting of chronic bacterial infections, immunosuppressive therapies have a narrow window of opportunity to reduce damaging inflammation without risking overwhelming infections. An alternative strategy may be to target suboptimal inhibition of p38α MAPK (as little as 10% may be sufficient), which would decrease neutrophil recruitment without completely impairing host defense mechanisms.

Acknowledgements

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All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

CHAPTER 3: Recognition of Mucoid and Non-mucoid *Pseudomonas aeruginosa* by Airway Epithelial Cells Pathogen Recognition Receptors

Adapted from [32]:

Beaudoin T, Lafayette S, Nguyen D, Rousseau S. **Mucoid Pseudomonas aeruginosa caused by mucA mutations result in activation of TLR2 in addition to TLR5 in airway epithelial cells.**
Biochem Biophys Res Commun. 2012 Nov 9;428(1):150-4.

Preface

The second major phenotypic change that occurs to *Pseudomonas aeruginosa* during the course of infection in the CF lung is a switch from non-mucoid to a mucoid phenotype. As was described in the general introduction, this switch is thought to confer resistance to phagocytosis via host macrophages, as well as providing resistance to antibiotics. While strains of Pseudomonas isolated early on in the course of infection are often 'environmental', non-mucoid strains (which are also more motile), somewhere between 70 and 96% of strains isolated from a chronically infected CF patient (6-12 years post initial infection) exhibit a mucoid phenotype. A switch to a mucoid phenotype often arises (in 70% of the cases of clinical *Pseudomonas* strains) from a mutation in a repressor of alginate biosynthesis. Interestingly, very little has been studied on how this switch is recognized by immune cells or the airway epithelium and weather mucoid strains are more or less inflammatory. Initial studies from our lab suggested that the clinical isolate we tested (which was indeed mucoid) were able to initiate p38 signaling through Toll-like receptor 2 (TLR2). Here we investigate whether the switch from a non-mucoid to a mucoid phenotype of Pseudomonas aeruginosa is able to activate TLR2.

Statement of Contribution

The main text of the article was prepared by Dr. Simon Rousseau. Materials and Methods and Figure legends were prepared by Trevor Beaudoin. Editing prior to submission was performed by all authors, but significant revisions were contributed by Trevor Beaudoin and Dao Nguyen. Correction of Proofs was done by Dr. Simon Rousseau and Trevor Beaudoin.

All Figures and data analyses were performed by Trevor Beaudoin.

Shantelle LaFayette confirmed the mucoid phenotype of the mucA mutant used in this study.

Abstract

The presence of mucoid *Pseudomonas aeruginosa* in patients with Cystic Fibrosis (CF) is a marker of poor survival. As CF lung disease results from chronic infection leading to airway inflammation, we determined whether the switch to a mucoid phenotype by *P. aeruginosa* has an impact on the inflammatory response of airway epithelial cells. Exposure of airway epithelial cells to non-mucoid and mucoid *P. aeruginosa*-derived material leads to p38α MAPK activation, a key protein kinase involved in transmitting inflammatory signals. However, while the non-mucoid strain PAO1 activates p38α MAPK pathway solely via TLR5, the mucoid strain PACF508 activates p38α MAPK via both TLR5 and TLR2. Inactivation of *mucA* (the gene responsible for the mucoid phenotype) in PAO1 leads to p38α MAPK activation by both TLR2 and TLR5, as observed in the clinical mucoid isolate PACF508. Therefore, the switch to mucoid phenotype may contribute to more inflammation via TLR2 activation in addition to TLR5. Our findings highlight an important and under recognized role for TLR2 in the response of airway epithelial cells to infection.

Introduction and Background

Cystic fibrosis (CF) is characterized by mucus hyper-secretion, chronic infection and inflammation associated with decreasing lung function.[406, 452] Therefore signals that increase inflammation in chronically infected CF patients may worsen decline in lung function. Markers of inflammation are increased at the onset of pulmonary exacerbations and, following these episodes, a net decline in lung function has been documented from pre-exacerbation state.[394, 421] Unfortunately, very little is known about pulmonary exacerbations. They are probably related to a complex relationship between host defense and airway microbiology. One model proposes that exacerbations are caused by the release and proliferation of planktonic bacteria from biofilm aggregates.[485]

Pseudomonas aeruginosa is the most significant pathogen in CF with up to 80% of patients eventually chronically infected with *P. aeruginosa*.[286] Moreover, clinically the presence of the mucoid phenotype of *P. aeruginosa* is a marker of poor survival in CF.[186, 334] The mucoid phenotype is typically attributed to mutations in the mucA gene, a negative regulator of the stress sigma factor AlgU. [297]

Activation of innate immunity in response to pathogens is mediated via pattern-recognition receptors (PRRs) expressed by host cells. The p38α mitogen-activated protein kinase (MAPK) is an important mediator of inflammatory signaling that plays a role in host defenses against *P. aeruginosa* in vertebrates.[410] Activation of p38α MAPK in response to *P. aeruginosa* has been linked to the flagellin receptor Toll-like receptor 5 (TLR5) expressed at the surface of airway epithelial cells.[535]

In this chapter, we investigated if the switch to a mucoid phenotype by P. aeruginosa has an impact on the activation of p38α MAPK in airway epithelial cells.

Materials and Methods

Chemicals

All chemicals were bought from Fisher Scientific (Fair Lawn, NJ, USA). Zeocin, Hygromycin, Blasticidin, Normocin, FSL-1, LPS from *P. aeruginosa* and *S. typhimurium* flagellin were bought from Invivogen (San Diego, CA, USA).

P. aeruginosa strains

As described in Chapter 2

P. aeruginosa diffusible material preparation

As described in Chapter 2

Antibodies

Neutralizing antibodies against TLR2, TLR4 and TLR5 were used at 5 ng/mL and purchased from Invivogen (CA, USA). Anti-GAPDH (used at a concentration of (1/4000) and anti-phospho p38 MAPK (Thr180/Thr182; used at 1/1000 dilution) were purchased from Millipore (Temecula, CA). Anti-p38 MAPK (used at a dilution of 1/ 1000) was purchased from Cell signaling (Boston, Ma). Goat anti-rabbit IgG DyLight™800 (35,571; 1:15,000) and Goat anti-mouse IgG DyLight™680 (35,518; 1:15,000) were bought from Thermo Scientific (Rockford, IL, USA).

Cell culture

BEAS-2B AECs were cultured as previously described. HEK-Blue TLR5 cells and HEK-Blue Null1 cells were purchased from Invivogen (San Diego, CA). HEK-Blue TLR5 were grown and maintained in DMEM supplemented with 10% FBS with 100 U/mL Penicillin G, 100 µg/mL of Streptomycin, 100 µg/mL Normacin, 100 µg/mL Zeocin and 30 µg/mL of Blasticidin. 24 h prior to stimulation, cells were starved in DMEM without antibiotics. HEK-Blue Null1 were grown and maintained in DMEM supplemented with 10% FBS with 100 U/mL Penicillin G, 100 µg/mL of Streptomycin, 100 µg/mL Normacin and 100 µg/mL Zeocin. 24 h prior to

stimulation, cells were starved in DMEM without antibiotics. Human airway epithelial cell line NuLi was derived from a normal lung of a 36-year-old male patient and CuFi airway epithelial cell line derived from lung of a 14-year-old female patient with cystic fibrosis homozygous for the CFTRDF508 mutation were cultures as previously described.

Cell lysis and immunoblotting

As described in Chapter 2

ELISA

As described in Chapter 2

Statistical analysis

Analyses of variance (ANOVA) followed by a multiple comparison test (Bonferroni) were used to test differences in mean between groups. p values <0.05 were considered significant.

Experimental Results

Activation of p38α by PsaDM occurs through TLR5

Because of the previously reported importance of *P. aeruginosa* flagellin in promoting pro-inflammatory cytokine secretion, we studied the ability for different preparations and various strains to induce p38α activation through TLR5 (the known receptor for flagellin). As seen in Figure 37A and B, the phosphorylation of p38α by a non-mucoid strain of

Pseudomonas aeruginosa occurs exclusively in a TLR5 dependent manner. A TLR5 neutralizing antibody is able to fully block the phosphorylation of p38α by PAO1. Further evidence is provided by using a filtrate of PsaDM derived from a PAO1 mutant lacking the flagellum. This filtrate was unable to stimulate p38α phosphorylation. Biofilm preparations of PsaDM where unaffected by the neutralizing antibodies, confirming data presented in the previous chapter. When stimulating airway epithelial cells with PsaDM derived from a clinical mucoid strain, neutralization of TLR5 only partially blocked the activation of p38α (Figure 38A). To further demonstrate the importance for TLR5 in this pathway, HEK cells, which normally do not express TLR5 were unable to respond to the filtrate, or flagellin. When TLR5 was stably transfected and expressed in these cells, p38α was able to be activated by the PsaDM filtrate (Figure 38B).

Importantly, biofilm materials were unable to initiate p38α activation via null or transfected HEK cells, further confirming that they do not activate p38α in a similar manner to planktonic material, as described in chapter two of this thesis.

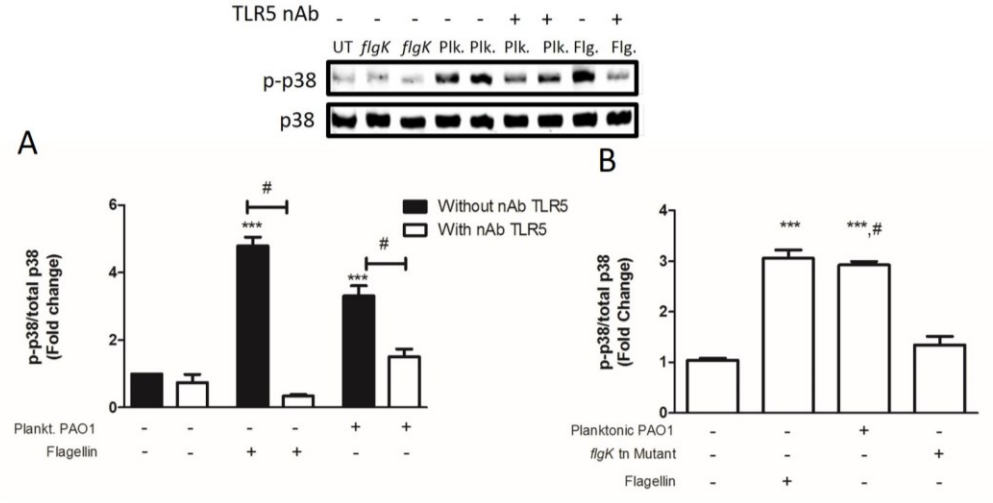


Figure 37: TLR5 leads to p38α MAPK activation in AECs exposed to Planktonic PsaDM.

BEAS-2B AECs were left untreated (-) or pretreated for 30 min without (Black bars) or with (White bars) 5 μg/mL neutralizing TLR5 antibody (white bars) followed by exposure (+) for 30 min to 0.4 μg/mL of flagellin or 1 μg/mL of planktonic PAO1 PsaDM (A). Following stimulation, cells were lysed and 20 μg of Triton-soluble materials was subjected to SDS–PAGE. After transfer to nitrocellulose, the membranes were probed with antibodies recognizing only the phosphorylated forms of p38α MAPK or antibodies that recognize all forms of p38α MAPK. Quantitative analysis of the signals from each antibody was performed using Li-Cor infrared Odyssey imaging system. (B) BEAS-2B AECs were left untreated (-) or stimulated (+) for 30 min with 1 μg/mL planktonic PsaDM from wildtype PAO1 or a *flgK* mutant.

The mean of three experiments is shown +/_ SEM. Statistics were performed using one-way ANOVA with Bonferroni post-test analysis. ***,**,* $p \leq 0.001$ or 0.01, respectively compared to unstimulated; #, $p \leq 0.05$ in absence of TLR5 nab compared to its presence. &, $p \leq 0.05$ wildtype PAO1 compared to PAO1 *flgK*.

The mucoid phenotype of *P. aeruginosa* leads to activation of TLR2

The results from Figure 38A would indicate that only part of the activation of p38α by the clinical isolate of *P. aeruginosa* was occurring through the TLR5 pathway. (In addition to TLR5, TLR2 and TLR4 have been shown to bind bacterial components and induce p38α activation, thus it was of interest to see if they played a role in p38α activation in the context of our study. As is seen in Figure 39A, a TLR2 neutralizing antibody was able to partially reduce the level of p38α activation by PACF508 PsaDM. TLR4 neutralizing antibody did not show the ability to reduce p38α induction by the planktonic PsaDM (Figure 39B). Neither neutralizing antibodies were effective in reducing biofilm PsaDM activation of p38α. Combining TLR2 and TLR5 antibodies completely eliminate p38α activation by the PACF508 clinical strain (Figure 40). The addition of TLR4 neutralizing antibody does not affect the level of p38α activation. Taken together, these results suggest that this clinical isolate is able activate p38α through TLR5 as well as TLR2 pathways. In the course of a chronic infection in the CF lung, *P. aeruginosa* isolates can acquire many different mutations. The majority of strains acquire mutations that lead to a 'mucoid' phenotype, while simultaneously downregulating the expression

of flagellum. The clinical isolate we used was indeed a stable mucoid strain of *P. aeruginosa.*

The activation of p38α was greater in a cell line that contained a mutated CFTR (CuFi cells, a common CF cell line) as noted before. In any case, similar results were seen, as planktonic PsaDM was reduced by both TLR2 and 5, while Biofilm PsaDM was unaffected (Figure 41). Because the slime layer produced could contain different lipopeptides, which have been shown to be agonists for TLR2, we wanted to see if the mucoid phenotype could account for the increased p38α expression. To do this, we took a PAO1 strain that had a transposon insertion mutant in the *mucA gene*, representing the most common mutation found in clinical CF isolates, to obtain the mucoid phenotype in PAO1. As seen in Figure 42, the activation of p38α by PAO1 wildtype strain was unaffected by TLR2 neutralizing antibody. In contrast, the *mucA* mutant showed a significant decrease in p38α activation in the presence of a TLR2 neutralizing antibody, in addition to TLR5 antibody. Adding both antibodies together result in a further decrease. These results suggest that blocking one, or the other pathway may be sufficient to reduce p38α activation, without the need to block both. They also suggest that a switch to a

mucoid phenotype creates an additional signaling event to activate p38α via TLR2.

Cytokine Production

In addition to the decrease in the activation of p38, blocking the TLR pathways decreases the amount of pro-inflammatory cytokine production form airway epithelial cells. The addition of TLR5 neutralizing antibody was able to block completely the production of IL-6 and CXCL8 from BEAS-2B cells in response to planktonic PACF508 PsaDM, but showed no effect on biofilm material (Figure 43). Similarly, a neutralizing antibody to TLR2 was able to partially block planktonic PsaDM cytokine producing effect on the BEAS-2B cells, while a TLR4 neutralizing antibody had no effect (Figure 44 and 45). Importantly, when a non-mucoid strain of *P. aeruginosa* is used, TLR2 neutralizing antibodies no longer effect cytokine production (Figure 46). Using the *mucA* mutant of PAO1 produces similar amounts of IL-6 and CXCL8 cytokine production as PAO1, however, both TLR 2 and TLR5 are important factors in driving this production, as blocking either one of them significantly reduces cytokine production and blocking both further reduces cytokine levels (Figure 46).

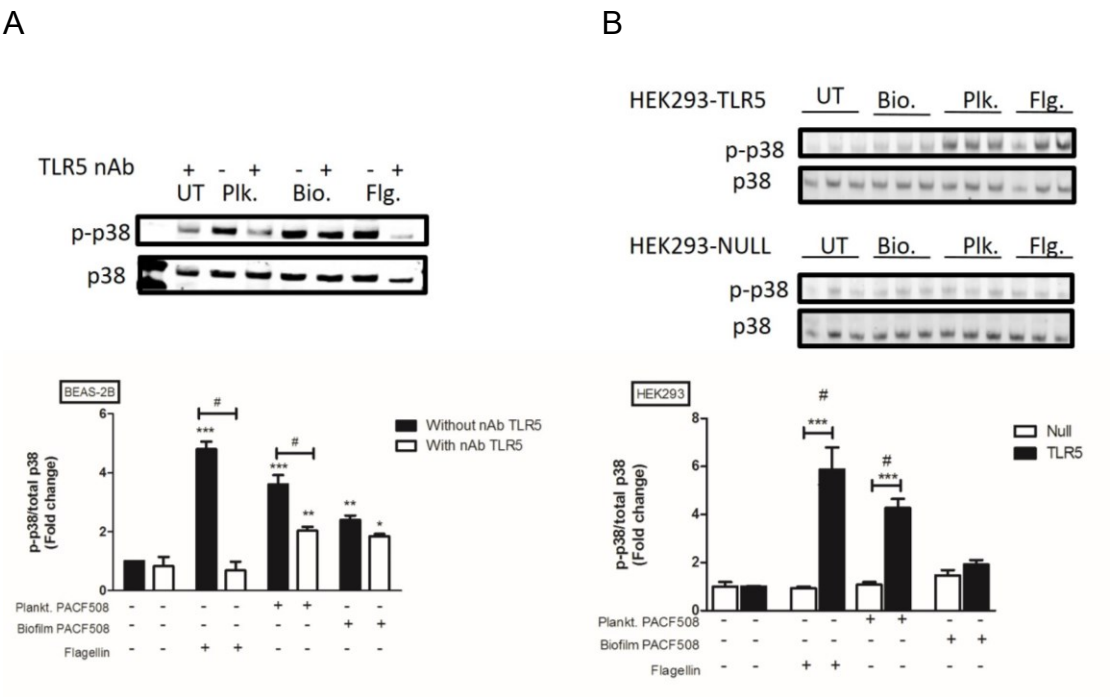


Figure 38: Activation of p38α by mucoid PsaDM

BEAS-2B AECs were left untreated (-) or pretreated for 30 min without (black bars) or with 5 μg/mL TLR5 neutralizing antibody (nAb) (white bars) followed by exposure (+) for 30 min to 0.4 μg/mL of flagellin or 1 μg/mL of planktonic PACF508 PsaDM. B) Human embryonic kidney (HEK) cells lacking (null, white bars) or expressing TLR5 (TLR5, black bars) were left untreated (-) or treated (+) with 0.4 μg/mL flagellin or 1 μg/mL of planktonic or biofilm PACF508 PsaDM grown in SCFM for 30 min. p38α MAPK activation was determined as previously described. The mean of three experiments is shown +/_ SEM. Statistics were performed using one-way ANOVA with Bonferroni post-test analysis. ***,**,* $p \leq 0.001$ or 0.01, respectively compared to unstimulated; #, $p \leq 0.05$ in absence of TLR5 nAb compared to its presence.

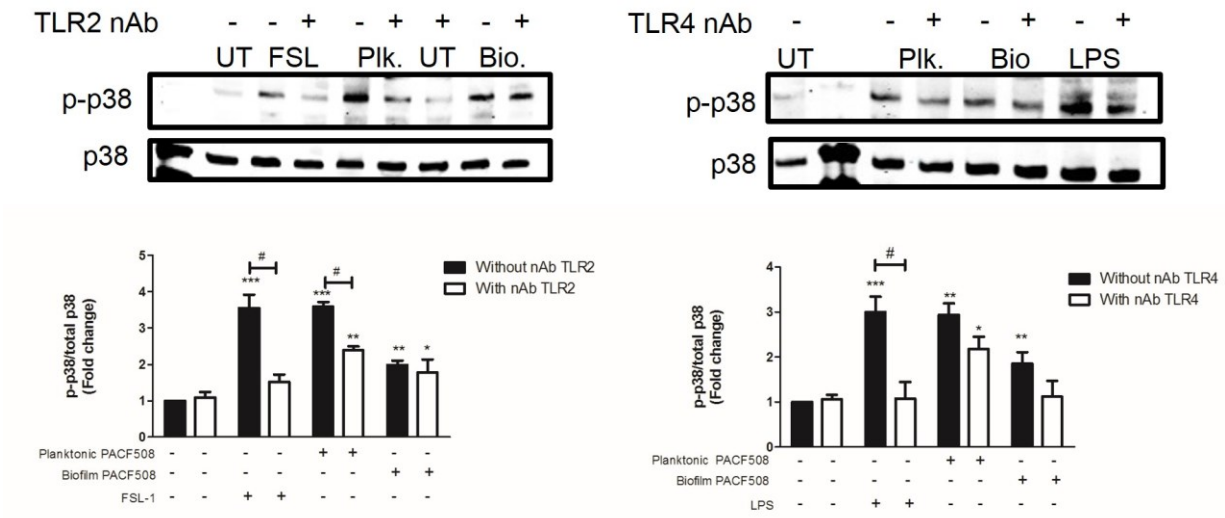


Figure 39: The mucoid phenotype of *P. aeruginosa* leads to activation of TLR2.

BEAS-2B AECs were left untreated (-) or pretreated for 30 min without (black bars) or with (white bars) 5 μg/mL TLR2 (A), TLR4 (B) neutralizing antibodies (nAB) followed by exposure (+) for 30 min to 1 μg/mL of planktonic PsaDM, or 0.4 μg/mL of FSL-1 (A) or 0.5 μg/mL LPS (B). p38α MAPK activation was determined as previously described. The mean of three experiments is shown +/_ SEM. Statistics were performed using one-way ANOVA with Bonferroni post-test analysis. ***,**,* $p \leq 0.001$ or 0.01, respectively compared to unstimulated; #, $p \leq 0.05$ in absence of nAb compared to its presence.

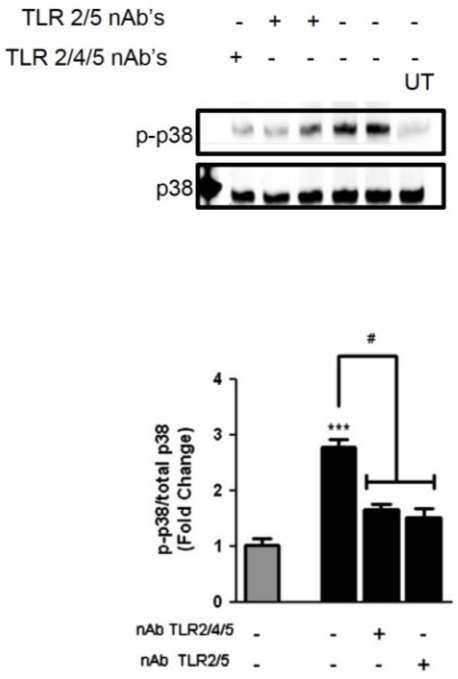


Figure 40: Phosphorylation of p38α by PsaDM of mucoid phenotype is completely blocked by TLR2 and TLR5 neutralizing antibodies.

BEAS-2B were left untreated (-) or pretreated with a combination of 5 μg/mL TLR2 and TLR5 neutralizing antibodies (nAb) (+) or a combination of 5 μg/mL TLR2, TLR4 and TLR5 (+) neutralizing antibodies for 30 min prior to stimulation with 1 μg/mL of planktonic PACF508 PsaDM. p38α MAPK activation was determined as previously described. The mean of three experiments is shown +/_ SEM. Statistics were performed using one-way ANOVA with Bonferroni post-test analysis. ***,**,* $p \leq 0.001$ or 0.01, respectively compared to unstimulated; #, $p \leq 0.05$ in absence of nAb compared to its presence.

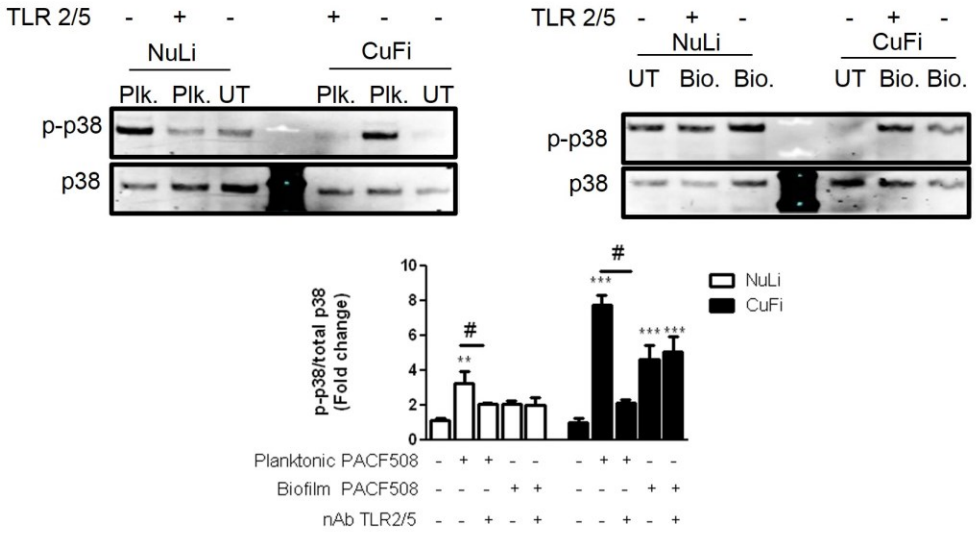


Figure 41: TLR2 and TLR5 lead to p38α MAPK activation in airway epithelial cells expressing wildtype CFTR and CFTRΔF508.

NuLi (non-CF, white bars) or CuFi (CF, black bars) AECs were left untreated (-) or pretreated with a combination of 5 μg/ mL TLR2 and 5 μg/mL TLR5 (+) before stimulation for 30 min with 1 μg/mL of planktonic PACF508 PsaDM grown in SCFM. p38α MAPK activation was determined as previously described. The mean of three experiments is shown +/_ SEM. Statistics were performed using one-way ANOVA with Bonferroni post-test analysis. ***,**,* $p \leq 0.001$ or 0.01, respectively compared to unstimulated; #, $p \leq 0.05$ in absence of nAb compared to its presence.

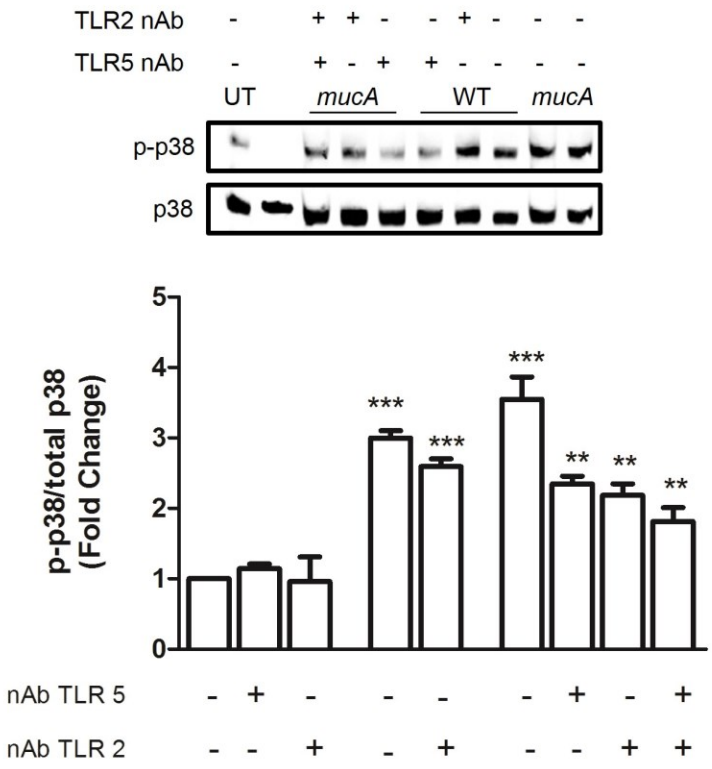


Figure 42: Mutation of the mucA gene in a non-mucoid PAO1 background induces phosphorylation of p38 through TLR2

BEAS-2B AECs were left untreated (-) or pretreated with (+) 5 μg/mL TLR2 and/ or 5 μg/mL TLR5 nAb for 30 min followed by exposure to wild type planktonic PAO1 PsaDM (PAO1 wt) or a PAO1 mucA mutant (PAO1 mucAtn). p38α MAPK activation was determined as previously described. The mean of three experiments is shown +/_ SEM. Statistics were performed using one-way ANOVA with Bonferroni post-test analysis. ***,**,* $p \leq 0.001$ or 0.01, respectively compared to unstimulated; #, $p \leq 0.05$ in absence of nAb compared to its presence.

A B

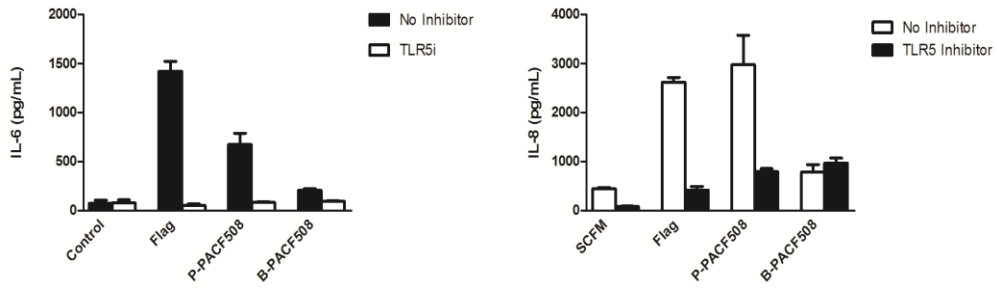


Figure 43: Pro-inflammatory cytokine production is increased in the presence of PsaDM and blocked via TLR5 neutralizing antibody.

BEAS-2B airway epithelial cells (AECs) were left untreated or pretreated with 5μg/mL of TLR5 nAb for 30 minutes before exposure to 1 μg/mL planktonic or biofilm PACF508 PsaDM grown in synthetic cystic fibrosis medium (SCFM) for 6 hours. After stimulation, the culture medium was collected, and the abundance of interleukin 6 (IL-6; A) or CXCL8 (B) was determined by enzyme-linked immunosorbent assay. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** $P \leq .001$, ** $P \leq .01$, and * $P \leq .05$, compared with control; #$P \leq .05$ for comparison between groups.

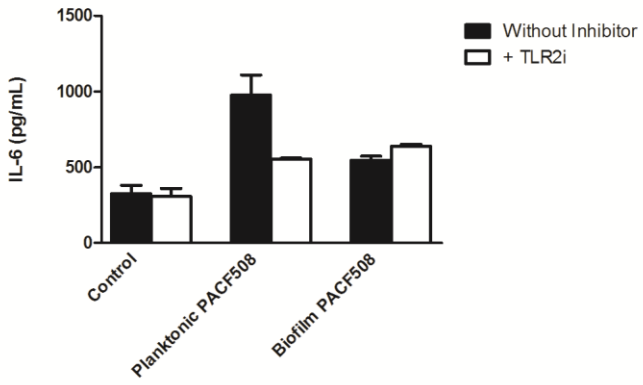


Figure 44: Pro-inflammatory cytokine production is increased in the presence of PsaDM and blocked via TLR5 neutralizing antibody.

BEAS-2B airway epithelial cells (AECs) were left untreated or pretreated with 5μg/mL of TLR2 nAb for 30 minutes before exposure to 1 μg/mL planktonic or biofilm PACF508 PsaDM grown in synthetic cystic fibrosis medium (SCFM) for 6 hours. After stimulation, the culture medium was collected, and the abundance of interleukin 6 (IL-6; A) or CXCL8 (B) was determined by enzyme-linked immunosorbent assay. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** P ≤ .001, ** P ≤ .01, and * P ≤ .05, compared with control; #P ≤ .05 for comparison between groups.

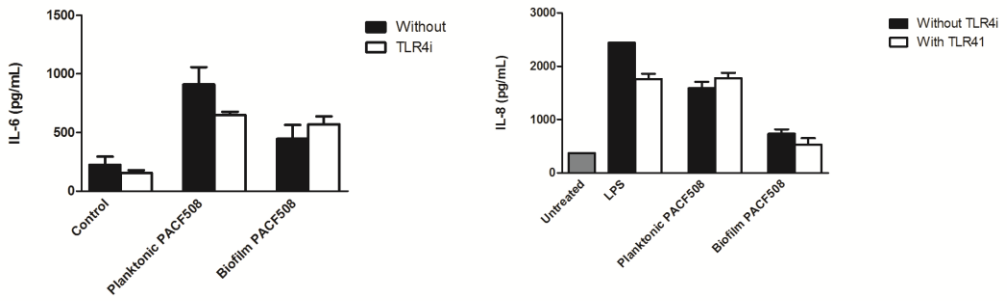
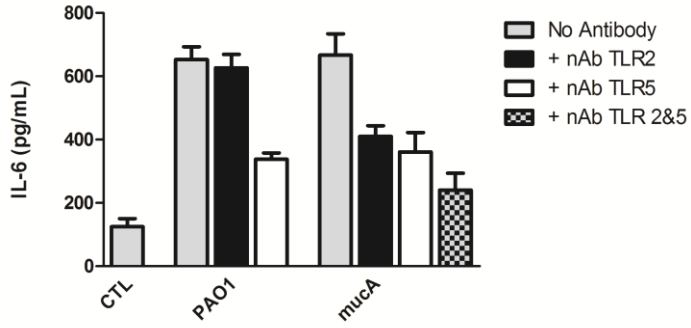


Figure 45: Blocking TLR4 has no effect on cytokine production in AECs in response to PsaDM

BEAS-2B airway epithelial cells (AECs) were left untreated or pretreated with 5μg/mL of TLR4 nAb for 30 minutes before exposure to 1 μg/mL planktonic or biofilm PACF508 PsaDM grown in synthetic cystic fibrosis medium (SCFM) for 6 hours. After stimulation, the culture medium was collected, and the abundance of interleukin 6 (IL-6; A) or CXCL8 (B) was determined by enzyme-linked immunosorbent assay. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** P ≤ .001, ** P ≤ .01, and * P ≤ .05, compared with control; #P ≤ .05 for comparison between groups.

A



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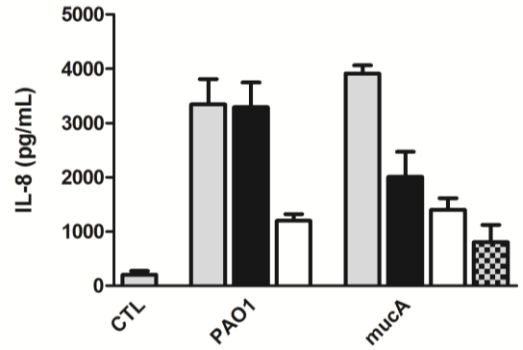

Figure 46: Increased cytokine production in response to mucoid PsaDM is blocked by TLR2 and 5 nAb

BEAS-2B airway epithelial cells (AECs) were left untreated (Grey bars) or pretreated with 5μg/mL of TLR2 (Black bars), TLR5 (White bars) or TLR2 and TLR5 (dotted bars) nAb for 30 minutes before exposure to 1 μg/mL planktonic PAO1 or *mucA* PsaDM for 6 hours. After stimulation, the culture medium was collected, and the abundance of interleukin 6 (IL-6; A) or CXCL8 (B) was determined by enzyme-linked immunosorbent assay. The mean values (± standard error of the mean) of at least 3 experiments are shown. Statistics were performed using one-way analysis of variance with Bonferroni post-test analysis. *** $P \leq .001$, ** $P \leq .01$, and * $P \leq .05$, compared with control; #$P \leq .05$ for comparison between groups.

Discussion

TLR5 leads to p38α MAPK activation in AECs exposed to planktonic

To compare the impact of the switch to a mucoid phenotype by *P. aeruginosa* on activation of host inflammatory pathway activation, two *P. aeruginosa* strains were studied: the non-mucoid common laboratory strain PAO1 and a mucoid clinical isolate of PA (PACF508). Moreover, to further simulate growth conditions relevant to pulmonary exacerbations in CF airways, the two strains were prepared as planktonic cultures in synthetic CF sputum medium (SCFM) that mimics the nutritional composition of CF sputum. Furthermore, since bacteria are found distal from airway surface, AECs likely interact and respond to diffusible bacterial product. Therefore, AECs were exposed to planktonic *P. aeruginosa* diffusible material (PsaDM) form the two strains and the activation of p38α MAPK measured in view of the critical role of this protein kinase in transmitting inflammatory signals.

Given that flagellin is recognized by TLR5 at the surface of mammalian cells and is a potent activator of p38α MAPK, we first checked the contribution of TLR5 to host activation. To test this, HEK293 cells with or without TLR5 expressed at their surface were challenged with PACF508 PsaDM. p38α MAPK activation was only detected in TLR5

expressing HEK293 cells exposed to planktonic PsaDM. This shows that like PAO1, PACF508 also activates TLR5. As would be suspected from our previous findings, activation of p38 by biofilm PsaDM was not affected by the presence of TLR neutralizing antibodies.

We next tested whether neutralizing TLR5 on AECs was sufficient to suppress p38α MAPK activation in response to planktonic PsaDM. As expected, neutralizing TLR5 prevented p38α MAPK activation in BEAS-2B AECs stimulated with flagellin in response to planktonic PsaDM from strain PAO1. This was further supported by our finding that a PAO1 mutant lacking a flagella due to a *flgK* gene mutation failed to activate p38α MAPK in BEAS-2B AECs.[353] Intriguingly, blocking TLR5 activation only reduced p38α MAPK activation by 46% in response to the planktonic PACF508 PsaDM. Therefore, in contrast to the common laboratory strain used in many studies investigating activation of host defense mechanism, a mucoid clinical isolate presents a more complex profile of activation; while flagellin-mediated TLR5-dependent mechanisms are dominant in PAO1, additional pathways contribute to p38α MAPK activation in the PACF508 clinical isolate, namely TLR2.

In addition to TLR5, TLR2 and TLR4 bind bacterial membrane constituents and activate the p38α MAPK pathways. As expected, both

TLR2 and TLR4 neutralizing antibodies prevented p38α MAPK activation by their respective ligands, FSL-1 (a synthetic TLR2/TLR6 agonist) and lipopolysaccharides (LPS). When stimulated with PACF508 PsaDM, inhibition of TLR2 reduced the p38α MAPK activation by 34% while TLR4 inhibition had a small reduction in p38α MAPK activation that was not found to be statistically significant. Again, biofilm PsaDM was unaffected by the presence of neutralizing antibodies.

These results were confirmed in two other airway epithelial cell lines, one expressing wildtype CFTR (NuLi) and one expressing the most common mutation leading to CF, CFTRDF508 (CuFi). Neutralizing TLR2 and TLR5 greatly reduced p38α MAPK activation by planktonic PACF508 PsaDM in both NuLi and CuFi AECs. It is worth noting that consistent with previous observations, p38α MAPK activation is greater in the CFTRΔF508 expressing cells.[43, 304])

The PACF508 strain is mucoid, a phenotype attributed to mutations in the mucA gene. While such mutation leads to over-production of alginate, it is also associated with increased expression of bacterial lipoproteins, which can be potent TLR2 agonists.[135] We therefore hypothesized that mucA mutations are responsible for the greater TLR2-dependent activation of p38α MAPK in mucoid strains. To test this

hypothesis, we used a PAO1-mucA-deficient strain and found that, in contrast to planktonic PsaDM from wildtype PAO1 where p38α MAPK activation was completely dependent on TLR5 and not TLR2, the mucA-deficient bacteria activated p38α MAPK in both a TLR5 and TLR2-dependent manner. Inhibition of TLR4 in addition to TLR2 and TLR5 did not further decrease p38α MAPK activation by planktonic PACF508 PsaDM. These results demonstrate that in addition to activating TLR5, mucoid strains activate TLR2, which contributes to p38α MAPK activation.

Conclusions

Overall, these results indicate that planktonic-derived material is not uniform in its capacity to stimulate host responses and that changes of bacterial gene expression modulate this response. These changes may occur at different moments in the evolution of the disease, for example during episodes of pulmonary exacerbations, where CF patients experience worsening of their respiratory symptoms.[421] The underlying mechanisms of these pulmonary exacerbations is unknown, but it has been proposed that they can be caused by the release of planktonic bacteria from the biofilm aggregates. In the planktonic form, these bacteria express flagellin, which binds and activates TLR5. Interestingly, TLR5 has been identified as a modifier gene in CF and proposed as an anti-

inflammatory target.[52, 53] Moreover, if these bacteria have mutations in their *mucA* gene, linked to a mucoid phenotype, they will also activate innate immune responses through TLR2. Figure 47 summarizes the responses that we have seen to various modes of growth for *P. aeruginosa* and the effects they elicit in airway epithelial cells in the context of CF, as described in chapters 2 and 3.

Since the presence of the mucoid phenotype of *P. aeruginosa* is a marker of poor survival in CF, our findings highlight an important and under recognized role for TLR2 in the response of airway epithelial cells to infection.[186, 334] These findings also raise the question whether mucoid *P. aeruginosa* can induce stronger pulmonary exacerbations than non-mucoid clinical isolates due to the engagement of both TLR2 and TLR5. It would also be interesting to test whether TLR2 is another CF modifier gene by itself or in combination with TLR5. Finally, in the optic of designing better treatments against mucoid infections, which are found to be deadlier in children suffering from CF, our report adds a novel avenue of decreasing lung inflammation.

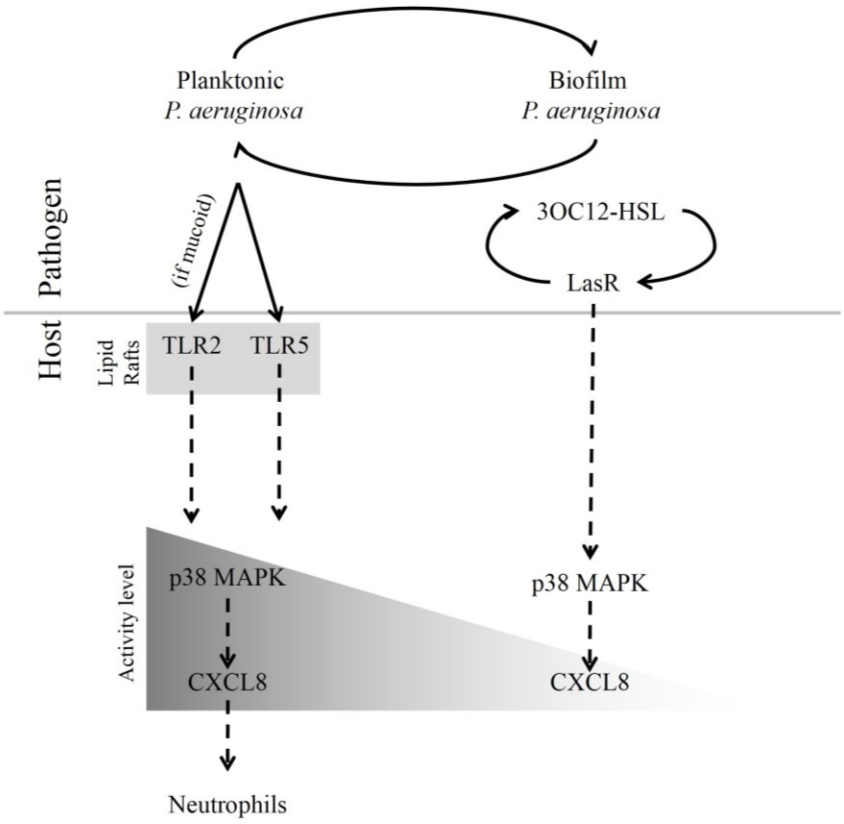

Figure 47: The level of p38α mitogen-activated protein kinase (MAPK) activation in airway epithelial cells determines the onset of innate immune responses to planktonic and biofilm microcolonies of *Pseudomonas aeruginosa.*

P. aeruginosa can be found in the airways growing as a planktonic organism or as part of organized microcolonies called biofilms. Planktonic *P. aeruginosa* diffusible material (PsaDM) induced stronger p38α MAPK activation as compared to biofilm PsaDM, via Toll-like receptor 5 (TLR5) and TLR2 found in lipid rafts. The involvement of TLR2 is associated with mucoid strains of P. aeruginosa. In contrast, biofilm PsaDM activates p38α MAPK in a TLR-independent fashion via the lasI/lasR quorum-sensing system, but this activation is insufficient to recruit neutrophils.

Acknowledgments

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Chapter 4: Roles or TLR1, TLR2 and TLR6 in response to Mucoid *Pseudomonas aeruginosa* Activation of Airway Epithelial Cells

To Be Submitted

Trevor Beaudoin, Shantelle LaFayette Dao Nguyen, Simon Rousseau

Preface

Since our previous findings indicated that TLR2 became important in activation of p38 MAPK in AEC after a switch to mucoidity in *P. aeruginosa* and this had not been previously reported, we intended to look more closely at this phenomenon. Because TLR2 can heterodimerizes with TLR1 (to recognize synthetic tri-acylated lipopeptides) or with TLR6 (to recognize synthetic di-acylated lipopeptides), we choose to create a bimolecular fluorescence complementation (BiFC) tool that would reconstitute fluorescence when the dimer pairs came in contact. To that end we made spit Venus constructs of TLR1/2/5/6. In addition, we created stably transfected HEK293 report cells lines for ATF2 (transcription factor downstream of p38) and NFκB. Thus, by transfecting the stably transfected reporter cell lines with the BiFC reporters, we could visualize TLR interactions and rapidly study the activation of p38α and NFκB to various ligands produced by mucoid and non-mucoid *P. aeruginosa*

Abstract

One of the hallmarks of chronic *Pseudomonas aeruginosa* infection in patients with Cystic Fibrosis is the presence of mucoid strains isolated from sputum. Because chronic inflammation leads to increased morbidity and mortality in these patients, we were interested in how the airway epithelium responds to this phenotypic switch in the bacteria. The airway epithelium is able to induce an inflammatory response through recognition of key bacterial components, known as pathogen associated molecular patterns (PAMP), through a number of receptors known as pathogen recognition receptors (PRR). The toll-like receptors are an important group of PRR. Because TLR 2 has been shown to be important in recognizing the bacterial PAMP lipopeptide, and because mucoid strains of Pseudomonas have been shown to upregulate lipopetide expression, we were interested in looking to further evaluate this pathway of activation. Our results have indicated that mucoid *P. aeruginosa* is able to activate both NFκB and ATF2 transcription factors mostly via a TLR2/6 dimerization.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that initiates chronic infection in the lungs of patients with CF. This infection induces a destructive cycle of increasing inflammation and lung tissue destruction. The initiation of innate immunity through activation or airway epithelium PRR is a key contributor to this process.

During the course of infection, *P. aeruginosa* isolates acquire many mutations that allow them to better adept to the CF host environment.[66, 192, 377] One major adaptation is acquisition of a ‘mucoid' phenotype, with 70% of all isolated strains from CF patients exhibiting this phenotypic change.[286] Along with acquisition of mucoidity, comes the differential regulation of different virulence genes, which can change the profile of recognition by the AEC.[135] Previous reports from our lab have shown that the switch to mucoidity by *P. aeruginosa* initiates a TLR2 response in addition to TLR5.[32] Mucoid strains show increased production of alginate and overproduction of a number of different lipoproteins that may or may not contribute to the downstream activation of various pro-inflammatory cytokine factors.[99, 141, 156]

TLR2 has been shown to heterodimerize with TLR1 or TLR6 in response to tri-acylated or di-acylated synthetic lipoproteins respectively.

Gram-negative bacteria such as *P. aeruginosa* are thought to mainly produce tri-acylated lipoproteins that are found on the outer membrane of these bacteria. Further, several studies have suggested that lipoproteins produced by mucoid *P. aeruginosa* can stimulate p38 MAPK expression and lead to increased cytokine production in AEC. [75, 77, 99, 131, 156, 254, 259, 262, 263, 285, 318] However, a detailed look at the TLR1/2/6 pathway and subsequent activation of p38 MAPK and NFκB has not yet been achieved. Because of the critical importance of *P. aeruginosa* infections in the morbidity and mortality of CF patients and the high prevalence of mucoid phenotype in these patients, we decided to elucidate the role of TLR1/2/6 signaling in the induction of an inflammatory response.

Materials and Methods

Materials

BIRB 0796 was kindly provided by Professor Sir Philip Cohen (MRC PPU, University of Dundee, UK). All chemicals were bought from Fisher Scientific (Fair Lawn, NJ, USA). FSL-1, LPS from *Pseudomonas aeruginosa* and *S. typhimurium* Flagellin were bought from Invivogen (CA, USA).

Pseudomonas aeruginosa strains

As described in Chapter 2

Bacterial Media Preparation

Bacteria were grown in 4% peptone (Fisher Chemical, Fair Lawn, NJ, USA).

P. aeruginosa diffusible material preparation

As described in Chapter 2

RNA extraction and cDNA synthesis

Total RNA was extracted using Trizol method (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol.

Cloning of Toll-like receptors

Toll-like receptors 2, 5 and 6 where cloned from total messanger RNA obtained from BEAS-2B cells as followed. Total RNA was extracted from BEAS-2B cells as described above and 0.5 µg of mRNA was amplified using the described primers (Table 9 below) using KOD hot start polymerase as per manufactures protocol (Fermentas). TLR1 was cloned from an image clone bought from (Invivogen) and amplified as above. These amplified fragments were cut using appropriate restriction enzymes, gel purified using a Fermentas kit for cloning into Venus constructs.

Table 9: TLR Cloning Primers

Primer	Sequence (5' to 3')	RS
TLR1-F	AATTGCGGCCGCCACCATGACTAGCATCTTCCATT	NotI
TLR1-R	CCCGGATCGATTTTCTTTGCTTGCTCTGTCAGC	ClaI
TLR2-F	AATTGCGGCCGCCACCATGCCACATACTTTGTGGA	NotI
TLR2-R	CCGCGGATCGATGGACTTTATCGCAGCTCTCAGAT	ClaI
TLR5-F	AATTGCGGCCGCCACCATGGGAGACC	NotI
TLR5-R	CCCGATCGATGGAGATGGTTGCTAC	ClaI
TLR6-F	AATTGCGGCCGCCACCATGACCAAAGACAAAGAAC	NotI
TLR6-R	CCGCGGATCGATAGATTTCACATCATTGTTTTCAG	ClaI

Antibodies

As described in Chapter 3

Cell lysis and Immunoblotting.

As described in Chapter 2

NFκB and ATF2 Reporter Assay

The NFκB consensus response element (GGGGACTTTCC) or ATF2 consensus response element (GTGACGTCA) was synthesized in 4

copies and cloned at the XhoI/BglII sites of the pGL4.28 vector (Promega, Madison, WI,USA). The resulting vector was transformed into DH5α bacterial strain and purified with Invitrogens Purelink maxi prep kit. (Invitrogen, Burlington, Ontario) pGL4.28NFκB or pGL4.28ATF2 was then stably transfected into HEK293 cells and maintained with DMEM as described previously with the addition of 200μg/mL of hygromycin. These cells were plated in 12 well plates (Corning) to confluency and then stimulated with agonists for 3 hours. Following stimulation, cells were washed twice with ice-cold phosphate-buffered saline, and 40 μL of reporter lysis buffer (Promega) was added per well. After 5 minutes, cells were scraped and collected into a microcentrifuge tube and spun down at 13 000 × g for 3 minutes. A total of 50 μL of supernatant was collected in a new tube and stored at −20°C. Twenty microliters of sample was used in 96-well plates for the reporter assay. A total of 25 μL of luciferase assay reagent (20 mM Tricine, 1.07 mM $(MgCO_3)\cdot 4H_2O$ $Mg(OH)_2\cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM ethylenediaminetetraacetic acid, 33 mM dithiothreitol, 270 μM coenzyme A, 0.477 mM D-luciferin, and 0.533 mM adenosine triphosphate) was added to each well, using an automatic injector. Emission units were read on a Tecan Infinite M1000 plate reader.

Transfection of HEK293 ATF2 and NFkB stable cell linses with TLR constructs.

Stably transfect HEK293 with ATF2 or NFκB reporter vector were plated at 30,000 cells per well on Puracol coated plates in 1 mL of 10% FBS, P/S DMEM with 200 μg/mL of Hygromycin and incubated overnight at 37°C and 5% CO_2 overnight to 40-60% confluency per well. Wells were then transfected with different combinations of TLR1,2,5 and 6 Venus constructs as follows. 1 μg of DNA, 2 μL of PEI reagent in 100 μL of DMEM. 0.5 μg of TLR construct was used for each well to be transfected. A mix of 1 mL per 12 well plate was made, vortexed for 10 seconds and incubated at room temperature for 30 min. 100 μl was added to each well and incubated overnight. After 16 hours, the media was removed and replaced with 0.5% FBS in DMEM for 24 hours, The cells were stimulated with PsaDM and observed under the microscope each hour for fluorescence. After three hours the total fluorescence was read on the TEACAN microplate reader, followed by cell lysis and luciferase assay as described above.

Experimental Results

Our previous work implicated that mucoid strains of *P. aeruginosa* were able to signal through TLR2 in addition to TLR5 to initiate p38 MAPK activation. Because TLR2 can interact with several other TLRs, namely

TLR1 and 6, to induce signalling, we were interested in identifying the required partner, if any, that was needed for the response to mucoid strains of *P. aeruginosa.* In order to determine the TLR2 pair involved in recognizing mucoid *P. aeruginosa*, we choose to create a series of TLR probes that contained the N terminus (502 plasmid) or C terminus (503 plasmid) of Venus fluorescent protein. Alone these probes would not fluoresce, but if the two halves came in close proximity, the protein Venus protein would fold, mature and fluoresce. In this way we could see if, in the presence of different ligands, an interaction between different TLRs would occur. Figure 48 summarizes this strategy, also known bimolecular fluorescence (BiFc). Further, we created stable ATF2 and NFκB luciferase reporter cell lines in HEK293 cells. HEK 293 cells express low levels of TLR1,2 and 6 and are deficient in TLR5. Thus, by transfecting the TLR probes into these cells and stimulating with different ligands we would have a functional response to stimulation with our various ligands.

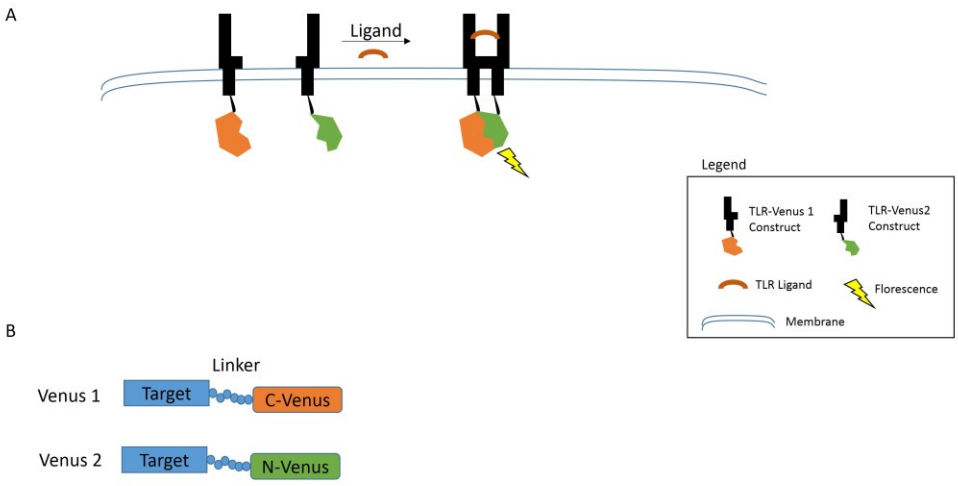

Figure 48: Bimolecular Fluorescence Complementation Assay Strategy

Bimolecular fluorescence complementation (BiFC) for strategy for toll-like receptors. Venus spilt constructs were created on the N-terminus of different TLR pairs. Upon ligand binding, the TLRs come in close contact, allowing the Venus protein to fold and inducing fluorescence at specific wavelengths (A). The target proteins (different TLR) were attached to the N and C terminus of Venus protein respectively, and separated by a 12 amino acid linker (B)

In order to confirm our previous findings and to test the probes, we transfected HEK293 cells with the TLR5-502 and TLR5-503 plasmid constructs. As can be seen in Figure 49A and B, the TLR5 probes are able to reconstitute fluorescence in the presence of PsaDM derived from planktonic PACF508 or in the presence of flagellin. Transfected cells left untreated had only low, background levels of fluorescence. In addition, this fluorescence could be read from a plate reader, and there was a much greater signal in the PsaDM and flagellin treated wells then from the untreated wells or transfected HEK 293 cells (Figure 49C).

In order to determine if these probes were functional and capable of inducing downstream signals, we transfected them into the HEK293 ATF2 and NFκB reporter cells. As can be seen by the western blot in Figure 50 A, HEK293 cells transfected with TLR5 were able to respond to PsaDM and to flagellin by increasing p38 phosphorylation, while untransfected HEK 293 cells were unresponsive. This was blocked in the presence of a TLR5 neutralizing antibody (nAb).

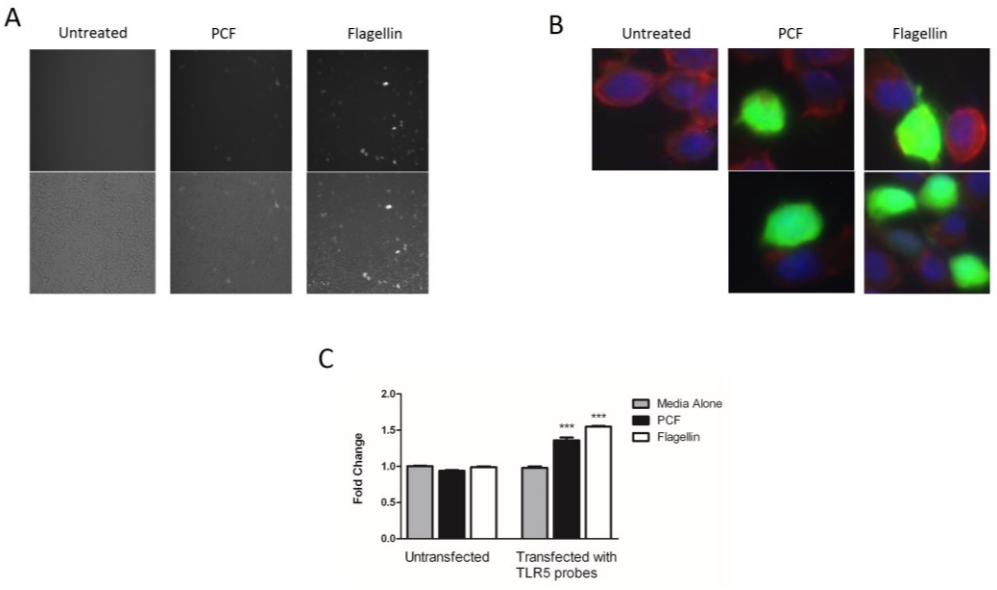

Figure 49: Mucoid *Pseudomonas aeruginosa* activates TLR5

HEK293 cells were transfected with TLR5-NVenus (N terminus of Venus YFP) and TLR5-CVenus (C-terminus of Venus YFP) constructs and left untreated or treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours and then visualized at 400X magnification using a fluorescent microscope laser (A) and luminescence was read (C). HEK293 cells were transfected with TLR5-NVenus (N terminus of Venus YFP) and TLR5-CVenus (C-terminus of Venus YFP) constructs and left untreated or treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 2 hours, followed by fixation and staining with Hoechst (Blue) and Phalloidin (Red) dyes. Cells were then visualized at 400X magnification using a fluorescent microscope laser (B).

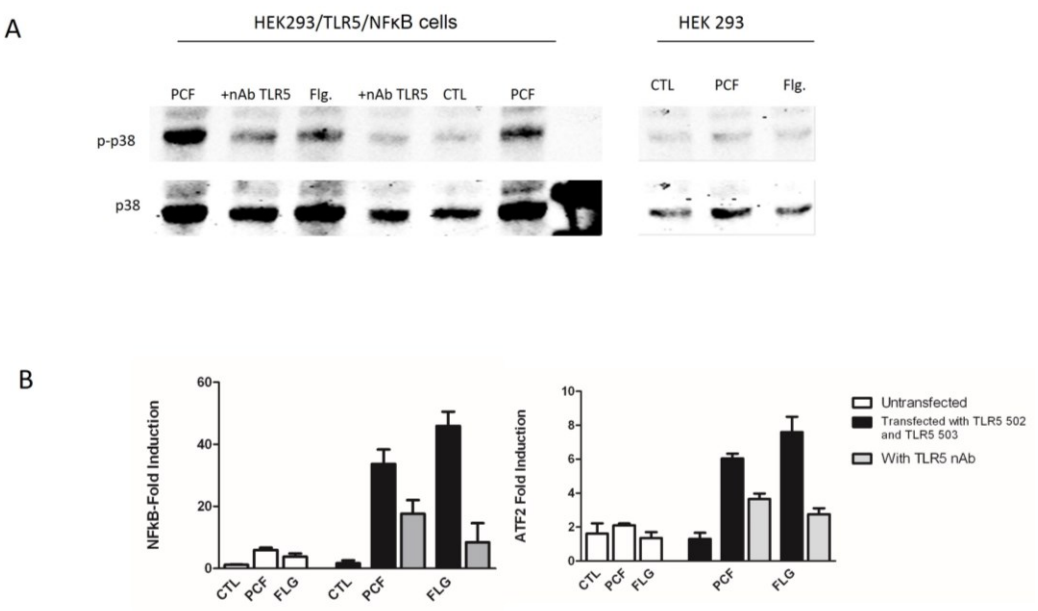

Figure 50: Mucoid *P. aeruginosa* activates TLR5 signaling to induce NFκB and ATF2

HEK293 cells were transfected with TLR5-NVenus (N terminus of Venus YFP) and TLR5-CVenus (C-terminus of Venus YFP) constructs and left untreated or were pretreated with 5 μg/mL TLR5 nAb for 30 minutes. Cells were then treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 30 minutes. p38α MAPK activation was determined as previously described.(A) Stably transfected HEK293 cells with NFκB or ATF2 reporter construct were transfected without or with TLR5 and treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours. The mean and SEM of 3 experiments are shown.

In addition, the NFκB and ATF2 reporter cells produce significantly more signal when treated with PsaDM or flagellin compared to untreated or untransfected control, and this increase was abrogated in the presence of a TLR5 nAb. Taken together, these results support that TLR5 homodimerizes in the presence of its ligand and this can be visualized using these probes. Furthermore, the functional assay of TLR signaling to induce NFκB and ATF2 activation was confirmed.

Similar results are shown for the transfection of TLR2/6 probe pairs in HEK 293 cells. Figure 51A shows that these probe pairs can produce fluorescence in HEK293 cells in the presence of a mucoid strain of *P. aeruginosa*, but this is blocked by addition of a TLR6 nAb. Further, p38 phosphorylation as well as ATF2 and NFκB activation is driven by TLR2/6 and can be completely abrogated by TLR2 nAb and partially blocked via TLR6 nAb. Interestingly, TLR6 nAb only partially blocks ATF2 and NFκB to mucoid PsaDM, suggesting a possible role for TLR1 or TLR2 homodimers in this activation.

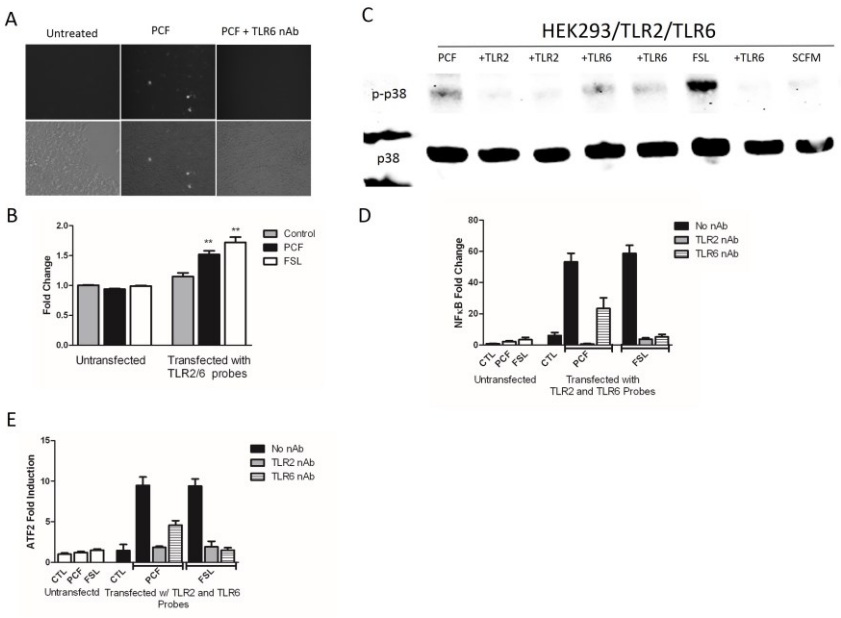

Figure 51: Mucoid *P. aeruginosa* activates p38α MAPK, NFκB and ATF2 in a TLR2/6 dependent manner.

HEK293 cells were transfected with TLR2-NVenus (N terminus of Venus YFP) and TLR6-CVenus (C-terminus of Venus YFP) constructs and left untreated or treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours and then visualized at 400X magnification using a fluorescent microscope laser (A) and luminescence was read (B). HEK293 cells were transfected with TLR2-NVenus (N terminus of Venus YFP) and TLR6-CVenus (C-terminus of Venus YFP) constructs and left untreated or were pretreated with 5 μg/mL TLR2 or TLR6 nAb for 30 minutes. Cells were then treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 30 minutes. p38α MAPK activation was determined as previously described (C). Stably transfected HEK293 cells with NFκB (D) or ATF2 (E) reporter construct were transfected without or with TLR2 and TLR6 and then pretreated with or without TLR2 or 6 nAb for 30 minutes. Cells were then treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours. The mean and SEM of 3 experiments are shown.

HEK293 cells transfected with TLR6-502 and TLR6-503 show increased fluorescence in both untreated and PCF PsaDM treated conditions that can be abrogated by TLR6 nAb (Figure 52A and B). Despite this increase in fluorescence, there is no increase in p38 phosphorylation, NFκB or ATF2 expression (Figure 52C, D and E respectively). This would indicate that TLR6 probes are able to make homodimers in HEK293 cells, but despite this, they do not seem to activate p38, ATF2 or NFκB.

When HEK293 cells were transfected with TLR1 502 and TLR2 503 probe pairs, little increase in fluorescence was observed (Figure 53A and B). Both NFκB (Figure 53C) and ATF2 (Figure 53D) showed an increase in activation compared to the untransfected condition. However, this activation was blocked by the presence of TLR 2nAb, but not be the presence of TLR1 nAb. This would suggest that TLR2 homodimers, or TLR2 and endogenously expressed TLR6 in HEK293, were responsible for the activation of NFκB and ATF2.

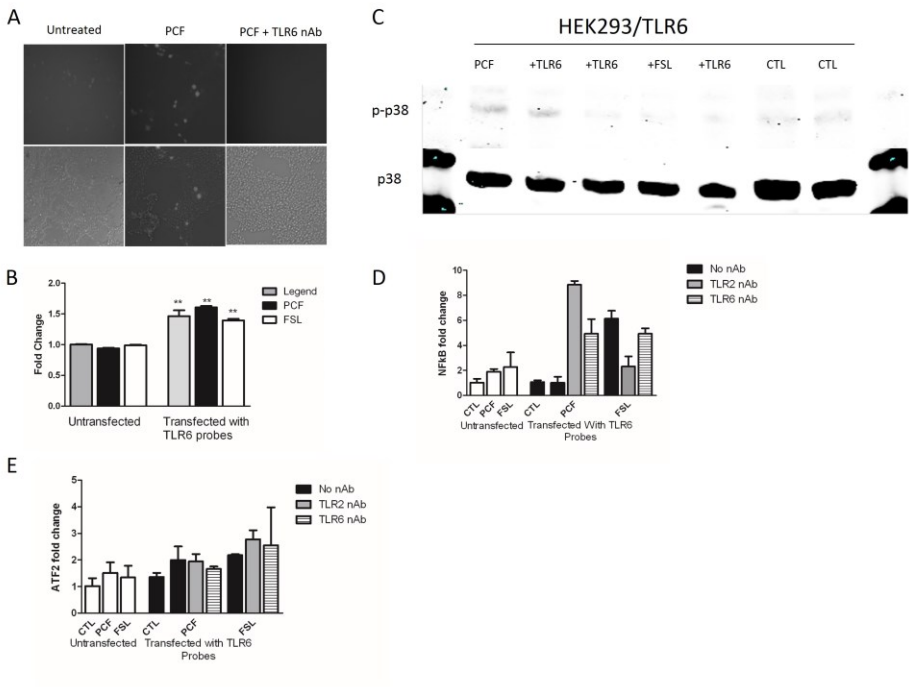

Figure 52: TLR6 homodimers do not activate p38α MAPK, NFκB or ATF2 in the presence of mucoid *P. aeruginosa*

HEK293 cells were transfected with TLR6-NVenus (N terminus of Venus YFP) and TLR6-CVenus (C-terminus of Venus YFP) constructs and left untreated or treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours and then visualized at 400X magnification using a fluorescent microscope laser (A) and luminescence was read (B). HEK293 cells were transfected with TLR6-NVenus (N terminus of Venus YFP) and TLR6-CVenus (C-terminus of Venus YFP) constructs and left untreated or were pretreated with 5 μg/mL TLR6 nAb for 30 minutes. Cells were then treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 30 minutes. p38α MAPK activation was determined as previously described (C). Stably transfected HEK293 cells with NFκB (D) or ATF2 (E) reporter construct were transfected without or with TLR6 and then pretreated with or without TLR6 nAb for 30 minutes. Cells were then treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours. The mean and SEM of 3 experiments are shown.

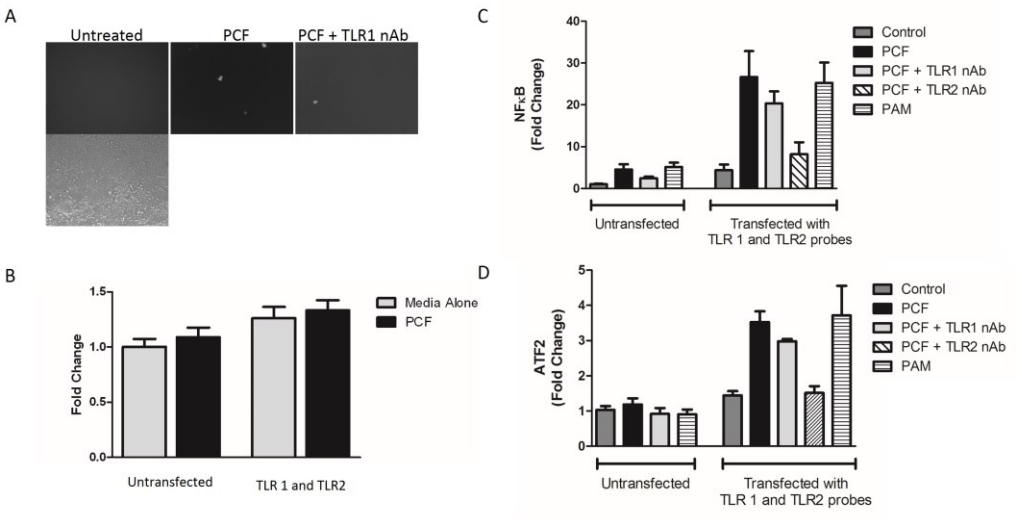

Figure 53: TLR1/2 does not activate p38α MAPK, NFκB or ATF2 in the presence of mucoid *P. aeruginosa*

HEK293 cells were transfected with TLR1-NVenus (N terminus of Venus YFP) and TLR2-CVenus (C-terminus of Venus YFP) constructs and left untreated or treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours and then visualized at 400X magnification using a fluorescent microscope laser (A) and luminescence was read (B). HEK293 cells were transfected with TLR1-NVenus (N terminus of Venus YFP) and TLR2-CVenus (C-terminus of Venus YFP) constructs and left untreated or were pretreated with 5 μg/mL TLR1 or TLR2 nAb for 30 minutes. Cells were then treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 30 minutes. p38α MAPK activation was determined as previously described (C). Stably transfected HEK293 cells with NFκB (D) or ATF2 (E) reporter construct were transfected without or with TLR1 and TLR2, pretreated with or without TLR6 nAb for 30 minutes. Cells were then treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours. The mean and SEM of 3 experiments are shown.

No fluorescence or activation of NFκB and ATF2 was seen in cells transfected with TLR 1 and 6 probe pairs. There is no presence of fluorescence when HEK293 were transfected with TLR 1and TLR6 constructs (Figure 54A and B) and no activation of ATF2 and NFκB (Figure 54C and D) occurs through these receptors. Figure 55 shows an overview of PCF stimulation of NFκB and ATF2 via PCF PsaDM in HEK293 transfected with various TLRs combinations.

In order to test whether or not the switch to a mucoid phenotype was important in TLR2/6 recognition, we used PAO1 (non-mucoid laboratory strain) and a *mucA* mutant strain, as previously described. Both of these strains were able to initiate signaling in HEK293 cells transfected with our TLR5 probe pairs, as seen in Figure 56. Additionally, TLR2_6 was activated by *mucA* mutants but not PAO1 (Figure 57), while TLR1_2 was not activated by either strain (Figure 58).

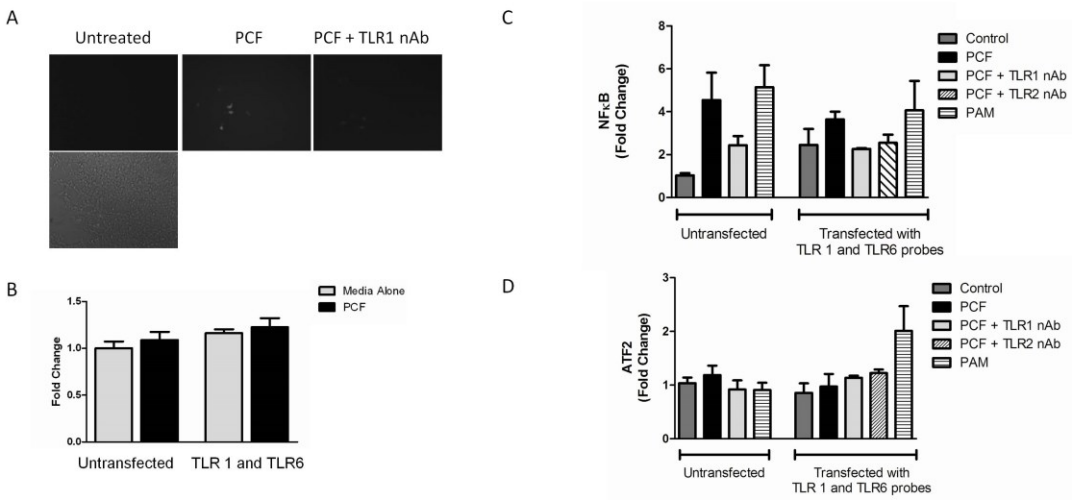

Figure 54: TLR1/6 does not activate p38α MAPK, NFκB or ATF2 in the presence of mucoid *P. aeruginosa*

HEK293 cells were transfected with TLR1-NVenus (N terminus of Venus YFP) and TLR6-CVenus (C-terminus of Venus YFP) constructs and left untreated or treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours and then visualized at 400X magnification using a fluorescent microscope laser (A) and luminescence was read (B). Stably transfected HEK293 cells with NFκB (C) or ATF2 (D) reporter construct were transfected without or with TLR1 and TLR6, pretreated with or without TLR 1 or TLR6 nAb for 30 minutes. Cells were then treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours. The mean and SEM of 3 experiments are shown.

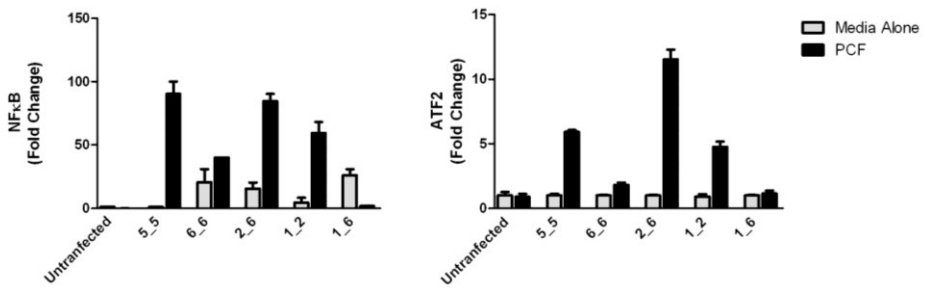

Figure 55: Overview of NFκB and ATF2 expression by mucoid *P. aeruginosa* via TLR1,2,5 and 6 pathways

HEK293 cells were transfected with TLRX-N Venus (N terminus of Venus YFP) and TLRY-C Venus (C-terminus of Venus YFP) constructs (depicted as X_Y on graph above) and left untreated or treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours and then visualized at 400X magnification using a fluorescent microscope laser (A) and luminescence was read (B). Stably transfected HEK293 cells with NFκB (C) or ATF2 (D) reporter construct were transfected without or with TLR1 and TLR6, pretreated with or without TLR 1 or TLR6 nAb for 30 minutes. Cells were then treated with 1μg/mL of planktonic PACF508 or 0.4 μg of flagellin for 3 hours. The mean and SEM of 3 experiments are shown.

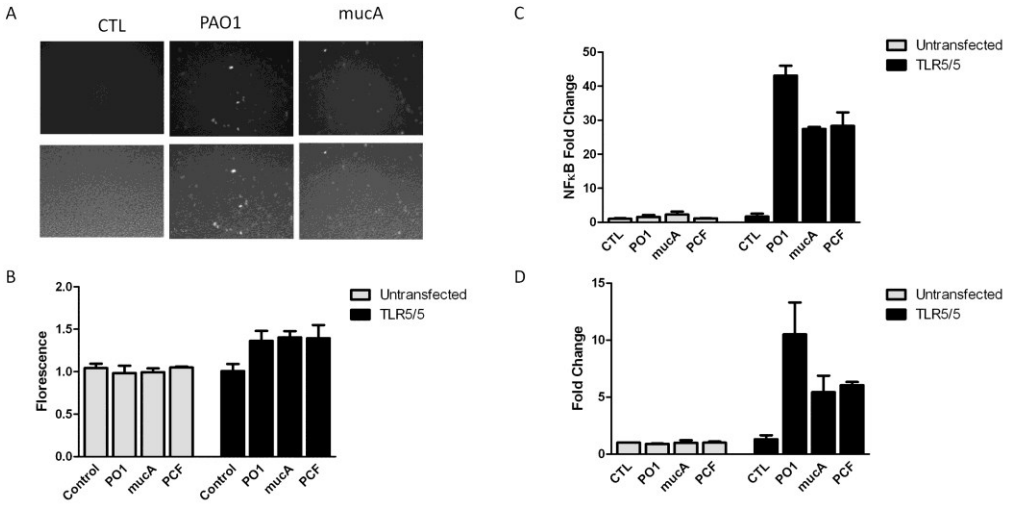

Figure 56: Activation of NFκB and ATF2 by mucoid and non-mucoid *P. aeruginosa* via TLR5

HEK293 cells were transfected with TLR5-NVenus (N terminus of Venus YFP) and TLR5-CVenus (C-terminus of Venus YFP) constructs and left untreated or treated with 1μg/mL of planktonic wildtype PAO1, a mucA mucoid PAO1 (mucA) or PACF508 (PCF) for 3 hours and then visualized at 400X magnification using a fluorescent microscope laser (A) and luminescence was read (B). Stably transfected HEK293 cells with NFκB (C) or ATF2 (D) reporter construct were transfected without or with TLR5. Planktonic wildtype PAO1, a mucA mucoid PAO1 or PACF508 for 3 hours. The mean and SEM of 3 experiments are shown.

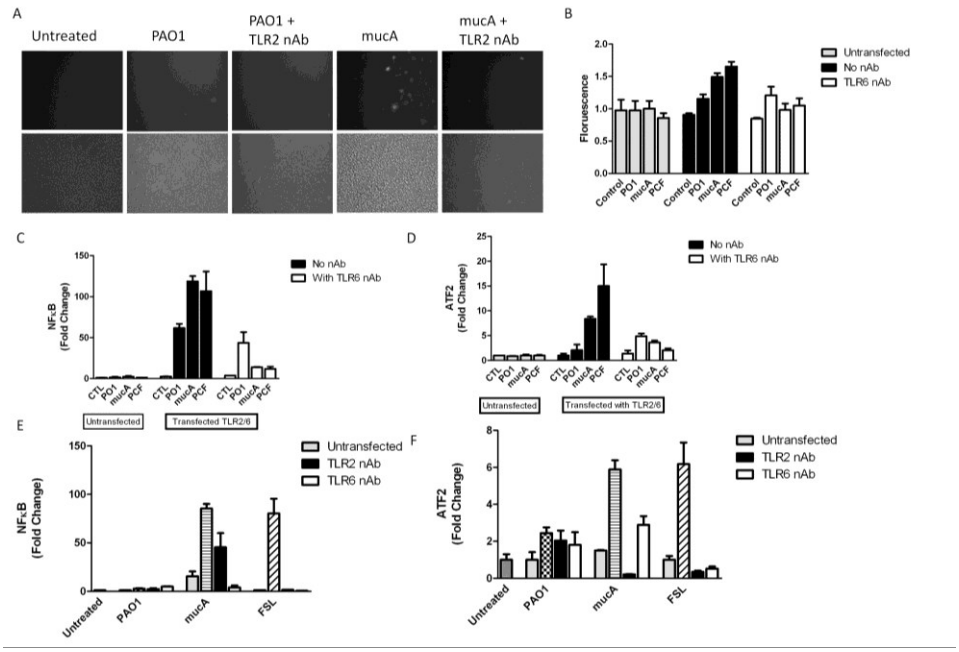

Figure 57: Activation of NFκB and ATF2 by mucoid and non-mucoid *P. aeruginosa* via TLR2/6

HEK293 cells were transfected with TLR2-NVenus (N terminus of Venus YFP) and TLR6-CVenus (C-terminus of Venus YFP) constructs and left untreated or pretreated with TLR6 nAb. Cells were then treated with 1μg/mL of planktonic wildtype PAO1, a mucA mucoid PAO1 (mucA) or PACF508 for 3 hours and then visualized at 400X magnification using a fluorescent microscope laser (A) and luminescence was read (B). Stably transfected HEK293 cells with NFκB (C) or ATF2 (D) reporter construct were transfected without or with TLR2 and TLR 6. Cells were pretreated with or without 5μg/mL of TLR6 nAb before being treated with planktonic wildtype PAO1, a mucA mucoid PAO1 or PACF508 for 3 hours.

Stably transfected HEK293 cells with NFκB (E) or ATF2 (F) reporter construct were transfected without or with TLR2 and TLR 6. Cells were pretreated with or without 5μg/mL of TLR2 or TLR6 nAb before being treated with planktonic wildtype PAO1, a mucA mucoid PAO1 or 0.4μg of FSL for 3 hours. The mean and SEM of 3 experiments are shown.

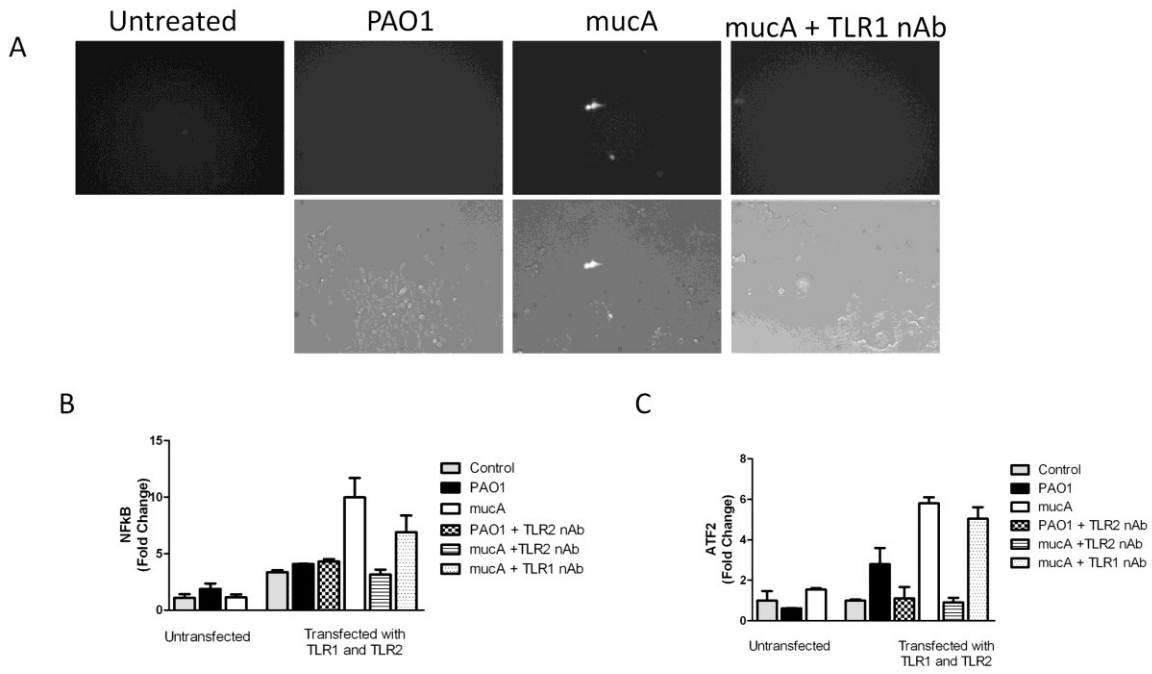

Figure 58: Activation of NFκB and ATF2 by mucoid and non-mucoid *P. aeruginosa* via TLR1 and TLR2

HEK293 cells were transfected with TLR1-NVenus (N terminus of Venus YFP) and TLR2-CVenus (C-terminus of Venus YFP) constructs and left untreated or pretreated with TLR1 nAb. Cells were then treated with 1μg/mL of planktonic wildtype PAO1 or a mucA mucoid PAO1 (mucA) for 3 hours and then visualized at 400X magnification using a fluorescent microscope laser (A). Stably transfected HEK293 cells with NFκB (B) or ATF2 (C) reporter construct were transfected without or with TLR1 and TLR2. Cells were pretreated with or without 5μg/mL of TLR1 or TLR2 nAb before being treated with planktonic wildtype PAO1 or a mucoid PAO1 (mucA) or PACF508 for 3 hours. The mean and SEM of 3 experiments are shown.

In order to confirm some of the results seen using the heterologous expression system, we used an immortalized bronchial airway epithelial cell line, BEAS-2B stimulated without filtrates. Using neutralizing antibodies to TLR6, but not TLR1, we are able to effectively abolish activation of p38 MAPK phosphorylation in response a clinical mucoid isolate of *P. aeruginosa*, as can be seen in Figure 59. Similarly, non-mucoid PAO1 was unaffected by either neutralizing antibody, while the mucA mutant was effectively blocked by TLR6 or TLR 2 neutralizing antibodies as seen in Figure 60.

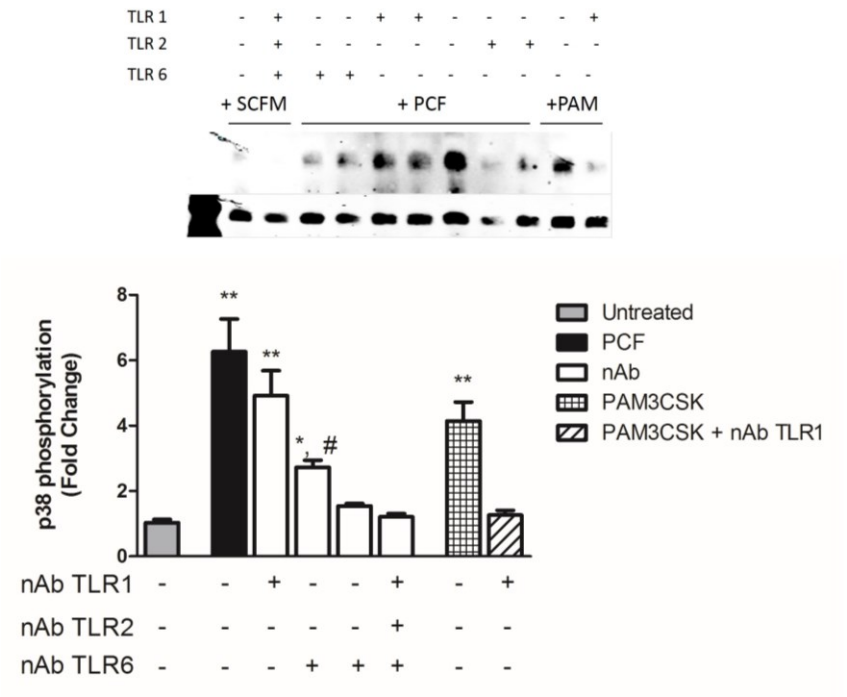

Figure 59: Blocking TLR2 or TLR6 but not TLR1 reduces p38α MAPK phosphorylation by Mucoid *Pseudomonas. Aeruginosa*

BEAS-2B AECs were left untreated (-) or pretreated with (+) 5 μg/mL TLR1, TLR2 or TLR6 nAb for 30 min followed by exposure to media alone (SCFM), planktonic PACF508 PsaDM (PCF) or PAM3CSK. p38α MAPK activation was determined as previously described. The mean of three experiments is shown +/_ SEM. Statistics were performed using one-way ANOVA with Bonferroni post-test analysis. ***,**,* $p \leq 0.001$ or 0.01, respectively compared to unstimulated; #, $p \leq 0.05$ in absence of nAb compared to its presence.

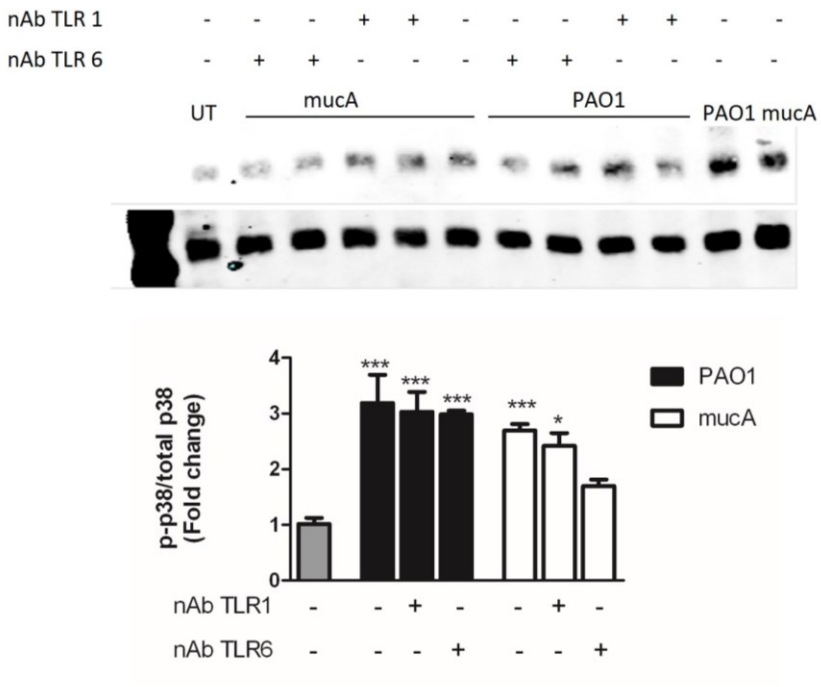

Figure 60: Mucoid strains of PAO1, but not wildtype, activate p38α MAPK via TLR6

BEAS-2B AECs were left untreated (-) or pretreated with (+) 5 μg/mL TLR1 or TLR6 for 30 min followed by exposure to wild type planktonic PAO1 PsaDM (PAO1) or a PAO1 mucA mutant (mucA). p38α MAPK activation was determined as previously described. The mean of three experiments is shown +/_ SEM. Statistics were performed using one-way ANOVA with Bonferroni post-test analysis. ***,**,* $p \leq 0.001$ or 0.01, respectively compared to unstimulated; #, $p \leq 0.05$ in absence of nAb compared to its presence.

Discussion

The majority of isolates of *Pseudomonas aeruginosa* extracted from patients with chronic infection is CF exhibit a mucoid phenotype. The high prevalence of this mutation is thought to be a protective mechanism by *P. aeruginosa* within the host in order to protect the bacteria from reactive oxygen species and phagocytosis by the host immune system.[161] The switch to mucoidity does, however, affect the virulence of the *P. aeruginosa* strain.[135, 299] And while mucoid strains have been shown to have a different virulence capacity, and differentially expresses numerous potential TLR ligands, there are very few studies looking at how the switch to mucoidity affects activation of the inflammatory response in AEC. Here we show, using a heterologous expression system as well as bronchial epithelia cell lines, that material produced from mucoid strain of *Pseudomonas aeruginosa* are able to activate p38α MAPK and NFκB through TLR2-6 heterodimers or TLR2 homodimer signaling.

The studies performed indicate an additional role of TLR2 involvement in the switch from non-mucoid to a mucoid phenotype. We have shown that activation of p38α by a non-mucoid laboratory strain of *P. aeruginosa* occurs exclusively through the TLR5/flagellin pathway. Upon a switch to a mucoid phenotype, TLR2 and TLR6 signaling become

important for p38α and NFκB activation.[32] Importantly, there is not a dramatic increase in p38α activation, despite the fact that these additional pathways are now involved (TLR2, TLR2/6 and TLR5). This could be due to the fact that mucoid *P. aeruginosa* often down-regulates flagellin as supported by some reports in the literature, and possibly indicates the redundancy of the pathways.[305] Further, the presence of TLR6 homodimers can be seen in our results, however the activation of p38α MAPK or NFκB does not seem to occur through these complexes. Whether this is an artifact, or there is a physiological role for these pairs (sequester TLR6 to allow more TLR2 homodimers/ make other interactions more favorable) will have to be investigated further.

Other labs have shown that clinical isolates that are non-mucoid still have a component that is able to activate TLR2.[262, 263] This led us to believe that one of the gene products that is up-regulated in mucoid *P. aeruginosa* might be up-regulated in these non-mucoid clinical isolates that are activating TLR2. Additionally, since TLR2 can heterodimerize with TLR1 and TLR6 and possible homodimerize, we wanted to elucidate which signaling partner was important for *P. aeruginosa.*[131] In order to test this hypothesis, we created tools to look at TLR dimerization

(Bimolecular fluorescence complementation) and downstream signaling pathways (NFκB reporter assay and ATF2 reporter assay for p38 function). We have validate this system using TLR5 constructs with flagellin and our filtrates. Our results show the importance of TLR5 coupling and signaling in the activation of p38αand NFκB. Using our constructs to test TLR 1,2 and 6 interactions with mucoid *P. aeruginosa*, our results indicate that mucoid strains are able to interact with TLR2/6 heterodimers and maybe TLR2 homodimers to drive NFκB and ATF2 activation.

Past reports had indicated that gram-negative bacteria produce tri-acyl lipopeptides and would thus signal through TLR1/2 homodimers, while gram-positive bacteria would form di-acyl lipopeptides and signal through TLR2/6 receptor dimers. [466, 518] Recent evidence has shown that gram-positive bacteria, such as *S. aureus*, can modulate there lipoprotein expression depending on their environmental conditions, producing tri-acyl lipopeptides in certain environments. A look at the biosynthetic pathway of *P. aeruginosa* doesn't preclude the production of di-acyl lipopeptides, and it is our contention that mucoid strains could potentially release di-acyl components under certain conditions. However, more studies must be done to confirm our early observations.

Interestingly, others have reported that the switch to a mucoid phenotype can induce the upregulation of lipopeptide genes that have a capacity to activate NFκB through TLR2.[135] The study by Fivored et al. showed the key role of a specific lipopeptide, derived from the LptA gene in inducing NFκB via TLR2. Other studies have implicated 7 key lipopeptide genes that are upregulated in mucoid *P. aeruginosa*, or when the bacteria come in contact with AEC. Our results suggest this modulation could have a great impact on the ability of the AEC to imitate a host immune response via TLR2. Our final set of experiments will use over expression of individual components of mucoid *P. aeruginosa* that are likely candidates for TLR2 activation (i.e. Over express the individual lipopeptides that are up regulated in the mucoid strain, as determined by previous microarray work, to see if an individual lipoprotein is responsible). If a single lipopeptide structure produced by the mucoid strains, or the general lipopeptide pathway can be identified as a key contributor to the inflammation seen in CF, these could be a potential avenue for anti-virulence strategies in treating *P. aeruginosa* infections in CF.

Chapter 5: General Discussion and Conclusions

Summary of Findings and Original Contributions

The purpose of this thesis was to identify how the airway epithelium is able to distinguish between different modes of bacterial growth that are commonly observed in chronic infection states, such as in CF patients. Particularly we were interested in looking at how planktonic and biofilm bacteria are distinguished, and if there was a differences in sensing mucoid vs. non-mucoid strains of bacteria. Both of these phenotypic switches are commonly observed in *Pseudomonas aeruginosa* found in the lungs of chronically infected CF patients.

In our first study, we were able to show that p38α MAPK is activated to a greater extent in normal airway epithelial cells by planktonic grown bacteria as compared to biofilm grown material. Importantly this lead to greater increase in pro-inflammatory cytokine production that lead to a greater capacity of the conditioned media to attract neutrophils. This occurred in both cell culture and ex-vivo nasal polyps tissue explant cultures. Our data suggest that there is a threshold level of p38α activation that is required to induce sufficient cytokine production to elicit neutrophil recruitment, a threshold that is only overcome by planktonic material in normal cells. Importantly, we also showed that biofilm material is able to induce increased p38α activation and lead to increased neutrophil

recruitment in CF cell lines. This would suggest that both planktonic and biofilm infections can contribute to the observed increase in cytokine production and neutrophil recruitment capacity of airway epithelial cells.

The second study focused on the ability of non-mucoid vs. mucoid *P. aeruginosa* to activate p38α MAPK. We show here that non-mucoid strains activate p38 in a distinct manner from mucoid strains. Namely, non-mucoid *P. aeruginosa* activates p38 α activation and cytokine production exclusively through TLR5, whilst mucoid strains activate p38 partially through TLR2 and partially through TLR5. In addition, we have shown that biofilm material signals through a non-TLR dependent pathway.

Our final study involves looking at the TLR interaction partners amongst TLR1/2/6 that are important for activating ATF2 and NFκB in response to mucoid and non-mucoid *P. aeruginosa.* Our goal was to develop tools to rapidly assess the TLR interacting partners (using BiFC) and downstream signaling (stable ATF2 and NFκB reporter HEK293 cell lines). Using these tools, we showed that TLR2/6, but not TLR1/2, heterodimers are activating ATF2 and NFκB in response to mucoid *P. aeruginosa*, a finding that is particularly interesting considering that TLR1/2 partners was initially predicted to be involved. In the future, we

plan on overexpressing individual lipopeptides (the ligands for TLR2/6) in non-mucoid *P. aeruginosa* to see if a single lipopeptide could be responsible, and thus might be an attractive virulence factor to target therapeutically. Thus, the original contributions of this thesis are summarized below:

(1) There exists a threshold of p38α MAPK activation required to trigger engage host defense mechanisms.

(2) Planktonic PsaDM induces a stronger p38α MAPK activation as compared to biofilm PsaDM.

(3) Biofilm PsaDM activates p38α MAPK via the product of the lasR gene distinct from lasI. Biofilm material (either mucoid or non-mucoid strains) activate p38α in a non-TLR dependent fashion.

(4) In AECs that do not express functional CFTR, the threshold of p38α MAPK activity required to recruit neutrophils is lower than that for their non-CF counterparts.

(5) Non-mucoid strains of *P. aeruginosa* activate AEC via a TLR5 pathway exclusively.

(6) Mucoid P. aeruginosa is able to activate AEC p38α via TLR2 pathway in addition to TLR5

(7) TLR2/6 heterodimers are activated by mucoid *P. aeruginosa* leading to ATF2 and NFκB activation.

General Discussion

This thesis elucidates how airway epithelial cells are able to identify various bacterial components and contribute to the overall inflammatory response seen in patients with Cystic Fibrosis. We aimed to discuss how common phenotypic switches exhibited by bacteria are able to be recognized by the airway epithelium and identify pathways leading to increased inflammation.

Bacterial Biofilms and Airway Inflammation

The chronic infections of *P. aeruginosa* found within the CF lung have been shown to be a result of biofilm-based infections for many years now.[96, 381, 441] Despite this, little has been shown on how the biofilm actually activates airway epithelial cells. Part of the problem is that bacteria will quickly kill epithelial cells, even those produced at an air-liquid interface.[320] This has limited researchers in their ability to study these interactions. Instead, they have been forced to use purified ligands, such as glycoprotein slime layers or 3-oxo-c12-homoserine lactone (HSL), to stimulate the AEC.[116, 304, 536] In our work, we used secreted materials from both planktonic and biofilm grown bacteria to stimulate the AEC. The

prevailing theory of infection is that the bacteria form biofilms that are trapped in a thick mucus layer, distal from the AEC themselves.[93] And so using bacterial derived products that could diffuse through this layer was a novel approach to studying the problem.

As described in Chapter 2, planktonic and biofilm derived materials elicit different levels of p38α MAPK activation, resulting in differential secretion of pro-inflammatory cytokines. Planktonic bacteria are able to overcome the necessary threshold of activation of p38α αactivation to drive inflammation in normal airway epithelial as well as CF airway epithelial, while biofilm material is only sufficient to drive inflammation in the context of CF. Together our data indicates that the therapeutic targets against *P. aeruginosa* are different between the planktonic and biofilm context of the infection. Importantly, targeting the HSL pathway in chronic Pseudomonas infections in CF lungs might be a valuable tool in treating *P. aeruginosa* driven inflammation.[210] Anti-quorum sensing compounds are being currently being developed to treat biofilm based infections, and our data suggest this could be useful in CF patients in conjunction with standard antibiotic treatments.[328]

Since the start of our project, at least two other studies have used similar methods to produce biofilm-derived materials to look at biofilm

activation of AEC. One studied looked at *S. aureus* activation of MAPK in keratinocytes.[430] These studies showed similar results that planktonic derived material drove more cytokine production and inflammation then biofilm derived material. Interestingly they speculated that biofilm material actually prevented p38 MAPK activation (reduced from baseline), a result that we did not find. However, this was a different pathogen and a different cell line so the results are not directly comparable. The second study used biofilm-derived material from *P. aeruginosa* to look at AEC stimulation in order to find a potential 'receptor' for the biofilm derived products. This study concluded that HSL produced from the biofilms was interacting with the T2R38 bitter taste receptor to drive cytokine production.[274, 275] Our data suggested that HSL as well as bacterial products that are under control of this system can drive cytokine production. More study on how inhibitors of this bacterial pathway affect AEC driven inflammation seems warranted.

Ultimately, understanding how biofilm material triggers an immune response should guide us as to how these infections should be handled. It is clear in the CF case, that both biofilm and planktonic bacteria are able to trigger an immune response. Thus, the eradication of *P. aeruginosa* infection may have a desirable effect. Currently, biofilms infections are

very difficult to eradicate due to increased resistance to the typical antibiotic regimens.[293] Identifying anti-biofilm therapeutics, in combination with standard treatment for planktonic bacteria, might lead to an increased success in treating the chronic infections CF patients.

Treatment of biofilms in the context of a non-CF host is less clear. In our studies, biofilms were able to stimulate p38α MAPK in non-CF cells, but not to an extent that drastically increased pro-inflammatory cytokine production or neutrophil recruitment. Other sites are prone to biofilm infection including the urinary tract, the ear (otitis media), implanted surgical devices, burn wounds etc. How biofilms interact with the local environment to initiate inflammation in these situations needs to be studied more in depth before conclusions can be made. Specifically targeting virulence molecules that are produced by the bacterial biofilms may be a beneficial strategy, even more so in an era of rapid antibiotic resistance. By reducing the selective pressure on the bacteria, it might be possible to reduce the inflammatory effects of the bacteria without developing further resistance to antibiotics. Using p38α as a marker to identify potential virulence factors produced by *P. aeruginosa* biofilm material is an attractive option to explore.

While our data shows the importance of switching from planktonic to biofilm in the virulence capacity of *P. aeruginosa* on AEC, several limitations exist and further experiments can be derived. Firstly, the *in vitro* system is less complex then would be optimal. While our *ex vivo* polyp system confirms some of the results shown, particularly in a 'non-CF AEC' context, cell lines can introduce artifacts and induce changes in the cells themselves. Further, the CF cell lines were not genetically matched and the effect of CFTR on the effect of the planktonic to biofilm switch can only be minimally suggested.[167] While several other CF cell lines showed similar results, a better CF system is needed. Also, many factors affect the expression of virulence genes within planktonic and biofilm states, and so growing our biofilms in the way that we did, may or may not reflect all virulence genes that would be expressed during chronic infections. Despite this, the type of experiments reported in this thesis are a needed first attempt to describe how the differences between planktonic and biofilm materials are sensed by their environment (herein the airway epithelium). Now that a difference has been identified and a pathway of importance found (p38α MAPK), further systems, including potentially an *in vivo* model could be used to add complexity to the situation. Furthermore, identifying the key virulence factors that are different

between the planktonic and biofilm filtrates through 2D-gel electrophoresis and mass spectrometry is of interest. An interesting study looking at the differences temporal expression of the proteome as *Pseudomonas aeruginosa* goes from planktonic to biofilm state.[357] This study identified 68 proteins present in planktonic *P. aeruginosa* and absent in biofilms and 58 proteins that are in biofilms and absent in planktonic sate. This study could be used as a basis for future experiments regarding virulence determinants of biofilm materials.

Mucoid Pseudomonas aeruginosa Infections

As has been described, the switch from a non-mucoid to a mucoid state is one of the most commonly exhibited switches found in chronic *Pseudomonas aeruginosa* infections of CF patients.[88, 161, 377] The environmental conditions of the CF lung and the protective nature of the mucoid strain selects for this adaptation. The switch to a mucoid phenotype induces a number of different virulence factors in *P. aeruginosa*, including the up regulation of alginate and several lipopeptides.[135, 142, 192, 426] Previous studies in our lab identified TLR2 as an important receptor for activation of pro-inflammatory cytokines, so naturally the increase in lipopeptides in mucoid strains made us curious as to whether the involvement of TLR2 was specific to mucoid strains.

Our data has shown that non-mucoid strains of *P. aeruginosa* act solely through the TLR5 pathway to activate p38α MAPK. Further, we have shown that TLR2 and TLR5 is activated in the presence of mucoid *P. aeruginosa*. Though this is the case, the overall induction of p38α in the presence of mucoid strains is not dramatically greater than non-mucoid strains. Because this switch is so commonly found in CF patients, understanding how to target or modulate this pathway could be of therapeutic benefit.[72] In fact, understanding how different bacteria affect the lipopeptide 'decorations' on the surface of bacteria has become an interesting topic of investigation in microbiology.[258, 259, 281, 331] While gram-positive and gram-negative bacteria have been thought to produce distinct types of lipopeptides, it is now becoming clear that these distinctions can be affected by different environmental conditions and enzymes present in the bacteria.[258, 259] Further, how these different lipopeptides and bacterial strains modulate the immune response via activation of different TLRs is once again of interest. The importance of understanding changes that bacteria undergo and how these changes affect the host pathogen interaction is of renewed interest. The tools created in the final chapter of my thesis can be used to further explore such interactions.

Again several limitations exist for the current course of study. While many of them are the same as described above, the BiFC assay adds some to the mix. Firstly, it is a heterologous expression system, producing overexpression of the TLRs in question. For this reason, non-specific TLR interactions or localization of the TLRs could be impaired. Despite this, the functional output of ATF2 and NFκB is of additional interest, and the system can be used to rapidly identify interactions of interest, which can then be further studied. It is, however, important to be aware of the limitations of the system, or any experimental procedure for that matter, and to subsequently confirm initial observations.

Mucoid strains of *P. aeruginosa* differentially express a number of different genes compared to their non-mucoid counterparts.[134] Seven of these genes are involved in producing distinct lipopeptides. Because of the importance of TLR2 in recognizing lipopeptides, future experiments involved expression of these individual lipopeptides into non-mucoid strains of *P .aeruginosa* to elucidate if a specific lipopeptide is responsible for p38 α activation. Further purification of lipopeptide and slime components of the filtrates is also of interest.

Treating Chronic Mucoid and Biofilm Infections

Our results indicate that treating chronic infections may be able to reduce the overall inflammatory response imitated by *P. aeruginosa.* Our studies confirm that blocking TLR could potentially reduce the overall inflammatory response that is seen in the lungs of CF patients. However, blocking a major receptor important in host-pathogen interaction is not an ideal solution. Rather, looking at ways to reduce the amount of secreted virulence factors, or modulate the switch from non-mucoid to mucoid phenotype is a potentially better option.

It is important to realize that chronic infection in the CF lung is often a result of both the switch to biofilm growth and mucoid strains of *P. aeruginosa.* These chronic infections can be treated via antibiotics to reduce bacterial load, but eradication is never achieved. Thus new strategies are required in order to achieve more positive results. Our data has shown that biofilm material does not induce AEC MAPK activation via the same mechanism as planktonic mucoid strains. One potential strategy maybe to disrupt the biofilm in chronic infections and then treat the underlying planktonic infection.

Future Directives

Our data indicated the importance at looking at how various phenotypic switches that occur during chronic infection can have an impact on how the host senses the infection. Because bacterial infections lead to increased inflammation, which results in increased morbidity in CF patients, it becomes important to identify and treat the causes of increased inflammation. In an era of increased antibiotic resistance, it important to find new approaches to this problem. While *Pseudomonas aeruginosa* was generally thought to be the major pathogen in CF patients, recent evidence suggest that the nature of CF lung is, in fact, a polymicrobial environment.[460] Further, the interaction between components of these polymicrobial 'communities' can directly affect the virulence and pathogenicity of other members.[24] Therefore, it would be very interesting to test the impact of polymicrobial interactions in the experimental model developed in this thesis.

In relation to Toll-like receptors, it is clear that more work must be done to identify the nature of ligand interactions with various components and how this affects TLR signaling and possible interactions. For instance, a recent studied identified that TLR2/6 interactions were able to synergize with the effects of TLR9 to provide protection against *P. aeruginosa* and

pneumonia.[124] The mechanism by which these TLR signaling pathways are able to enrich their downstream targets and how it provides protection is of future interest.

The work done in this thesis provides a framework to future studies regarding changes in phenotype of bacteria and how they affect the host. By studying the activation of p38α by various *P. aeruginosa* mutants, different modes of bacterial growth or even supernatants that are produced by polymicrobial communities, it could be possible to identify critical changes that the host responds to and develop therapeutics against these virulence targets.

Conclusions

These studies shine light on some of the important host-pathogen interactions that are occurring in the CF lung. I have shown the importance of p38α MAPK activation in AEC in the production of a pro-inflammatory response. Further I have demonstrated the importance of planktonic and biofilm modes of growth in the induction of this activation. Finally, I have shown that mucoid strains of *P. aeruginosa* are able to activate p38 α via TLR2, a distinct pathway from non-mucoid *P. aeruginosa.*

With an ever-increasing antibiotic resistance profiles seen in bacteria and a greater awareness of biofilm based infections it is clear that novel strategies to treat different infections will be needed in the future. Further, these studies implicate the importance of different environmental conditions and aspects of infection that must be accounted for during experimental design in order to better mimic conditions seen by the host.

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