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The Role of Alginate in the Inhibition of Macrophage Phagocytosis of Mucoid Pseudomonas aeruginosa

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THE ROLE OF ALGINATE IN THE INHIBITION OF MACROPHAGE PHAGOCYTOSIS
OF MUCOID PSEUDOMONAS AERUGINOSA

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor
of Philosophy

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Dedication

To my family and friends.

This would not be possible without your encouragement, love, and support.
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List of Abbreviations

σ22, sigma 22, AlgT/U
Alg, alginate
APG, acylpolygalactoside
ASMase, acid sphingomyelinase
BALF, bronchoalveolar lavage fluid
CF, cystic fibrosis
CFTR, cystic fibrosis transmembrane conductance regulator
CFU, colony forming units
CR3, CD11b/CD18, MAC-1
CR4, CD11c/CD18
Flg, flagella
GAP, GTPase-activating protein
GFP, green fluorescent protein
HAI, hospital acquired Infection
HMGB1, high mobility group box 1
IκBα, inhibitor of NF-κB alpha
ICAM, intercellular adhesion molecule
IL, interleukin
LB, Luria-Bertani
LPG, lipophosphoglycan
LPS, lipopolysaccharide
mC, mCherry
MEK, MAPK/ERK kinase
MFI, mean fluorescence intensity
MLCK, myosin light chain kinase
MOI, multiplicity of infection
MR, mannose receptor
PAMP, pathogen-associated molecular pattern
PI(3)P, phosphatidylinositol 3-phosphate
PI(3,4,5)P₃, phosphatidylinositol-3,4,5-trisphosphate
PI(4,5)P₂, phosphatidylinositol-4,5-bisphosphate
PI3K, phosphatidylinositol 3-kinase
PKC, protein kinase C
PLC, phospholipase C
PMNL, polymorphonuclear leukocyte
PS, phosphatidylserine
SR, scavenger receptor
TLR, Toll-like receptor
TPA, 12-O-tetradecanoylphorbol-13-acetate
Abstract

THE ROLE OF ALGINATE IN THE INHIBITION OF MACROPHAGE PHAGOCYTOSIS OF MUCOID PSEUDOMONAS AERUGINOSA

Warren James Rowe III, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Virginia Commonwealth University, 2013

Directors:
Dennis E. Ohman
Professor and Chair, Microbiology and Immunology
Deborah A. Lebman
Associate Professor, Microbiology and Immunology

During colonization of the cystic fibrosis airway Pseudomonas aeruginosa converts from non-mucoid to a mucoid phenotype, characterized by the production of the exopolysaccharide alginate. Alginate production has been shown to enhance survival by promoting biofilm formation, evading complement killing, and resisting phagocytosis. The mechanism by which alginate protects P. aeruginosa from phagocytosis is unclear. To investigate the role of alginate in the inhibition of phagocytosis, a human monocytic cell line (THP-1) and a murine alveolar macrophage cell line (MH-S) were used to determine the effects of alginate on macrophage binding,
signaling, and phagocytosis. Phagocytosis assays using the mucoid cystic fibrosis clinical isolate FRD1, and its non-mucoid isogenic \textit{algD} mutant FRD1131, revealed that alginate inhibits opsonic and non-opsonic phagocytosis. The inhibitory effect of alginate production is intrinsic to the bacteria as exogenous alginate was unable to protect non-mucoid FRD1131 from phagocytosis. Decreased binding of FRD1 compared to FRD1131 was also demonstrated by using the actin polymerization inhibitor cytochalasin D to inhibit phagocytosis. Furthermore, studies using blocking antibodies to CD11b and CD14 found that both of these receptors were important for the phagocytosis of FRD, and it is likely that these receptors are blocked by alginate. Alginate production by \textit{P. aeruginosa} may reduce lipid raft formation, however, it was not found to affect acid sphingomyelinase activity, which is important for ceramide formation within the lipid raft. Decreased binding led to decreased signaling in macrophages demonstrated by reduction in level and alteration in kinetics of phosphorylation of AKT and ERK1/2 kinases. Signaling pathway inhibitors revealed that PI3K, but not MEK, activation was critical for phagocytosis of \textit{P. aeruginosa}. Despite altered intracellular signaling in murine macrophages, both mucoid and non-mucoid \textit{P. aeruginosa} induced similar levels of IL-8 and MIP-2 from human and murine macrophages, respectively. By understanding the pathways involved in mediating efficient phagocytosis of clinical isolates, it may be possible to develop a treatment to promote clearance by the resident alveolar macrophages. These experiments may serve as a model to evaluate the effectiveness of such treatments. This approach also provides valuable insight into previously unknown mechanisms of phagocytosis of \textit{P. aeruginosa}.
Chapter 1
Introduction

*Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a gram-negative, biofilm forming, aerobic rod. *P. aeruginosa* is commonly found throughout the environment in soil, surface water, sewage, plants, and foods (70). While it is a common human intestinal bacterium, *P. aeruginosa* is an opportunistic pathogen, which is responsible for a variety of infections (70). These infections include keratitis, skin infections, urinary tract infections, infections of the upper and lower respiratory tract, and of the bloodstream (45). Immunocompromised patients as well as those on mechanical ventilation are at risk of serious infections by *P. aeruginosa* (70). Immunocompromised patients include those with cancer or HIV infection, transplant recipients, and burn patients. Nosocomial infection by *P. aeruginosa* presents an important risk for these patients as it increases the mortality rate by 40% (70). Recent studies have found that while the chance of *P. aeruginosa* infection in U.S. hospitals is 4%, it is responsible for 10% of all hospital-acquired infections (HAI) (70). Acute infection of the respiratory tract by *P. aeruginosa* often leads to septic shock, multiple organ dysfunction, and has a mortality rate of up to 48% (70). Chronic infection of the airway is very different from acute infections. Chronic infection of the airway of cystic fibrosis (CF) patients causes significant inflammation and tissue damage, which is a major cause of morbidity and mortality (70). *P.*
*P. aeruginosa* infection is very common among patients with cystic fibrosis and is currently found in 58% of all patients. *P. aeruginosa* strains isolated from patients with CF are phenotypically different from those isolated from the environment and these adaptations promote survival by allowing *P. aeruginosa* to evade the host immune system. CF strains often lack flagellin and pilin expression. These CF adapted strains also produce ‘rough’ lipopolysaccharide (LPS), which does not have the long O-side chains characteristic of ‘smooth’ LPS (70). This change in LPS structure could alter the innate immune response by affecting binding to phagocytes and inflammatory signaling.

*P. aeruginosa* virulence factors include antibiotic resistance, biofilm formation, alginate production, and secreted effectors. Clinical isolates have been found to have pathogenicity islands which directly contribute to virulence. Secreted effectors such as elastase, alkaline proteases, hemolysins, cytotoxins, and siderophores promote tissue invasion. These effectors also protect against the innate and adaptive immune system by cleaving important receptors, signaling molecules and by inactivating complement and antibodies (16, 70). *P. aeruginosa* also produces pyocyanin, which has a proinflammatory effect in the lungs of CF patients. Pyocyanin also disrupts antioxidant functions within the lung which leads to oxidative damage to the lung epithelium (70). *P. aeruginosa* also secretes several effector proteins via a type III secretion system including ExoS, ExoT, ExoU, and ExoY (16). Type III secretion involves a molecular nanomachine to inject effectors directly into target cells (16). These effectors are known to disrupt intracellular signaling, which can affect phagocytosis and inflammation (9, 16). For example, ExoS has been shown function as a GTPase-activating protein (GAP) to
stimulate GTP hydrolysis by RhoA, which regulates cytoskeletal rearrangement and is involved in PI3K-mediated signaling pathways (9, 16, 22).

**Pseudomonas aeruginosa infection in cystic fibrosis**

A large amount of research is directed at *P. aeruginosa* respiratory infections in patients with cystic fibrosis. Cystic fibrosis is an important respiratory disease that affects 70,000 people worldwide with 1000 people being diagnosed every year (12). CF is an autosomal recessive disease caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which produces a cyclic AMP-regulated chloride ion channel (18). Over 1,500 possible mutations in the CFTR gene have been identified, typically leading to the production of a non-functional, and subsequently degraded, protein (18, 36, 72). The loss of the channel results in defective chloride ion transport, which has many systematic effects including the production of highly adhesive mucus (67). This altered mucus production inhibits bacterial clearance and promotes respiratory infections. A recent study found that 97% of children with CF were colonized with *P. aeruginosa* by the age of 3 years and that among all patients over 58% were infected with *P. aeruginosa* (70). *P. aeruginosa* infection in CF is associated with increased morbidity and mortality (29). Due to the widespread presence of *P. aeruginosa* in the environment and the high incidence of hospital acquired infections, it is important to understand the survival mechanisms responsible for its persistence within the CF lung (31, 70).
Alginate regulation and synthesis

\textit{P. aeruginosa} produces three extracellular polysaccharides called Psl, Pel, and Alginate. Psl is produced by a fifteen-gene operon and is important for attachment as well as the maintenance of biofilm structure (69). Psl was found to be required for adherence to mucin-coated surfaces and airway epithelial cells. Pel is a glucose-rich matrix polysaccharide produced by a seven-gene operon (69). Pel is important for pellicle formation and biofilm structure, similar to Psl. However, Pel is biochemically and genetically distinct from Psl. During colonization of the CF airway, \textit{P. aeruginosa} converts from non-mucoid to a mucoid phenotype, characterized by the production of a capsular exopolysaccharide alginate (13, 46). High levels of alginate have been measured in the sputum of patients with CF who are chronically infected with \textit{P. aeruginosa} (18). Alginate is composed of D-manuronate and L-guluronate, of which the D-manuronate is O-acetylated. The \textit{P. aeruginosa} alginate operon is composed of 12 genes controlled by a single promoter, which is ultimately regulated by $\sigma^{22}$ (AlgT/U) (34). $\sigma^{22}$ activation leads to the production of AlgR, AmrZ and AlgB, which bind to the alginate promoter allowing for transcription. $\sigma^{22}$ is negatively regulated by the membrane bound anti-sigma factor, MucA. The conversion from non-mucoid to mucoid phenotype may be caused by many factors including cell wall stress and oxygen radicals (11, 13, 53, 87).

Alginate synthesis begins in the cytoplasm and modifications continue while it is transported through the inner and outer membranes (34, 35). Alginate production begins with fructose-6-phosphate, a common sugar generated during glycolysis. Fructose-6-phosphate is rearranged by the phosphomannose isomerase activity of AlgA
producing mannose-6-phosphate. Phosphomannose mutase, produced by algC, then moves the phosphate group on mannos-6-phosphate to form mannose-1-phosphate. The GDP-mannose phosphorylase activity of AlgA uses mannose-1-phosphate, along with the cofactor GTP, to form GDP-mannose. GDP-mannose dehydrogenase, produced by algD, then oxidizes the C6 hydroxyl group of GDP-mannose to a carboxylic acid to form GDP-mannuronic acid. GDP-mannuronic acid is then transported across the inner membrane by the polymerase, produced by alg8. During this process, GDP-mannuronic acid is polymerized by Alg8 with the help of Alg44 to form polymannuronate. Polymannuronate is then epimerized at the C5 carbons by D-mannuronate C5-epimerase, produced by algG, to form a polymer of D-mannuronate and L-guluronate subunits. D-mannuronate subunits are O-acetylated by a complex formed by AlgI, AlgJ, and AlgF. The O-acetyltransferase, produced by algI, transfers an acetyl group from acetyl-CoA across the inner membrane to AlgJ and AlgF, to be added to D-mannuronate. The acetylated D-mannuronate-L-guluronate polymer is transported across the periplasm with the help of several scaffold proteins produced by algX, algL, and algK. AlgE, recruited by AlgK, forms a porin in the outer membrane to export the alginate that has been transported across the periplasm (34, 35, 39, 60, 66).

Mucoid conversion of P. aeruginosa in the CF airway is a slow process; therefore, alginate production is not likely to be responsible for the initial immunological failure leading to infection and colonization of the CF lung by P. aeruginosa. However, alginate production by mucoid P. aeruginosa may contribute to maintenance of the chronic infection by inhibiting its clearance by alveolar macrophages. Alginate production has been shown to enhance survival through various means including
promoting biofilm formation and evading complement killing (6, 8, 43). $\sigma^{22}$ has also been shown to promote expression of AmrZ, which inhibits the master regulator of flagellum expression, FleQ (78, 79). 39% of P. aeruginosa CF isolates were found to be non-motile and several studies have shown that the non-motile phenotype may enhance the survival of P. aeruginosa in the CF lung by evading phagocytosis (1, 47, 50, 51). It has been reported that mucoid P. aeruginosa is resistant to opsonic and non-opsonic phagocytosis, and that alginate production by P. aeruginosa confers protection against IFN-γ mediated opsonic killing of a P. aeruginosa biofilm (8, 43, 46, 63, 68). There are low levels of opsonins found in the lung environment, and the effects of alginate production on phagocytosis by macrophages in a serum-free environment have not previously been extensively studied (29).

**Phagocytosis**

Phagocytosis is an actin-dependent process that involves the use of multiple cell surface receptors to bind pathogens and cell debris (16, 84). Macrophages also extend actin-dependent projections to search the surrounding environment for pathogens (16). This internalization requires a complicated series of membrane remodeling, cytoskeletal rearrangement, and intracellular signaling events. The result of these events is the formation of a phagosome, the engulfment of the pathogen, and the release of inflammatory cytokines. Multiple receptors come together and form multimers within lipid rafts to enhance binding capacity. This is necessary because bacterial movement, due to Brownian motion and flagellar motility, will cause the bacteria to detach if there are only low affinity interactions (16).
Phagosome formation begins with an increase in phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) concentration in the cell membrane. Phosphatidylinositol 3-kinase (PI3K) stimulates phospholipase C (PLC)-γ which modifies PI(4,5)P₂ into phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃) until the phagosome has sealed. After sealing, PI(3,4,5)P₃ is once again modified to become phosphatidylinositol 3-phosphate (PI(3)P) which is the primary lipid in early phagosomes (16). These early phagosomes bind to early endosomes and eventually late endosomes and lysosomes to form late phagosomes and late phagolysosomes (16). This results in the acidification of the phagosome and destruction of the pathogen.

Phagocytosis involves two broad categories of receptors, opsonic receptors and pattern-recognition receptors. Opsonic phagocytosis requires receptors that bind an intermediary molecule which binds to the target bacteria. Opsonic receptors include Fc receptors such as FcγRI, FcγRII, and FcγRIII and complement receptors such as CR1, CR3 (CD11b/CD18, MAC-1), and CR4 (CD11c/CD18) (16). For opsonic phagocytosis Fcγ receptors bind various IgG antibodies, while complement receptors bind mannann-binding lectin (MBL) and various complement fragments. However, further research has revealed that these opsonic receptors are also capable of binding bacterial ligands directly and that these interactions are critical for host defense. For example, complement receptor 3 is able to bind numerous ligands including LPS, LPG (lipophosphoglycan), acylpolygalactoside (APG), β-glucans, intercellular adhesion molecules (ICAM)-1, ICAM-2, ICAM-3, β-amyloid, high mobility group box 1 (HMGB1), and oligodeoxynucleotide (7, 89). As a result, CR3 has been found to be important for cellular binding and internalization by murine macrophages (27). Pattern-recognition
receptors recognize a broad range of pathogen-associated molecular patterns (PAMPs). These receptors include Toll-like receptors (TLR) and scavenger receptors (SR) which are also critical to host defense (16, 84). Both SR-A and TLR-4 (with CD14) are able to bind LPS (16). However, it is possible that alterations to LPS structure may result in altered signaling through these receptors. Several macrophage receptors have been implicated in the phagocytosis of *P. aeruginosa*. These receptors include TLR2, TLR4, TLR5, mannose receptor (MR), CR3, CR4, SR-A, MARCO and Fc receptors (4, 16, 29, 38, 70, 74). However, the contribution of each receptor varies depending on the *P. aeruginosa* strain that is used. Although, antibodies to alginate are generated by CF patients with chronic *P. aeruginosa* infections, these antibodies have been shown to be unable to mediate opsonic killing in vitro (69).

Phagocytosis plays an important role in clearing cell debris, including apoptotic cells (16). Phosphatidylserine (PS) is a lipid that is restricted to the inner leaflet of the plasma membrane. Apoptosis exposes this lipid as a marker for clearance. MFG-E8 (lactadherin) binds PS which is then bound by several phagocytic receptors including αVβ3 integrin, CD36, and CD68 (16). While phagocytosis of pathogens induces the production of inflammatory cytokines, phagocytosis of apoptotic cells induces anti-inflammatory cytokines to prevent tissue damage (16).

Activation of the above receptors results in the activation of a wide range of interconnected signaling pathways that lead to the formation of the phagocytic cup and the release of inflammatory cytokines and chemokines. Efficient phagocytosis of *P. aeruginosa* is dependent on PI3K signaling, leading to the phosphorylation and activation of certain signaling molecules, such as AKT and NF-κB (37). NF-κB is a
transcription factor that is activated when the inhibitor of NF-κB alpha (IκBα) is phosphorylated, leading to its degradation (42). Once free from IκBα, NF-κB translocates to the nucleus and promotes the expression of inflammatory cytokines including IL-8, TNF-α, and IL-6. AKT inhibition has resulted in inhibited phagocytosis of PAO1 by murine macrophages (37). ERK activation may also contribute to phagocytosis of *P. aeruginosa* as it has been shown that ERK activation leads to phosphorylation of myosin light chain kinase (MLCK) in polymorphonuclear leukocyte (PMNL) and epithelial cells (52, 58). Phosphorylation of MLCK leads to actin rearrangement, which is necessary for the formation of the phagocytic cup. Actin rearrangement during phagocytosis is also dependent on the Rho GTPase Rac (16). Prior to these signaling events, ceramide accumulation and lipid raft formation within the phagocytic cup may also be critical to the phagocytosis of *P. aeruginosa* (23).

**The macrophage response to *Pseudomonas aeruginosa* in the cystic fibrosis lung**

Significant numbers of macrophages are found in the CF airway (2). However, macrophages isolated from *P. aeruginosa* infected CF patients exhibit a M2 phenotype, which can mediate chronic inflammation (28). The mechanism by which alginate protects *P. aeruginosa* from opsonic and non-opsonic phagocytosis is unclear. When the murine macrophage cell line RAW264.7 is exposed to alginate, production of inflammatory cytokines, such as TNF-α and Interleukin 6 (IL-6), are up-regulated (88). CF is also associated with increased Interleukin 8 (IL-8) production within the lung (77). This suggests that alginate is able to affect macrophage activation, which could affect
inflammatory cytokine production in the CF airway. The CFTR mutation may further complicate inflammatory signaling within the CF lung. A mutation in the CFTR gene has been shown to lead to an up-regulation of inflammatory cytokine production by airway epithelial cells (49). Inflammatory cytokines released by epithelial cells, and to a lesser extent macrophages, exacerbate the disease by recruiting large numbers of neutrophils to the airway and causing tissue damage (11). This is likely due to constitutively active NF-κB (49). The recruited neutrophils are unable to clear the infection and further contribute to the disease by providing a DNA and F-actin rich environment for *P. aeruginosa* growth (6). *P. aeruginosa* utilizes polymers of DNA and F-actin, provided by the neutrophils, to form biofilms in the CF airway (86). These neutrophils are also a source of oxygen radicals, which may induce mucoid conversion in *P. aeruginosa* (53).

**Scope of this project**

Understanding how alginate production by *P. aeruginosa* leads to enhanced survival in the context of the CF lung is important for the treatment of CF lung infections. By understanding which receptors and signaling pathways are critical to the phagocytosis of mucoid *P. aeruginosa*, it may be possible to stimulate the alveolar macrophages to enhance clearance of an established infection. Prior to this dissertation, the mechanism by which alginate inhibits macrophage phagocytosis of *P. aeruginosa* was not well characterized. This was accomplished by using a combination of microbiological and immunological assays to determine the role of alginate in inhibiting phagocytosis.
To address this problem, I first determined the effect of alginate on opsonic and non-opsonic phagocytosis by human and murine macrophages. I found that alginate was able to inhibit phagocytosis in both types of macrophages, under opsonic and non-opsonic conditions. I followed this up by determining how and when alginate is able to inhibit phagocytosis, as well as determining how other factors influence phagocytosis of *P. aeruginosa*. This revealed that alginate production has a local inhibitory effect by blocking binding to the macrophage. I continued by investigating the inhibitory effect of alginate on macrophage receptor binding, intracellular signaling, and extracellular signaling. This revealed important phagocytosis receptors, the blocking of which causes altered activation of critical phagocytosis signaling pathways and reduced phagocytosis. This research could provide important information leading to a better understanding of the chronic lung infection in CF as well as providing information on the mechanisms involved in the phagocytosis of *P. aeruginosa* by human and murine macrophages.
Chapter 2
Materials and Methods

Bacterial culture media and growth conditions

All *Pseudomonas aeruginosa* and *Escherichia coli* strains were grown on Luria-Bertani (LB) agar plates. Following growth on solid media, all strains were grown in LB liquid media at 37°C with shaking. Antibiotics were used when necessary for plasmid maintenance at the following concentrations: ampicillin, 100 µg/ml; carbenicillin, 150 µg/ml; kanamycin, 30 µg/ml.

Bacterial strains and plasmids

The bacterial strains used in this study are described in Table 1. Plasmids used in this study are described in Table 2. FRD1 is a cystic fibrosis clinical isolate in which alginate production is constitutively active due to a mucA mutation, which prevents MucA from sequestering σ^22-. FRD1131 is an isogenic mutant of FRD1 that contains an algD::Tn501-33 mutation. algD is the first gene in the alginate operon and the transposon is polar on all downstream genes, so FRD1131 is unable to produce alginate, regardless of σ^22- regulation. PAO1 is a clinical wound isolate that does not produce alginate. PDO300 is a PAO1 mucA mutant in which alginate production is constitutively active.
GFP-expressing strains were constructed by transferring pMF230 into the recipient strain by conjugation. The mCherry plasmid was constructed by PCR amplifying the gene from pMP7605 with the following primers: 5’-gatcaagcttttactgtacagctgcatc-3’ and 5’-gatctctagaatgtgagcaagggcagg-3’ (44). DNA was ligated to the pMF54 plasmid, a broad-host range expression vector, and transformed into competent DH5α cells. The completed plasmid was then transferred into the recipient strains by conjugation.

For purposes of judging multiplicity of infection (MOI) for all experiments, colony counts were used. Overnight cultures of bacterial strains were diluted 1:10 and grown for 2 hours until mid-log phase growth (0.8 at OD600). 1 ml of each strain was centrifuged at 14,000 RPM for 3 minutes. Supernatants were removed and the pellet was resuspended in 1 ml of RPMI. The number of resuspended bacteria was determined by serially diluting 100 µl 1:10, and spreading 100 µl of the dilutions on LB agar plates. After overnight incubation at 37°C, colonies were counted to determine colony forming units (CFU). This CFU count was used to determine the MOI for subsequent experiments.

**Strain construction**

Sequence-defined transposon insertions in PAO1 from a mutant library were moved by transduction to PAO and FRD strains (30). Phage F116L plate lysates were made on a library mutant. The filter sterilized lysate was incubated for 4 hours with the recipient strain and then plated onto LB-agar with tetracycline (100 µg/ml) to select for inheritance of the transposon by homologous recombination.
Macrophage culture

THP-1 and MH-S macrophages were purchased from ATCC (TIB-202 and CRL-2019, respectively) and grown in complete RPMI containing 10% FBS (Gibco), 1% penicillin and streptomycin, and 1% L-glutamine. Cells were counted by hemocytometer prior to plating for each experiment. THP-1 macrophages were differentiated with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma, final concentration 3.2 x 10^{-7} M) for 2 days prior to infection experiments (82).

Flow cytometry based phagocytosis assay

2 x 10^5 THP-1 or MH-S macrophages were grown in 12-well plates prior to infection. Overnight cultures of GFP-expressing bacterial strains were diluted 1:10 and grown for 2 hours until mid-log phase growth (0.8 at OD600). 1 ml of each strain was centrifuged at 14,000 RPM for 3 minutes. Supernatants were removed and the pellet was resuspended in 1 ml of RPMI. 10 µl of the resuspended bacterial strains were added to the macrophages, resulting in an MOI of 50, and incubated at 37°C in 5% CO₂ for 30 minutes. Macrophages were then washed and harvested with 0.02% EDTA in PBS and analyzed by flow cytometry to determine the amount of internalized GFP-expressing bacteria. Macrophage gating was determined first by forward scatter versus side scatter to eliminate dead cells and debris. Secondary gating was determined by GFP expression versus red autofluorescence to count the number of macrophages containing GFP-expressing bacteria (41, 64, 75, 81).
Table 1. Description of bacterial strains

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype and/or Phenotype</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
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<tr>
<td>FRD1</td>
<td><em>mucA22 Alg</em> (^+) LPS-O(^-)</td>
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<td>FRD1131</td>
<td>FRD1<em>algD::Tn501-33 Alg</em></td>
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<tr>
<td>FRD1131C'</td>
<td>FRD1131(pAlg2) <em>Cb</em> (^+) Alg*</td>
<td>This study</td>
</tr>
<tr>
<td>FRD1 GFP</td>
<td>FRD1(pMF230) <em>Cb</em> (^+) GFP*</td>
<td>This study</td>
</tr>
<tr>
<td>FRD1131 GFP</td>
<td>FRD1131(pMF230) <em>Cb</em> (^+) GFP*</td>
<td>This study</td>
</tr>
<tr>
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<td>FRD1*flgB::Tn mariner Tc(^+) Flg(^-)</td>
<td>Ohman lab strain</td>
</tr>
<tr>
<td>FRD1131 Flg-</td>
<td>FRD1131*flgB::Tn mariner Tc(^+) Flg(^-)</td>
<td>Ohman lab strain</td>
</tr>
<tr>
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<tr>
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<td>(53)</td>
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<td>DH5(\alpha)(pMF230) <em>Ap</em> (^+) GFP*</td>
<td>Ohman lab strain</td>
</tr>
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<td>DH5(\alpha)(pRK2013) *Tra+ Km(^+)</td>
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<tr>
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Table 2. Description of bacterial plasmids

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<td>pRK2013</td>
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<tr>
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<td>(44)</td>
</tr>
<tr>
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<td>pMF54 P&lt;sub&gt;trc&lt;/sub&gt;-mCherry (mC&lt;sup&gt;+&lt;/sup&gt;) Cb&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>
**Gentamicin protection assay**

2x10^5 THP-1 or MH-S macrophages were grown in 12-well plates prior to infection. Overnight cultures of bacterial strains were diluted 1:10 and grown for 2 hours until mid-log phase growth (0.8 OD600). 1 ml of each culture (1 x 10^5 cells/ml) was centrifuged at 14,000 RPM for 3 minutes. Supernatants were removed and the pellet was resuspended in 1 ml of RPMI. Macrophages were infected with 10 µl of the resuspended bacterial strains (1 x 10^7 cells), resulting in a MOI of 50, and incubated at 37°C in 5% CO_2 for 1 hour. MH-S cells were washed with cold PBS, treated with gentamicin (2 mg/ml) for 30 minutes to kill extracellular bacteria, washed twice with cold PBS, and subsequently lysed with 0.25% SDS to release intracellular bacteria. Cell lysates were serially diluted 1:10 and 100 µl of the dilutions were spread on LB agar plates. Plates were incubated at 37°C overnight and colonies were counted to determine CFU. For some experiments, blocking antibodies were added 15 minutes prior to infection and signaling inhibitors were added 30 minutes prior to infection, and both remained present during phagocytosis. Controls including wells containing only bacteria were treated with gentamicin (2 mg/ml) for 30 minutes or 0.25% SDS which resulted in 0% or 100% CFU, respectively, compared to untreated wells (14, 17, 55).

**Intracellular survival assay**

2x10^5 THP-1 macrophages were grown in 12-well plates prior to infection. Overnight cultures of bacterial strains were diluted 1:10 and grown for 2 hours until mid-log phase growth (0.8 OD600). 1 ml of each strain was centrifuged at 14,000 RPM for 3 minutes. Supernatants were removed and the pellet was resuspended in 1 ml of RPMI.
Macrophages were infected with 10 µl of the resuspended bacterial strains, resulting in a MOI of 50, and incubated at 37°C in 5% CO₂ for 2 hours. Macrophages were washed with cold PBS and treated with gentamicin (2 mg/ml) for 30 minutes. Following gentamicin treatment (Time 0) macrophages were incubated at 37°C in 5% CO₂ for 2 hours. At varying time points throughout the 2 hour incubation, macrophages were washed twice with cold PBS and lysed with 0.25% SDS to release intracellular bacteria. Cell lysates were serially diluted 1:10 and 100 µl of the dilutions was spread on LB agar plates. Plates were incubated at 37°C overnight and colony counts were performed (55).

**Microscopy**

THP-1 or MH-S macrophages were grown in chamber slides or glass bottom dishes prior to infection. Overnight cultures of bacterial strains were diluted 1:10 and grown for 2 hours until mid-log phase growth (0.8 OD600). 1 ml of each strain was centrifuged at 14,000 RPM for 3 minutes. Supernatants were removed and the pellet was resuspended in 1 ml of RPMI. For fluorescent microscopy, bacterial strains expressing GFP were added at a MOI of 500 for 2 hours and visualized with a Zeiss Axiovert 200 using a green filter. For confocal microscopy, MH-S macrophages were labeled using Alexa488-Cholera Toxin B (Molecular Probes) to label ganglioside GM1 in the membranes for 15 minutes prior to the addition of mCherry-expressing bacteria. Macrophages were washed and fixed with 4% formaldehyde for 15 minutes. Formaldehyde was removed and macrophages were washed once with PBS.
Macrophages and associated bacteria were visualized using a Zeiss LSM 710 confocal microscope at 63x magnification (33, 37).

**Swimming motility assay**

Overnight cultures of bacterial strains were diluted 1:10 and grown for 2 hours until mid-log phase growth (0.8 OD600). 0.5% LB agar plates were point inoculated with 1 µl of the mid-log phase bacterial culture and incubated at 37°C overnight. 24 hours later, the diameter of the resulting growth was measured (21, 48).

**Alginate isolation and purification**

Strain FRD1 was grown overnight in 10 ml of LB liquid media at 37°C with shaking, and 1 ml was used to inoculate 100 ml of fresh LB, which was incubated overnight at 37°C with shaking. The culture was then centrifuged at 10,000 rpm for 60 minutes. Supernatants were moved to a sterile beaker where 2 volumes of cold ethanol were added and mixed. The precipitate was removed with a sterile glass plate spreader and placed into a petri dish and dried at 37°C. The precipitate was dissolved in 100 ml of 1 M NaCl overnight at 5°C with agitation. 100 ml of cold isopropanol was added and the solution was centrifuged at 10,000 RPM and 5°C for 60 minutes to collect the precipitate. The resulting precipitate was removed with a sterile glass plate spreader and dissolved in 20 ml saline, overnight at 37°C with shaking. Once dissolved, trypsin (0.5 mg/ml) was added to eliminate contaminating proteins, and incubation continued for 2 hours at 37°C with shaking. 1 g of NaCl was added followed by 1 volume of cold isopropanol. The solution was centrifuged at 10,000 RPM and 5°C for 60 minutes. The
resulting precipitate was dissolved in 20 ml saline. The solution was dialyzed against distilled water (19).

**Alginate concentration**

A borate stock was prepared by dissolving 2.474 g of \( \text{H}_3\text{BO}_3 \) in 4.5 ml of 4 M KOH and diluting to 10 ml with distilled water. A sulfuric acid-borate reagent was prepared by diluting 500 µl of borate stock up to 20 ml with \( \text{H}_2\text{SO}_4 \). A carbazole stock was prepared by adding 10 mg of carbazole to 10 ml ethanol. An alginate stock solution was prepared by adding 20 mg of sodium alginate (Sigma) to 10 ml saline.

1 ml of the sulfuric acid-borate reagent was added to a glass tube on ice. 100 µl of an alginate sample solution or control was layered on top of the sulfuric acid-borate reagent and the tube was vortexed and placed on ice for 3 minutes. 33 µl of carbazole reagent was added and the tube was vortexed and placed on ice for 3 minutes. The tubes were incubated in a 55°C water bath for 30 minutes. Following incubation, samples were removed and analyzed by spectrophotometry. Absorbance was measured at 530 nm. A standard curve was generated using the alginate stock solution and was assayed in duplicate. Standard curve data was graphed and alginate concentration was calculated by determining the equation of the resulting trend line (40).

**Western blot analysis**

2x10^6 MH-S macrophages were grown in 6-well plates prior to infection. Overnight cultures of bacterial strains were diluted 1:10 and grown for 2 hours until mid-log phase
growth (0.8 OD600). 1 ml of each strain was centrifuged at 14,000 RPM for 3 minutes. Supernatants were removed and the pellet was resuspended in 1 ml of RPMI. Macrophages were starved in serum-free RPMI for 6 hours and subsequently infected with 100 µl of the resuspended bacterial strains, resulting in a MOI of 50, and incubated at 37°C in 5% CO₂ for various time points. After infection, macrophages were washed twice with cold PBS and lysed with 70 µl RIPA (Cell Signal) containing 1 mM PMSF. Samples were centrifuged at 14,000 x g for 10 minutes and supernatants were frozen at -80°C for storage prior to analysis. Protein concentration was determined using a Bradford protein assay, and 20 µg of protein was separated on a 12% polyacrylamide gel in Tris-glycine electro-blotting buffer (National Diagnostics ec-870) for 90 minutes at 150 V. Proteins were transferred to PVDF with a semi-dry transfer cell (Bio-Rad) in Tris-glycine-SDS buffer (National diagnostics ec-880) at 25 V for 30 minutes. The blot was blocked with 2% BSA in TBS/Tween (0.05%, Amresco K873) for 1 hour. Primary antibodies to various macrophage proteins (Cell Signal, 4060, 4691, 4370, 4695) were used at 1:1000 in TBS/Tween with 2% BSA overnight, at 4°C with shaking. The blot was washed 3 times, for 10 minutes each, with TBS/Tween and secondary antibodies were added. α-Rabbit IgG-peroxidase secondary antibody (Sigma A9169) was used at 1:5000 in TBS/Tween with 2% BSA for 45 minutes at room temperature with shaking. The blot was washed 4 times, for 10 minutes each, with TBS/Tween. West Pico chemiluminescence substrate (Thermo Sci 34080) was added for 5 minutes prior to film exposure. To strip the blot to allow for additional probes, stripping buffer (Thermo Sci 21059) was added for 15 minutes at room temperature with shaking. The blot was washed with TBS/Tween and blocked with 2% BSA for 1 hour.
**Acid sphingomyelinase activity**

$2 \times 10^6$ MH-S macrophages were grown in 6-well plates prior to infection. Overnight cultures of bacterial strains were diluted 1:10 and grown for 2 hours until mid-log phase growth ($0.8 \ OD600$). 1 ml of each strain was centrifuged at 14,000 RPM for 3 minutes. Supernatants were removed and the pellet was resuspended in 1 ml of RPMI. Macrophages were serum starved for 6 hours prior to infection. 100 µl of the resuspended bacterial strains were added, resulting in a MOI of 50, and incubated at 37°C in 5% CO$_2$ for 15 minutes. The macrophages were washed twice with cold PBS and scraped using water with 1mM PMSF. Cells were harvested and lysed by three freeze-thaw cycles in liquid nitrogen. Samples were spun at 14,000 x g for 10 minutes to remove cell membranes and debris, and supernatants were frozen at -80°C for storage prior to analysis. Protein concentration was determined using a Bradford protein assay and ASMase activity in 2 µg of protein was measured using an ASMase activity assay kit (Echelon K-3200).

**IL-8 and MIP-2 ELISA**

$2 \times 10^5$ THP-1 or MH-S macrophages were grown in 12-well plates prior to infection. Overnight cultures of bacterial strains were diluted 1:10 and grown for 2 hours until mid-log phase growth ($0.8 \ OD600$). 1 ml of each strain was centrifuged at 14,000 RPM for 3 minutes. Supernatants were removed and the pellet was resuspended in 1 ml of RPMI. MH-S and THP-1 cells were infected with 10 µl of the resuspended bacterial strains, resulting in a MOI of 50, and incubated at 37°C in 5% CO$_2$ for 8 hours.
Supernatants were collected and centrifuged to remove any remaining bacteria. IL-8 or MIP-2 levels were determined by ELISA Quantikine kit (R&D Systems d8000c, mm200).
Chapter 3
Alginate Production by *Pseudomonas aeruginosa* Inhibits Opsonic and non-Opsonic Phagocytosis by Human and Murine Macrophages

*Mucoid Pseudomonas aeruginosa* inhibits phagocytosis by human macrophages

Previous studies indicated that mucoid *Pseudomonas aeruginosa* are protected from non-opsonic phagocytosis (8, 43, 68). However, these findings came from visual observations and were not compared to proper controls. The effect of alginate production on non-opsonic phagocytosis of planktonic *P. aeruginosa* by macrophages has not been quantitatively studied and may contribute to the survival of *P. aeruginosa* within the CF lung. To understand the effect of alginate on phagocytosis of *P. aeruginosa*, I first wanted to establish a time course for phagocytosis using the human monocytic cell line, THP-1. *P. aeruginosa* strain FRD1, an alginate producing (Alg+) CF isolate, and its isogenic *algD::Tn501-33* (Alg-) mutant were compared. GFP-expressing FRD1 and FRD1131 were incubated with THP-1 macrophages and were analyzed by flow cytometry at various time points up to 4 hours (Figure 1). At the earliest time point, phagocytosis of FRD1131 exceeded that of FRD1 by 25%. This trend continued out to 4 hours despite increased phagocytosis of both strains.
Figure 1. Time course of phagocytosis of FRD1 and FRD1131 by THP-1 macrophages. THP-1 macrophages were infected with GFP-expressing FRD1 (Alg⁺) or FRD1131 (Alg⁻) at a MOI of 500 for various time points up to 4 hours. Percent phagocytosis indicates the percent of macrophages that are associated with GFP-expressing *P. aeruginosa* as detected by flow cytometry. Data are shown as mean and standard deviation of two samples.
GFP-expression was detectable by flow cytometry, and I wanted to determine if there was enough GFP-expression to observe phagocytosis by fluorescent microscopy. After establishing that phagocytosis could be observed as early as 30 minutes post infection, I visualized the difference at that time point. GFP-expressing FRD1 and FRD1131 were added to THP-1 macrophages and visualized using fluorescent microscopy (Figure 2). An overlay of the fluorescent *P. aeruginosa* on a light image of the macrophages revealed many more Alg^−^ FRD1131 within, and associated with, the macrophages compared to the Alg^+^ FRD1.

**Alginate^+^ Pseudomonas aeruginosa reduce the proportion of human macrophages that phagocytize and the total amount of bacteria phagocytized**

Using the time point at which phagocytosis was maximal, flow cytometry was repeated to determine if alginate production significantly affects phagocytosis. After a 30 minute incubation, alginate production by FRD1 significantly reduced the amount of phagocytosis by THP-1 macrophages (Figure 3). This indicates that alginate production is protective under non-opsonic conditions.
FRD1 (Alg⁺)

FRD1131 (Alg⁻)
Figure 2. Visualization of THP-1 macrophages following phagocytosis of GFP-expressing FRD1 and FRD1131. Images show GFP-expressing P. aeruginosa overlayed on a light image of THP-1 macrophages. THP-1 macrophages were infected with GFP-expressing FRD1 (Alg⁺) or FRD1131 (Alg⁻) for 30 minutes and visualized by fluorescent microscopy. Reduced phagocytosis and association with macrophages are observed with the FRD1 compared to the FRD1131 strain.
Figure 3. Percentage of THP-1 macrophages that have phagocytized FRD1 and FRD1131 at 30 minutes. THP-1 macrophages were infected with GFP-expressing FRD1 (Alg⁺) or FRD1131 (Alg⁻) for 30 minutes. Percent phagocytosis indicates the percent of macrophages that are associated with GFP-expressing *P. aeruginosa* as detected by flow cytometry. Phagocytosis of FRD1 was significantly inhibited compared to FRD1131. Data are shown as mean and standard deviation of three samples. Statistical significance is determined by Students *t*-Test (*; *P* < 0.05, **; *P* < 0.01, ***; *P* < 0.001).
It is possible for some bacteria to remain on the outside of the macrophages despite washing prior to flow cytometric analysis. Therefore, too ensure that the observed difference in phagocytosis was indeed due to internalized bacteria, an alternative phagocytosis assay was used. In this assay, FRD1 or FRD1131 were incubated with THP-1 macrophages for 1 hour and macrophages were subsequently washed and treated with gentamicin to kill extracellular bacteria. After lysing the macrophages and plating the lysate, colony counts were performed. It is important to note that this assay measures the number of bacteria phagocytosed, whereas the flow cytometric assay measures the number of macrophages that have phagocytized bacteria. As in the previous assay, alginate production by FRD1 significantly inhibited phagocytosis by THP-1 macrophages (Figure 4). To ensure that the inhibition of phagocytosis was due to the production of alginate alone, I complemented algD in FRD1131 (FRD1131C') to restore alginate production. Alginate production by FRD1131C', as with FRD1, was able to significantly inhibit non-opsonic phagocytosis, suggesting that alginate alone was responsible for the inhibition of phagocytosis that was observed. Together these data indicated that alginate production by *P. aeruginosa* significantly reduced the proportion of macrophages that phagocytosed *P. aeruginosa*, as well as the total number of bacteria phagocytized by the macrophages.
Figure 4. Number of bacteria phagocytized by THP-1 macrophages after 1 hour.

THP-1 macrophages were infected with FRD1 (Alg⁺), FRD1131 (Alg⁺), FRD1131C’ (Alg⁺), or HfrH (positive control) for 1 hour. CFU indicates the number of colony-forming units recovered from macrophages. Phagocytosis of FRD1 and FRD1131C’ was significantly inhibited compared to FRD1131. Data are shown as mean and standard deviation of a representative triplicate experiment. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Intracellular survival of *Pseudomonas aeruginosa* is unaffected by alginate production

It is possible that alginate enhances survival of *P. aeruginosa* by altering intracellular survival after phagocytosis. To determine the amount of intracellular survival, a modified phagocytosis assay was used. THP-1 macrophages were cultured with FRD1 or FRD1131 for 2 hours. Instead of lysing the macrophages immediately after gentamicin treatment, macrophages were cultured for up to two additional hours and lysed at 30 minute intervals. This allowed me to determine the number of *P. aeruginosa* that have survived within the macrophages over 2 hours (Figure 5). Survival rates between the two strains remained similar at each time point throughout the 2 hour period. Thus, alginate production by FRD1 did not appear to enhance intracellular survival within macrophages.
Figure 5. Time course of intracellular survival of FRD1 and FRD1131 within THP-1 macrophages. THP-1 macrophages were infected with FRD1 (Alg⁺) or FRD1131 (Alg⁻) for 2 hours and incubated for additional time following gentamicin treatment. Percent survival indicates the number of colony-forming units recovered from macrophages at the indicated time point relative to time 0. Survival of FRD1 was similar to that of FRD1131 at each time point. Data are shown as mean and standard deviation of two separate experiments.
Alginate\(^+\) *Pseudomonas aeruginosa* reduce the proportion of murine alveolar macrophages that phagocytize and the total amount of bacteria phagocytized

In cystic fibrosis, *P. aeruginosa* will encounter alveolar macrophages within the lung. Alveolar macrophages differ from peripheral macrophages in several important ways including different receptor expression, altered activation, and survival (25, 56). Therefore, it was important to determine if alginate production also affected phagocytosis by alveolar macrophages. To investigate this, the murine alveolar macrophage cell line MH-S was used. As with THP-1, I first wanted to establish a time line for phagocytosis. Again, substantial phagocytosis was observed as early as 30 minutes post infection and continued out to 1 hour (Figure 6). There was also an observable difference in the phagocytosis of FRD1 (Alg\(^+\)) compared to FRD1131 (Alg\(^-\)).

To confirm that alginate production significantly inhibited phagocytosis by MH-S macrophages, flow cytometry was used. MH-S macrophages were incubated with FRD1 or FRD1131 for 30 minutes. Similarly to THP-1 macrophages, alginate production by *P. aeruginosa* significantly inhibited non-opsonic phagocytosis by MH-S (Figure 7).

The gentamicin uptake assay was also repeated using MH-S macrophages to confirm the inhibition observed in the flow cytometric assay. FRD1, FRD1131, or FRD1131C' were added to MH-S macrophages for 1 hour. Colony counts revealed the significant inhibitory effect of alginate production by FRD1 on phagocytosis (Figure 8). Additionally, the Alg\(^+\) complemented strain, FRD1131C', was also phagocytized significantly less than Alg\(^-\) FRD1131, suggesting that alginate production alone is responsible for the inhibition of phagocytosis that is observed.
Figure 6. Time course of phagocytosis of FRD1 and FRD1131 by MH-S macrophages. MH-S macrophages were infected with GFP-expressing FRD1 (Alg⁺) or FRD1131 (Alg⁻) for various time points up to 1 hour. Percent phagocytosis indicates the percent of macrophages that are associated with GFP-expressing *P. aeruginosa* as detected by flow cytometry. Reduced phagocytosis of FRD1 compared to FRD1131 is observed as early as 30 minutes.
Figure 7. Percentage of MH-S macrophages that have phagocytized bacteria at 30 minutes. MH-S macrophages were infected with GFP-expressing FRD1 (Alg⁺), FRD1131 (Alg), or HfrH for 30 minutes. Percent phagocytosis indicates the percent of macrophages that are associated with GFP-expressing bacteria as detected by flow cytometry. Phagocytosis of FRD1 was significantly inhibited compared to FRD1131. Data are shown as mean and standard deviation of three separate experiments. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Figure 8. Number of bacteria phagocytized by MH-S macrophages after 1 hour.

MH-S macrophages were infected with FRD1 (Alg⁺), FRD1131 (Alg⁻), FRD1131C' (Alg⁺), or HfrH for 1 hour. CFU indicates the number of colony-forming units recovered from macrophages. Phagocytosis of FRD1 and FRD1131C' was significantly inhibited compared to FRD1131. Data are shown as mean and standard deviation of three separate experiments. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Alginate+ *Pseudomonas aeruginosa* inhibit opsonic phagocytosis by human and murine macrophages

Previous studies investigated the role of alginate structure in protecting planktonic *P. aeruginosa*, as well as *P. aeruginosa* in a biofilm, from opsonic killing (43, 46, 63). However, because these studies only addressed bacterial survival rather than phagocytosis directly, I wanted to repeat them with our macrophage cell lines. First it was necessary to determine the optimal concentration of human serum necessary for opsonic phagocytosis. MH-S macrophages were incubated with FRD1 and FRD1131 for 30 minutes with increasing concentrations of human serum (Figure 9). At all concentrations of serum tested, phagocytosis of Alg+ FRD1 appeared much lower than Alg− FRD1131, suggesting that alginate inhibits opsonic phagocytosis. 0.5% human serum was chosen for further experiments as this was the lowest concentration that enhanced phagocytosis.

To determine if alginate significantly inhibited opsonic phagocytosis, previous assays were repeated using the established concentration of 0.5% human serum. THP-1 macrophages were incubated for 1 hour with various bacterial strains in the presence of human serum. After treatment with gentamicin, colony counts were performed to determine the amount of phagocytosis (Figure 10). Both Alg+ FRD1 and the Alg+ complementation strain, FRD1131C′, were phagocytized significantly less than Alg− FRD1131.
Figure 9. Time course showing the percentage of MH-S macrophages that have phagocytized FRD1 or FRD1131 with increasing concentrations of human serum. MH-S macrophages were infected with GFP-expressing FRD1 (Alg\textsuperscript{+}) or FRD1131 (Alg\textsuperscript{-}) for 30 minutes in the presence of increasing concentrations of human serum. Percent phagocytosis indicates the percent of macrophages that are associated with GFP-expressing *P. aeruginosa* as detected by flow cytometry. An increase in the phagocytosis of FRD1 compared to FRD1131 is observed beginning at 0.5% human serum.
CFU

FRD1  FRD1131  FRD1131C'  HfrH

+0.5% Human Serum

Strain
Figure 10. Number of bacteria phagocytized by THP-1 macrophages after 1 hour in the presence of 0.5% human serum. THP-1 macrophages were infected with FRD1, FRD1131, FRD1131C’, or HfrH for 1 hour in the presence of 0.5% human serum. CFU indicates the number of colony-forming units recovered from macrophages. Opsonic phagocytosis of FRD1 and FRD1131C’ were significantly inhibited compared to FRD1131. Data are shown as mean and standard deviation of a representative triplicate experiment. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Inhibition of opsonic phagocytosis was assessed in MH-S macrophages as well. MH-S macrophages were cultured with GFP-expressing FRD1, FRD1131, or HfrH in the presence of 0.5% human serum for 30 minutes and analyzed by flow cytometry (Figure 11). Similarly to THP-1, opsonic phagocytosis of FRD1 was significantly less than that of FRD1131.

The inhibitory effect of alginate on phagocytosis by murine macrophages is not limited to the FRD strain

As an additional control for strain specific effects to ensure that alginate is responsible for the inhibitory effect on phagocytosis, another *P. aeruginosa* strain was utilized. PAO1 is a clinical wound isolate routinely used in many labs. PDO300 is a mutant strain of PAO1 with a *mucA22* mutation, similar to FRD1, causing constitutive alginate production. MH-S macrophages were cultured with FRD1, PAO1, or PDO300 for 1 hour. After treatment with gentamicin, colony counts were performed to determine the amount of phagocytosis (Figure 12). As with FRD1, Alg⁺ PDO300 was phagocytized significantly less than Alg⁻ PAO1. Therefore, the inhibitory effect of alginate production on phagocytosis is not limited to the FRD strain.

Taken together, this data is the first to demonstrate that alginate production by *P. aeruginosa* has the potential to significantly inhibit phagocytosis by both human and murine macrophages, in opsonic and non-opsonic conditions.
Figure 11. Percentage of MH-S macrophages that have phagocytized bacteria after 30 minutes in the presence of 0.5% human serum. MH-S macrophages were infected with GFP-expressing FRD, FRD1131, or HfrH for 30 minutes in the presence of 0.5% human serum. Percent phagocytosis indicates the percent of macrophages that are associated with GFP-expressing bacteria as detected by flow cytometry. Opsonic phagocytosis of FRD1 was significantly inhibited compared to FRD1131. Data are shown as mean and standard deviation of three separate experiments. Statistical significance is determined by Students $t$-Test (*; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$).
Figure 12. Number of PAO1 or PDO300 phagocytized by MH-S macrophages after 1 hour. MH-S macrophages were infected with FRD1 (Alg⁺), PAO1 (Alg⁻), or PDO300 (Alg⁺) for 1 hour. CFU indicates the number of colony-forming units recovered from macrophages. Phagocytosis of PDO300 was significantly inhibited compared to PAO1. Data are shown as mean and standard deviation of a representative triplicate experiment. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Chapter 4
Additional Mediators of Phagocytosis of *Pseudomonas aeruginosa*

The role of LPS on the phagocytosis of *Pseudomonas aeruginosa*

Figure 12 indicates that while alginate production by the PAO strain significantly decreased phagocytosis, it did not reduce it to the level of FRD1. Therefore there must be an additional factor, or factors, inhibiting phagocytosis. Like many CF-adapted strains, FRD1 has a mutation that blocks the production of O-side chains on LPS, which are present in PAO. To explore the role of differing LPS structures in the phagocytosis of *P. aeruginosa*, strain FRD1810 was compared to FRD1. FRD1810 is a FRD1 recombinant in which LPS O-side chain production has been restored. MH-S macrophages were cultured with Alg⁺ FRD1 or Alg⁺ FRD1810 for 1 hour. After treatment with gentamicin, colony counts were performed to determine the amount of phagocytosis (Figure 13). Restored O-side chain production resulted in the phagocytosis of significantly more FRD1810 than FRD1. This indicated that LPS was able to have some effect on phagocytosis in the presence of alginate.
Figure 13. Number of FRD1 or FRD1810 phagocytized by MH-S macrophages after 1 hour. MH-S macrophages were infected with FRD1 (Alg<sup>+</sup>) or FRD1810 (Alg<sup>+</sup>) for 1 hour. CFU indicates the number of colony-forming units recovered from macrophages. Phagocytosis of FRD1810 was significantly increased compared to FRD1. Data are shown as mean and standard deviation of a representative triplicate experiment. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
The role of motility and flagellum on the phagocytosis of *Pseudomonas aeruginosa*

It has been reported that both flagellum and motility are critical for promoting the phagocytosis of *P. aeruginosa* (1, 47, 50, 51). To ensure that the inhibitory effects of alginate production on phagocytosis are not due to changes in motility, a motility assay was used. Soft agar plates, which allow for the movement of motile bacteria, were used to measure motility. Each plate was inoculated with a log phase culture of FRD1, FRD1131, FRD1131C', PAO1, or HfrH and, after incubation, the diameter of the resulting growth was measured (Figure 14). All FRD strains tested had similar, minimal growth compared to the motile *E. coli* strain HfrH, indicating non-motility for all strains. Non-motility of these FRD strains was also visually confirmed by microscopy.

It is possible that flagellum is expressed by *P. aeruginosa* despite being non-motile and this expression could enhance phagocytosis. To address this, flagellum mutants were constructed for FRD1, FRD1131, and PAO1 and phagocytosis experiments were repeated using these strains (Figure 15). PAO1*flg-* was phagocytized significantly less than PAO1. Likewise, FRD1*flg-* was phagocytized significantly less than FRD1, suggesting the presence of flagellum on FRD1. Surprisingly, phagocytosis of FRD1131*flg-* was significantly increased relative to FRD1131. This is possibly due to the one or more stimulatory genes being introduced in the process of disabling flagellum expression. Conversely, it is possible that an inhibitory gene was disrupted in the process. The presence of flagellum does appear to affect the phagocytosis of *P. aeruginosa*, however, the contribution of flagellum to the phagocytosis of FRD1131 is not yet known.
**Figure 14. Growth diameter of bacteria in 0.5% agar plates.** 0.5% agar plates were inoculated with FRD1, FRD1131, FRD1131C’, PAO1, or HfrH and incubated at 37°C overnight. The diameter of the resulting growth was measured. FRD1, FRD1131, and FRD1131C’ demonstrated a significant lack of motility relative to the motile PAO1 and HfrH. Data are shown as mean and standard deviation of three separate experiments. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Figure 15. Number of bacteria with and without flagellum phagocytized by MH-S macrophages. MH-S macrophages were infected with FRD1, FRD1131, PAO1, and corresponding Flg- mutants for 1 hour. CFU indicates the number of colony-forming units recovered from macrophages. Phagocytosis of FRD1 and PAO1 Flg- mutants was significantly decreased. Phagocytosis of FRD1131 Flg- was significantly increased compared to FRD1131. Data are shown as mean and standard deviation of a representative triplicate experiment. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Chapter 5
Local Alginate Production is Required to Inhibit Phagocytosis

Exogenous alginate is unable to inhibit phagocytosis of non-mucoid Pseudomonas aeruginosa by murine macrophages

To begin to understand the role of alginate in the inhibition of phagocytosis I next determined if the addition of exogenous alginate would protect FRD1131 from phagocytosis. Previous publications have suggested that exogenous alginate may protect planktonic and biofilm P. aeruginosa (46, 68). Alginate was isolated from FRD1 and its concentration determined. MH-S macrophages were cultured with FRD1131 in the presence of increasing concentrations of FRD1 alginate (Figure 16). The addition of FRD1 alginate did not protect FRD1131 from phagocytosis. It is unlikely that alginate is inducing an inhibitory signal within the macrophages; as such an effect would still be possible from exogenous alginate. Instead, it is possible that alginate is preventing an interaction between the macrophage and P. aeruginosa. Surprisingly, the addition of 200 µg/ml of FRD1 alginate significantly increased phagocytosis of FRD1131. This is likely due to LPS contamination of the alginate during the isolation procedure.
Figure 16. Percentage of MH-S macrophages that have phagocytized FRD1131 after 30 minutes in the presence of FRD1 alginate. MH-S macrophages were infected with GFP-expressing FRD1 or FRD1131 for 30 minutes with increasing concentrations of FRD1 alginate. Percent phagocytosis indicates the percent of macrophages that are associated with GFP-expressing bacteria as detected by flow cytometry. Phagocytosis of FRD1131 (Alg⁻) was not significantly inhibited with the addition of FRD1 alginate. Data are shown as mean and standard deviation of three separate experiments. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
To determine if LPS contamination could be responsible for the enhanced phagocytosis seen in Figure 16, the experiment was repeated using seaweed alginate. Although seaweed alginate is free from LPS contamination, it is not acetylated like FRD1 alginate. Again, an increasing concentration of alginate was added with FRD1131 to MH-S macrophages (Figure 17). As with FRD1 alginate, seaweed alginate was unable to protect FRD1131 from phagocytosis. The enhanced phagocytosis observed when using FRD1 alginate was eliminated with the use of seaweed alginate suggesting that LPS contamination was likely the cause of the additional stimulation.

Mucoid *Pseudomonas aeruginosa* is unable to protect non-mucoid *Pseudomonas aeruginosa* from phagocytosis by murine macrophages

Since exogenous alginate was unable to protect *P. aeruginosa* from phagocytosis, my hypothesis was that the alginate produced by FRD1 would not protect FRD1131 from phagocytosis. FRD1 and FRD1131 were added either individually or together to MH-S macrophages for 1 hour. After treatment with gentamicin, colony counts were performed to determine the amount of phagocytosis (Figure 18). FRD1 and FRD1131 have visually distinct colony morphologies allowing for each strain to be distinguished from the other when a mixed culture was used. When both strains were added to macrophages together there was no significant difference between the amount of phagocytosis of FRD1131, when mixed with FRD1, compared to FRD1131 alone. Alginate production by FRD1 was unable to inhibit the phagocytosis of FRD1131 suggesting that alginate only benefits the individual bacteria that produced it. Likewise, FRD1 was phagocytized equally regardless of the presence of FRD1131.
Figure 17. Percentage of MH-S macrophages that have phagocytized FRD1131 after 30 minutes in the presence of seaweed alginate. MH-S macrophages were infected with GFP-expressing FRD1131 for 30 minutes with increasing concentrations of seaweed alginate. Percent phagocytosis indicates the percent of macrophages that are associated with GFP-expressing bacteria as detected by flow cytometry. Phagocytosis of FRD1131 (Alg\(^-\)) was not significantly inhibited with the addition of seaweed alginate. Data are shown as mean and standard deviation of three samples. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Figure 18. Number of FRD1 or FRD1131 phagocytized by MH-S macrophages after 1 hour when infected separately or together. MH-S macrophages were infected with FRD1 (Alg⁺), FRD1131 (Alg⁻), or both strains combined for 1 hour. CFU indicates the number of colony-forming units recovered from macrophages. Phagocytosis of FRD1131 was not significantly inhibited by the addition of FRD1. Data are shown as mean and standard deviation of three separate experiments. Statistical significance is determined by Students t-Test (∗; P < 0.05, **; P < 0.01, ***; P < 0.001).
Chapter 6
Alginate Confers Protection to *Pseudomonas aeruginosa* by Blocking Important Phagocytosis Receptors and Inhibiting Binding

Alginate inhibits phagocytosis of *Pseudomonas aeruginosa* by murine macrophages by reducing binding

The first step in phagocytosis is the binding of bacterial ligands to receptors on the macrophage surface. Since exogenous alginate and alginate produced in a mixed culture were unable to inhibit phagocytosis this suggests that alginate must be present on the surface of *P. aeruginosa* to inhibit phagocytosis. It is possible that alginate on the bacterial surface could interfere with binding to the macrophage. To assess binding, cytochalasin D was used to inhibit actin polymerization and therefore prevent phagocytosis. Various strains were added to MH-S macrophages in the presence of cytochalasin D for 1 hour. After incubation, macrophages were washed to remove unbound bacteria and lysed to free any bound bacteria (Figure 19). Alg⁺ strains FRD1 and FRD1131C’ were bound significantly less than the non-mucoid FRD1131, suggesting that alginate production does significantly inhibit binding to macrophages.

Taken together, these results show that alginate inhibits phagocytosis by locally reducing binding to the macrophage. Alginate only protects the bacterium that produced it and the inhibition of phagocytosis is not due to the presence of alginate in the media.
Figure 19. Number of bacteria bound to MH-S macrophages after 1 hour in the presence of cytochalasin D. MH-S macrophages were infected with FRD1 (Alg⁺), FRD1131 (Alg''), or FRD1131C' (Alg⁺) for 1 hour in the presence of cytochalasin D to inhibit phagocytosis. CFU indicates the number of colony-forming units recovered from macrophages. Binding of FRD1 and FRD1131C' was significantly inhibited compared to FRD1131. Data are shown as mean and standard deviation of a representative triplicate experiment. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Mucoid *Pseudomonas aeruginosa* induces lipid raft formation in murine macrophages

Lipid raft formation is important for the phagocytosis of *P. aeruginosa*, and it is possible that alginate inhibits lipid raft formation (37). To assess lipid raft formation, MH-S macrophages were serum starved for 6 hours and stained with Alexa488-cholera toxin B to visualize lipid rafts within the macrophage membrane. Cholera toxin B is an effective stain because it binds to ganglioside GM1 within lipid rafts (23). Macrophages were then cultured with mCherry-expressing *P. aeruginosa* for 30 minutes and visualized by confocal microscopy (Figure 20). Compared to the uninfected control, both FRD1 and FRD1131 induced lipid raft formation in the macrophages. However, FRD1131 (Alg') showed qualitatively more lipid raft staining. A decrease in lipid raft formation may contribute to the reduced binding demonstrated in Figure 19.
Figure 20. Confocal images showing rested MH-S macrophages labeled with cholera toxin interacting with FRD1 or FRD1131. Serum starved MH-S macrophages were stained with Alexa488 labeled cholera toxin B (green) which binds to ganglioside GM1, to label lipid rafts. Macrophages were infected with mCherry-expressing FRD1 or FRD1131 (red) for 30 minutes. Compared to uninfected, resting macrophages both FRD1 (Alg\(^+\)) and FRD1131 (Alg\(^-\)) were able to induce lipid raft formation. However, the Alg\(^-\) strain showed qualitatively more lipid raft staining.
CD11b and CD14 are important in the phagocytosis of *Pseudomonas aeruginosa* by murine macrophages and their binding is likely blocked by alginate

My previous studies indicated that alginate production decreased binding of *P. aeruginosa* to murine macrophages with a trend towards reduced lipid raft formation. This is possibly due to blocking specific receptor(s) critical to phagocytosis. The cell surface receptors CD11b (CR3) and CD14 have both been shown to be important for the phagocytosis of *P. aeruginosa* by macrophages and dendritic cells (29). To address the hypothesis that alginate interferes with receptor activation, I investigated how alginate affects interaction between *P. aeruginosa* and CD14 and CD11b. To determine if CD11b and CD14 are critical to the phagocytosis of mucoid and non-mucoid CF strains, MH-S macrophages were pretreated with specific blocking antibodies or an isotype control. Following antibody treatment, FRD1 or FRD1131 were added for 1 hour. After treatment with gentamicin, colony counts were performed to determine the amount of phagocytosis (Figure 21). When pretreated with α-CD11b or α-CD14, phagocytosis of FRD1131 was significantly inhibited but not entirely blocked. Phagocytosis of FRD1 was not inhibited further by the addition of receptor blocking antibodies, suggesting that these receptors may already be blocked by alginate. While these results do confirm the importance of CD11b and CD14 in the phagocytosis of FRD, this does not rule out the involvement of additional receptors for phagocytosis.
Figure 21. Number of FRD1 or FRD1131 phagocytized by MH-S macrophages in the presence of CD11b, CD14, or isotype-control blocking antibodies. MH-S macrophages were infected with FRD1 (A) or FRD1131 (B) for 1 hour in the presence of CD11b or CD14 blocking antibodies. CFU indicates the number of colony-forming units recovered from macrophages. Both antibodies significantly inhibited phagocytosis of FRD1131 compared to the isotype control. However, phagocytosis of FRD1 was not significantly inhibited by either antibody. Data are shown as mean and standard deviation of a representative triplicate experiment. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
To determine if additional receptors are involved in the phagocytosis of FRD CD11b and CD14 blocking antibodies were used together (Figure 22). Interestingly, while each antibody alone was able to inhibit phagocytosis of FRD1131 in Figure 21, the combination of these two antibodies significantly enhanced phagocytosis of both FRD1 and FRD1131. It is possible that by blocking both receptors, there is a synergistic signaling response that enhances phagocytosis.
Figure 22. Number of FRD1 or FRD1131 phagocytized by MH-S macrophages in the presence of CD11b and CD14, or isotype-control blocking antibodies. MH-S macrophages were infected with FRD1 (A) or FRD1131 (B) for 1 hour in the presence of CD11b and CD14 blocking antibodies. CFU indicates the number of colony-forming units recovered from macrophages. When treated with both antibodies together, phagocytosis of FRD1 and FRD1131 was significantly increased compared to the isotype controls. Data are shown as mean and standard deviation of three samples. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Intracellular signaling of murine macrophages is delayed and reduced by mucoid

*Pseudomonas aeruginosa*

It is possible that as a result of alginate inhibiting the binding of *P. aeruginosa* to macrophages, downstream signaling was affected. Previous studies have implicated both the PI3K and MAPK pathways in phagocytosis (37). Furthermore, CD11b and CD14 can contribute to PI3K signaling (54, 76, 80). To determine if alginate expression interferes with activation of signaling pathways involved in phagocytosis, the effect of FRD1 and FRD1131 on phosphorylation of AKT and ERK was determined. Alg+ FRD1 and Alg- FRD1131 were incubated with MH-S macrophages for various time points up to 1 hour. Cell lysates were prepared and activation of AKT was assessed by Western blot (Figure 23). FRD1131 induces p-AKT in MH-S as early as 15 minutes post infection whereas FRD1-induced activation is delayed to 30 minutes. The duration of AKT activation is also significantly lower with FRD1 compared to FRD1131 activated macrophages which maintained activation at 60 minutes post infection. Alginate production modulates AKT activation in MH-S macrophages and may contribute to the inhibition of phagocytosis that is observed.
Figure 23. Western blot indicating p-AKT and total AKT from MH-S macrophages after phagocytosis of FRD1 or FRD1131. Serum starved MH-S macrophages were infected with FRD1 (Alg⁺) or FRD1131 (Alg⁻) for up to 1 hour. Cell lysates were collected and a Western blot was performed using anti-p-AKT and anti-pan-AKT antibodies. FRD1 infection resulted in delayed AKT activation at 30 minutes compared to 15 minute activation with FRD1131. Data shown is a representative triplicate experiment.
In addition to AKT, ERK activation was also assayed by Western blot. Similarly to AKT activation, infection with FRD1 resulted in delayed and decreased ERK activation compared to infection with FRD1131 (Figure 24). While p-ERK was faintly detectable at 30 minutes in the FRD1 samples, FRD1131 induced ERK activation as early as 15 minutes post infection.

Taken together, these results show that alginate production by FRD1 leads to delayed and depressed activation of key signaling pathways relating to efficient phagocytosis.

**Phagocytosis of *Pseudomonas aeruginosa* by murine macrophages is dependent on PI3K, but not MEK, activation**

To confirm that AKT and ERK pathways are indeed important for the phagocytosis of *P. aeruginosa* by alveolar macrophages, the effect of specific pathway inhibitors on phagocytosis was evaluated. To confirm that the selected inhibitors effectively inhibited phosphoinositide 3-kinase (PI3K) and MAPK/ERK kinase (MEK) activation, MH-S macrophages were pretreated with wortmannin, LY294002, or U0126, for 30 minutes before the addition of FRD1131 for an additional 30 minutes. Cell lysates were prepared and activation of AKT and ERK was assessed by Western blot (Figure 25). The PI3K inhibitors, wortmannin and LY294002, successfully inhibited AKT activation at both concentrations tested. The MEK inhibitor, U0126, was able to inhibit ERK activation.
Figure 24. Western blot indicating p-ERK2 and total ERK1/ERK2 from MH-S macrophages after phagocytosis of FRD1 or FRD1131. Serum starved MH-S macrophages were infected with FRD1 (Alg⁺) or FRD1131 (Alg⁻) for up to 30 minutes. Cell lysates were collected and a Western blot was performed using anti-p-ERK and anti-pan-ERK antibodies. FRD1 infection resulted in decreased ERK activation at 30 minutes compared to FRD1131. Data shown is a representative triplicate experiment.
Figure 25. Western blots showing p-ERK2, total ERK1/ERK2, p-AKT, and total AKT from MH-S macrophages after phagocytosis of FRD1 or FRD1131 in the presence of wortmannin, LY294002, or U0126. Serum starved MH-S macrophages were infected with FRD1131 for 30 minutes in the presence of wortmannin (A), LY294002 (A), or U0126 (B). Cell lysates were collected and a Western blot was performed using anti-p-AKT (A), anti-pan-AKT (A), anti-p-ERK (B), and anti-pan-ERK (B) antibodies. Both wortmannin and LY294002 successfully inhibited AKT activation. Despite low baseline phosphorylation, U0126 was able to inhibit ERK activation.
MH-S macrophages were pretreated with wortmannin, LY294002, or U0126, for 30 minutes before the addition of FRD1, FRD1131, or PAO1. After treatment with gentamicin, colony counts were performed to determine the amount of phagocytosis (Figure 26). Both of the AKT inhibitors, wortmannin and LY294002, significantly inhibited phagocytosis of all *P. aeruginosa* strains tested. However the ERK inhibitor, U0126, did not inhibit phagocytosis of FRD1, FRD1131, or PAO1.

It is possible that ERK signaling may be dispensable for efficient phagocytosis of these strains. However, there are additional explanations for this observation. The threshold for ERK to contribute to phagocytosis could be very low or could be redundant, as there are many interacting signaling pathways. PI3K, and subsequently AKT, signaling is likely critical to phagocytosis of *P. aeruginosa*. Thus, alginate production by *P. aeruginosa* leads to reduced binding and depressed PI3K signaling, leading to inhibited phagocytosis.
Figure 26. Number of bacteria phagocytized by MH-S macrophages after 1 hour in the presence of AKT or ERK inhibitors. MH-S macrophages were infected with FRD1 (A), FRD1131 (B), or PAO1 (C) for 1 hour in the presence of wortmannin, LY294002, or U0126. CFU indicates the number of colony-forming units recovered from macrophages. Both AKT inhibitors, wortmannin and LY294002, significantly inhibited phagocytosis of FRD1, FRD1131 and PAO1. However, the ERK inhibitor U0126 was unable to inhibit phagocytosis of any strain tested. Data are shown as mean and standard deviation of a representative triplicate experiment. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Acid sphingomyelinase activity in murine macrophages is unaffected by mucoid *Pseudomonas aeruginosa*

Increased ceramide concentration within lipid rafts has been implicated as a critical mediator of phagocytosis of *P. aeruginosa* by epithelial cells and of latex beads by murine macrophages (23, 57). Ceramide is produced by acid sphingomyelinase (ASMase) and ASMase activity is increased during infection (23). However, another study showed that high ceramide concentration was associated with decreased *P. aeruginosa* killing in mice (3). *P. aeruginosa* LPS-induced Acid sphingomyelinase activity, and subsequently ceramide concentration, has been shown to be NF-κB dependent (85). As shown in Figure 23, AKT activation, which leads to NF-κB activation, is altered in the presence of alginate. Therefore, it is possible for ASMase activity to be affected by alginate production. To determine ASMase activity, MH-S macrophages were first serum starved for 6 hours, and then cultured with FRD1, FRD1131 or PAO1 for 15 minutes. Previous studies have indicated that ASMase activity peaks around 10 to 20 minutes following infection (23). Cell lysates were generated and ASMase activity was measured (Figure 27). ASMase activity was not significantly affected by the addition of *P. aeruginosa*, or the presence of alginate. Consequently, ceramide concentrations are likely to be unaffected by the presence of alginate. It is possible that while ASMase activity is NF-κB dependent, NF-κB may be sufficiently activated despite reduced AKT signaling.
Acid sphingomyelinase activity (pmol/hr/μg)

Strain

MΦ<sub>only</sub> FRD1 FRD1131 PAO1
Figure 27. Acid sphingomyelinase activity from MH-S macrophages after phagocytosis for 15 minutes. Serum starved MH-S macrophages were infected with FRD1, FRD1131, or PAO1 for 15 minutes. Macrophage lysates were collected and analyzed to determine ASMase activity. No significant difference in ASMase activity was induced by any strain compared to resting macrophages alone. Data are shown as mean and standard deviation of three separate experiments. Statistical significance is determined by Students t-Test (⋆; P < 0.05, **; P < 0.01, ***; P < 0.001).
IL-8 production by human macrophages is unaffected by mucoid *Pseudomonas aeruginosa*

Cytokine production plays an important role in promoting lung damage in cystic fibrosis (2, 6, 77). Large amounts of neutrophils traffic to the lung, leading to lung damage despite their inability to clear the infection. IL-8 is a chemokine produced by macrophages that functions as a major neutrophil chemoattractant. Mucoid *P. aeruginosa* inhibits AKT activation which could affect downstream activation of NF-κB activation. NF-κB is responsible for IL-8 production, therefore, cytokine production could be altered in macrophages due to altered binding, and signaling, from the presence of alginate (88). To determine if IL-8 production was affected by alginate production, an IL-8 ELISA kit was used. First, a time course of IL-8 production was established. THP-1 macrophages were cultured with FRD1, FRD1131 or 1 mg/ml zymosan for up to 8 hours. Supernatants were removed at 2 hour intervals and frozen for analysis (Figure 28). At every time point measured, IL-8 induced by FRD1 and FRD1131 appeared similar.
Figure 28. Time course of IL-8 produced by THP-1 macrophages after phagocytosis of FRD1 or FRD1131. THP-1 macrophages were infected with FRD1, FRD1131, or treated with zymosan for up to 8 hours. Supernatants were collected at various time points and analyzed by ELISA for IL-8 production. At each point measured, both FRD1 and FRD1131 induced similar amounts of IL-8.
To confirm the effect of alginate on the production of IL-8 by THP-1 macrophages, the ELISA was repeated using 8 hour cultures (Figure 29). As indicated, there was no significant difference in the amount of IL-8 induced by FRD1 compared to FRD1131. It is possible that the majority of IL-8 produced by macrophages was contact independent. If this were the case, the inhibited binding due to alginate would not affect IL-8 production. To determine if contact with *P. aeruginosa* was necessary for IL-8 production, FRD1 and FRD1131 were cultured in RPMI for 8 hours. After 8 hours supernatants were collected and applied to THP-1 macrophages for an additional 8 hours. As shown in Figure 29, the *P. aeruginosa* supernatants were able to induce IL-8 in THP-1 macrophages. There was no significant difference in the amount of IL-8 induced by the supernatants compared to the amount induced by the *P. aeruginosa* strains themselves. Therefore IL-8 production by THP-1 macrophages appeared to be contact independent and was unaffected by the production of alginate.
Figure 29. Amount of IL-8 produced by THP-1 macrophages 8 hours after phagocytosis of FRD1, FRD1131, or in the presence of bacterial supernatants. THP-1 macrophages were infected with FRD1, FRD1131, or treated with FRD1 or FRD1131 supernatants for 8 hours. Supernatants were collected and analyzed by ELISA for IL-8 production. No significant difference was found between the amount of IL-8 induced by FRD1 compared to FRD1131. Similarly, no significant difference was found between the amount of IL-8 induced by FRD1 and FRD1131 compared to IL-8 induced by the respective supernatants. Data are shown as mean and standard deviation of three separate experiments. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
MIP-2 production by murine macrophages is unaffected by mucoid *Pseudomonas aeruginosa*

Murine macrophages do not produce IL-8 and instead produce a similar neutrophil chemoattractant called MIP-2. I next wanted to assess MH-S macrophages for MIP-2 production in response to FRD1 and FRD1131. MH-S macrophages were cultured with FRD1 or FRD1131 for 8 hours. After incubation, supernatants were frozen for analysis by MIP-2 ELISA kit (Figure 30). As with IL-8 production, there was no significant difference in the amount of MIP-2 produced between FRD1 and FRD1131. Eight hour supernatants were again tested to determine contact dependence of MIP-2 production. There was no significant difference in MIP-2 production induced by the supernatants compared to the strains themselves.
Figure 30. Amount of MIP-2 produced by MH-S macrophages 8 hours after phagocytosis of FRD1, FRD1131, or in the presence of bacterial supernatants. MH-S macrophages were infected with FRD1, FRD1131, or treated with FRD1 or FRD1131 supernatants for 8 hours. Supernatants were collected and analyzed by ELISA for MIP-2 production. No significant difference was found between the amount of IL-8 induced by FRD1 compared to FRD1131. Similarly, no significant difference was found between the amount of IL-8 induced by FRD1 and FRD1131 compared to IL-8 induced by the respective supernatants. Data are shown as mean and standard deviation of three separate experiments. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
A possible problem with the use of 8 hour supernatants for an additional 8 hours is that the soluble, stimulatory factors in the supernatants would be highly concentrated immediately, whereas the wells with the *P. aeruginosa* strains added directly would slowly build up to that high concentration over the 8 hour incubation. As a more direct assessment of the contact dependence of MIP-2 production, 0.4 µm filter inserts were used to separate the added *P. aeruginosa* from the macrophages while allowing for soluble factors to diffuse throughout the media (Figure 31). There remained no significant difference in the amount of MIP-2 produced between FRD1 and FRD1131 with, or without, the filter. However, the addition of the filter significantly reduced the amount of MIP-2 produced by both FRD1 and FRD1131. The filter did not entirely eliminate MIP-2 production, suggesting that MIP-2 production is partially contact independent. Furthermore, based on the high concentration supernatants used in Figure 29 and 30, soluble factors can compensate for the lack of contact.
Figure 31. Amount of MIP-2 produced by MH-S macrophages 8 hours after phagocytosis of FRD1, FRD131, or in the presence of bacterial supernatants from bacteria separated by a filter. MH-S macrophages were infected with FRD1 or FRD131 either directly or above a filter for 8 hours. Supernatants were collected and analyzed by ELISA for MIP-2 production. No significant difference was found between the amount of IL-8 induced by FRD1 compared to FRD131. However, the amount of IL-8 induced by FRD1 was significantly higher than the amount induced by FRD1 when separated by a filter. Similarly, the amount of IL-8 induced by FRD131 was significantly higher than the amount induced by FRD131 when separated by a filter. Data are shown as mean and standard deviation of three separate experiments. Statistical significance is determined by Students t-Test (*; P < 0.05, **; P < 0.01, ***; P < 0.001).
Chapter 8
Discussion and Future Studies

The goal of this research was to determine the mechanism by which alginate, an exopolysaccharide secreted by mucoid Pseudomonas aeruginosa, inhibits phagocytosis by macrophages. The mucoid phenotype has been shown to enhance survival of P. aeruginosa and infection of the lung significantly increases the mortality rate of CF patients (8, 43, 68, 70). Therefore, understanding how alginate protects P. aeruginosa from phagocytosis is important for the treatment of cystic fibrosis lung infections. To address my goal, I investigated the effect of alginate by using two cell line models for phagocytosis. Initially, since cystic fibrosis is a human disease, the human monocytic cell line THP-1 was used as model for phagocytosis. Since alveolar macrophages have altered receptor expression, activation and survival compared to peripheral macrophages, I used the murine alveolar macrophage cell line MH-S to investigate phagocytosis by macrophages more appropriate to the lung infection environment (25, 56). The FRD strain of P. aeruginosa was used to address the effect of alginate on phagocytosis by these two cell lines. FRD1 is a mucoid cystic fibrosis clinical isolate and FRD1131 is a non-mucoid isogenic algD::Tn501-33 mutant. The only difference between these mutants is the production of alginate which allowed me to study the effect of alginate on phagocytosis. This research demonstrates for the first time that alginate on the bacterial surface inhibits phagocytosis by reducing binding to
macrophages and interfering with important phagocytosis receptors, resulting in decreased intracellular signaling and reduced phagocytosis.

Previous studies attempted to understand the role of alginate in resisting the immune response. Several groups suggested that mucoid P. aeruginosa are protected against non-opsonic phagocytosis (8, 43, 68). Likewise, Leid et al. (46) showed that mucoid P. aeruginosa have significantly enhanced biofilm survival against opsonic killing. These publications, however, did not quantitatively address the role of alginate in the non-opsonic phagocytosis of planktonic P. aeruginosa. My research demonstrates that alginate production by P. aeruginosa significantly protects against both opsonic and non-opsonic phagocytosis by human and murine macrophages. Phagocytosis was assessed through two assays that measured different aspects of phagocytosis. The total number of bacteria phagocytized was determined by lysing macrophages and counting CFUs. The fraction of macrophages engaged in phagocytosis was determined by using flow cytometry. It is also possible to use the mean fluorescence intensity (MFI) from the flow cytometry data to determine the relative number of GFP-expressing bacteria phagocytized by each macrophage. However, when performing the experiments presented here, the MFI data often revealed very little difference amongst the different strains or the negative control. It is possible that the system is unable to detect the difference in fluorescence from just a few bacteria. Another explanation could be that the amount of GFP within each bacterium could be inconsistent, depending on how long the protein has been expressed by an individual bacterium.

Alginate production and flagellum expression are inversely expressed in P. aeruginosa. Therefore, when considering the role of alginate in phagocytosis, it is also
important to consider the role of flagellum (78, 79). Mahenthiralingam et al. (50, 51) have shown that expression of flagellum significantly enhances phagocytosis by macrophages and conclude that flagellum is critical to internalization, but not binding. However, other studies indicated that motility, independent of flagellum expression and TLR signaling, is responsible for evasion of phagocytosis (1, 47). While I did observe decreased phagocytosis in the absence of flagellum expression, significant levels of phagocytosis occur in the absence of flagellum expression and motility. My results suggest that flagellum is expressed by FRD1, which enhances phagocytosis. This would mean that despite alginate production, indicating active σ\textsuperscript{22} and the down regulation of flagellum expression, flagellum is expressed enough to enhance phagocytosis. These results also disagree with the findings by the Berwin lab (1, 47), which conclude that motility, and not flagellum, is responsible for stimulating phagocytosis. It is possible that this disagreement is due to differences in the macrophages and \textit{P. aeruginosa} strains studied. While this study uses human and murine macrophages cell lines, their studies used human peripheral blood monocyte-derived macrophages. Primary human macrophages could express different amounts of TLR5, which could affect the amount of phagocytosis. For the motility studies, Lovewell et al. (47) and Amiel et al. (1) both use the \textit{P. aeruginosa} strain PA14. It is possible that the expression of ligands involved in phagocytosis, such as pilin or serotypes of LPS, could account for the observed differences.

Interestingly, when cultured together, alginate produced by FRD1 was unable to inhibit phagocytosis of non-mucoid FRD1131. This implies that alginate did not induce a negative signal within the macrophages because the presence of alginate in the media
would inhibit phagocytosis if this were the case. Also, alginate added exogenously was unable to protect non-mucoid *P. aeruginosa* from phagocytosis. Again, this suggests that alginate is not negatively regulating macrophages or else this would inhibit phagocytosis of non-mucoid FRD1131. Since alginate decreases *P. aeruginosa* binding to murine macrophages it is more likely that alginate interferes with a receptor-ligand interaction that is necessary for promoting phagocytosis.

In an attempt to identify a receptor(s) that may be blocked by alginate, I investigated the contribution of CD11b and CD14, which have been reported to facilitate phagocytosis of *P. aeruginosa* (29). CR3 has been shown to bind to a number of ligands associated with *P. aeruginosa* including LPS, APG, and β-glucan (7, 89). Blocking either CD11b or CD14 led to a significant decrease in phagocytosis of non-mucoid *P. aeruginosa* by murine macrophages, but this effect was absent with mucoid *P. aeruginosa*. This suggests that both of these receptors were being blocked completely by alginate and that further blockade with the antibodies was ineffective. Furthermore, phagocytosis of FRD1 or FRD1131 was not inhibited when both CD11b and CD14 antibodies were used together. It is possible that the antibodies did not completely block the receptors. This would allow for some function to remain and allow for phagocytosis, but this does not explain why phagocytosis was significantly enhanced when both antibodies were used. It is possible that the combination of antibodies resulted in stimulating or activating the macrophages. In an infection, it is likely that both of these receptors, and many others, would be simultaneously stimulated in a pathogen rich environment.
I also investigated the effects of alginate on the downstream signaling events contributing to efficient phagocytosis. Both CD11b and CD14 can activate PI3K and MEK1/2. CD14/TLR4 signaling through MyD88, IRAK1/4, and TRAF6 activate PI3K and MEK1/2 (71). CR3 can also lead to PI3K and MEK activation through PLC and protein kinase C (PKC) (26, 71, 83). AKT and ERK phosphorylation were used to measure PI3K and MEK1/2 activation. Both ERK and AKT activation were decreased by alginate production. While PI3K activation was found to be critical for phagocytosis of *P. aeruginosa*, loss of MEK activation had no significant effect. Reduced ERK activation by alginate may lead to altered inflammatory signaling despite not being necessary for phagocytosis. NF-κB activation occurs downstream of AKT phosphorylation and leads to ASMase activity (85). However, I found that ASMase activity was not altered in murine macrophages infected with mucoid *P. aeruginosa* compared to non-mucoid strains. This could be due to high ASMase activity in murine macrophages, or that the cells were not allowed to rest long enough before phagocytosis. In either case the ASMase activity in the negative control would be higher than expected. Similarly, I found no significant difference in the amount of IL-8 or MIP-2 induced by mucoid and non-mucoid *P. aeruginosa*. This could be because MIP-2 induction was found to be partially contact-independent, which is often the case in cytokine induction (84). It is possible that while ASMase activity and IL-8 production are NF-κB dependent, NF-κB may be sufficiently activated despite reduced AKT signaling. It is also possible that NF-κB activity is stimulated through a PI3K-independent pathway. This could be accomplished through other scavenger and pattern recognition receptor signaling.
While this dissertation presents many new findings, several avenues of research remain open relating to the effect of alginate production on phagocytosis. First, the addition of LPS O-side chain production to the FRD stain (FRD1810) and the addition of alginate production to the PAO strain (PDO300) do not lead to equal phagocytosis. Despite both strains producing alginate and LPS containing O-Side chains, PDO300 is phagocytized significantly more than FRD1810. There may be additional stimulatory factors present in PDO300, or addition inhibitory factors present in FRD1810, leading to altered phagocytosis. Further analysis of surface receptors present on both strains could reveal additional receptors present or lacking in the two strains. Additionally, I observed a trend of reduced induction of lipid raft formation by Alg⁺ FRD1 compared to Alg⁻ FRD1131. While I found no significant difference in the amount of ASMase activity induced between the two strains, it may be possible to quantify a difference in lipid raft formation or ceramide concentration. Furthermore, it would be interesting to determine if actin polymerization, and the formation of the phagocytic cup, is decreased when phagocytizing mucoid P. aeruginosa. Additionally, Leid et al. (46) established that alginate production also protects P. aeruginosa in a biofilm from opsonic phagocytosis. The experiments presented here could be repeated in the context of mucoid and non-mucoid P. aeruginosa in a biofilm (62). Finally, the research presented in this dissertation has focused on phagocytosis by human and murine macrophage cell lines. It would be beneficial to extend these studies by using primary human and murine macrophages. Primary human alveolar macrophages could be isolated from sputum of healthy donors and cystic fibrosis patients (73). Likewise, bronchoalveolar lavage fluid (BALF) can be acquired from mice and used to generate primary murine alveolar
macrophages (65). This would also allow for studies using CFTR knockout mice to evaluate phagocytosis by CFTR-deficient alveolar macrophages (24). Macrophages from CD11b and CD14 single, or double knockout mice could also be useful. This would allow for phagocytosis assays which could identify other important phagocytosis receptors. Other receptors found this way could again be confirmed by using macrophages from knockout mice.

Alginate may also relate to the clearance of apoptotic cells. It is possible that alginate interferes with binding of the lactadherin-phosphatidylserine complex to the αVβ3 integrin. αVβ3 integrin and CR3 have both been shown to bind HMGB1 (20, 89). It is conceivable then that if alginate is able to interfere with CR3, as I have shown to be possible in this study, then it could interfere with αVβ3 integrin as well. By doing so, alginate would inhibit phagocytic clearance of apoptotic cells (16). Thus, alginate could exacerbate the ineffective clearance of apoptotic neutrophils in the cystic fibrosis lung.

In summary, this dissertation demonstrated that alginate production by the cystic fibrosis clinical isolate of Pseudomonas aeruginosa, FRD1, inhibited phagocytosis by significantly decreasing binding to murine macrophages. Both CD11b and CD14 appear to be important for phagocytosis and may be blocked by alginate. Decreased binding leads to reduced downstream mediators of phagocytosis, including decreased AKT and ERK activation. To have this protective effect, alginate must be produced directly by the bacteria and did not appear to benefit other local non-mucoid bacteria. Understanding how alginate protects P. aeruginosa from phagocytosis is important for the treatment of lung infections commonly found in cystic fibrosis patients. By understanding the pathways involved in mediating efficient phagocytosis of clinical isolates, it may be
possible to develop a treatment to promote clearance by the resident alveolar macrophages. These experiments may also serve as a model to evaluate the effectiveness of such treatments. This approach also provides valuable insight into previously unknown macrophage phagocytosis mechanisms.
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Vita

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