Pseudomonas aeruginosa Evasion of Neutrophil Antibacterial Functions in Early Cystic Fibrosis Lung Infection

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Abstract

Chronic *Pseudomonas aeruginosa* (PA) airway infections in Cystic Fibrosis (CF) patients are associated with lung function decline and worse clinical outcomes. To prevent chronic infection, inhaled tobramycin (TOB) is commonly used to eradicate new-onset PA infections in CF children. However, up to 28-40% of patients fail TOB eradication therapy and the reasons for these treatment failures are still unclear. Neutrophils are the primary immune cells that are critical for PA eradication. PA clinical isolates can display strain-specific phenotypes that modulate innate immunity. For example, overproduction of exopolysaccharides (EPS) such as Psl, Pel and alginate, and loss of twitching motility have been shown to impede neutrophil-mediated clearance. In our studies, we tested PA isolates collected from a cohort of 39 CF children with new-onset PA infections who underwent TOB eradication therapy, where 30 patients had eradicated and 9 patients had persistent infection following TOB. We hypothesized that PA isolates' resistance to neutrophil antibacterial functions is associated with persistent infection in CF patients with new-onset infection, and that targeting strain-specific bacterial determinants of persistent PA isolates restores neutrophil antibacterial functions to improve PA eradication.

First, we assayed PA isolates for *in vitro* neutrophil phagocytosis and intracellular bacterial killing (OPK) using immortalized human dHL-60 cells. We observed that isolates from patients with persistent infections were more resistant to *in vitro* OPK than those with eradicated infections. Multivariable analysis showed that loss of twitching motility and mucoidy were significantly associated with resistance to *in vitro* OPK, and *in vitro* phagocytosis was a significant independent predictor of failed TOB.

Second, we further characterized *in vitro* neutrophil antibacterial functions and *in vivo*

clearance elicited by two pairs of representative Persistent (565P, 505P) and Eradicated isolates (513E, 558E). Using bone marrow-derived neutrophils, we observed lower bacterial adhesion to neutrophils in Persistent vs. Eradicated isolates, in conjunctions with impaired neutrophil OPK and degranulation. Using an *in vivo* murine lung infection model, 565P and 505P-infected mice failed to clear and resulted in higher bacterial burden at 48 and 96 h post-infection, compared to infections with Eradicated isolates (513E, 558E), which cleared completely by 96h. In addition, no differential neutrophil recruitment was detected in Persistent vs. Eradicated isolate infections. Our results thus suggest that Persistent isolates evade neutrophil-mediated antibacterial functions *in vitro* and fail to be cleared *in vivo*.

Finally, we tested the effect of monoclonal antibodies (mAb) for their ability to enhance neutrophil-mediated OPK against a subset of Persistent (n=7) and Eradicated (n=8) isolates. Psl0096, which targets EPS Psl, and MEDI3902, a bispecific mAb that targets both Psl and the type three secretion system PcrV, were equally effective at potentiating *in vitro* OPK. Moreover, using the same *in vivo* PA infection model, MEDI3902 treatment prior to infection with Persistent isolates (565P, 505P) resulted in reduced bacterial burden compared to IgG control. This suggests that MEDI3902 could be optimized as a potential therapeutic treatment to improve PA eradication.

Altogether, identifying bacterial determinants that contribute to persistence following TOB in early PA infection may help predict outcomes of PA eradication in CF children and provide a better understanding of host-pathogen interactions that are important for PA clearance. Our work also suggests the possibility that novel non-antibiotic therapies that potentiate neutrophil-PA interactions could be a treatment strategy to improve the outcome of PA eradication in CF patients.

Résumé

Les infections chroniques des voies respiratoires à *Pseudomonas aeruginosa* (PA) chez les patients atteints de fibrose kystique (FK) sont associées à une dégradation de la fonction pulmonaire et à une détérioration des résultats cliniques. La tobramycine inhalée (TOB) est utilisée pour éradiquer les nouvelles infections à PA chez les enfants FK. Cependant, jusqu'à 28-40% des patients échouent au traitement d'éradication.

Les neutrophiles sont les cellules immunitaires essentielles à l'éradication de PA. Les isolats cliniques de PA peuvent présenter des phénotypes spécifiques qui altèrent l'immunité innée. Il a été démontré que la surproduction d'exopolysaccharides (EPS) tels que Psl, Pel et alginate, et la perte de motilité par contraction entravent la clairance médiée par les neutrophiles. Nous avons testé des isolats PA provenant d'une cohorte de 39 enfants FK ayant subi un traitement d'éradication au TOB. 30 patients ont été éradiqués et 9 patients ont présenté une infection persistante après la TOB. Nous avons émis l'hypothèse que la résistance des isolats PA aux fonctions antibactériennes des neutrophiles est associée à la persistance de l'infection chez les patients FK et en ciblant des déterminants bactériens spécifiques à la persistance nous pourrons rétablir les fonctions antibactériennes des neutrophiles.

Nous avons d'abord testé *in vitro* la phagocytose des neutrophiles et la destruction bactérienne intracellulaire (OPK) sur des cellules humaines immortalisées dHL-60, à partir d'isolats PA. L'analyse a montré que la perte de motilité par contraction et la mucoïdie étaient significativement associées à la résistance à l'OPK, et que la phagocytose était un facteur prédictif indépendant significatif a l'échec de la TOB.

Ensuite, nous avons caractérisé l'OPK *in vitro* des neutrophiles et la clairance *in vivo* provoquée par deux paires d'isolats persistants (565P, 505P) et éradiqués (513E, 558E). En

utilisant des neutrophiles dérivés de la moelle osseuse, nous avons observé une plus faible adhésion bactérienne aux neutrophiles chez les isolats persistants par rapport aux isolats éradiqués, une altération de l'OPK et de la dégranulation des neutrophiles. En utilisant un modèle d'infection pulmonaire murine, les souris infectées par les isolats persistants n'ont pas éliminé les bactéries 48 et 96 heures après l'infection, par rapport aux souris infectées par les isolats éradiqués, qui ont complètement éliminé l'infection en 96 heures. Aucun recrutement différentiel de neutrophiles n'a été détecté dans les infections d'isolats persistants ou éradiqués. Nos résultats suggèrent que les isolats persistants échappent aux fonctions antibactériennes des neutrophiles *in vitro* et ne sont pas éliminés *in vivo*.

Enfin, nous avons testé l'effet des anticorps monoclonaux (mAb) pour leur capacité à augmenter l'OPK médié par les neutrophiles contre d'isolats persistants et éradiqués. Psl0096, qui cible l'EPS Psl, et MEDI3902, un mAb bispécifique qui cible à la fois Psl et le système de sécrétion de type 3 PcrV, ont été aussi efficaces l'un que l'autre pour potentialiser l'OPK *in vitro*. En utilisant le même modèle d'infection PA *in vivo*, le traitement par MEDI3902 avant l'infection d'isolats persistants a permis de réduire la charge bactérienne par rapport au contrôle IgG. Cela suggère que MEDI3902 pourrait être optimisé en tant que traitement thérapeutique potentiel pour améliorer l'éradication de PA.

Dans l'ensemble, l'identification des déterminants bactériens qui contribuent à la persistance de PA après la TOB peut aider à prédire les résultats de l'éradication de PA chez les enfants FK et permettre de mieux comprendre les interactions hôte pathogène qui sont importante pour la clairance de PA. Nos travaux suggèrent également que de nouvelles thérapies non antibiotiques qui potentialisent les interactions neutrophiles-PA puissent être une stratégie de traitement pour améliorer les résultats de l'éradication de PA chez les patients FK.

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First and foremost, I thank my supervisor, Dr. Dao Nguyen, for supporting me through my entire degree with endless patience and enthusiasm. You have taught me so much in the past 7 years during my Ph.D, from critical and analytical thinking regarding my projects to scientific writing. Under your guidance, I have grown from an ambitious nervous graduate student to finally able to slow down, think, and process information properly before speaking and acting. All the while, I have learned to take the initiative in everything in order to achieve certain goals or to push our projects forward. I greatly appreciate all our conversations about what it takes to be a good scientist.

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Last but not least, I would like to thank my family for their tremendous support throughout my many years of studies and my life. Thank you, my best friend Aparna Dintakurti, Mellissa Gaudet, John Lin, and Paul J. Przytocki. I cannot thank you all enough!

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List of abbreviations

ΔF508: the deletion of phenylalanine

AdIkBdn: the dominant negative IkBα adenovirus

AEC: airway epithelial cell

ANOVA: analysis of variance

ASL: airway surface liquid

ATP: adenosine triphosphate

AUC: area under the curve

BALF: bronchoalveolar lavage fluid

BCA: bicinchoninic acid

BMDM: bone marrow-derived macrophages

BMDN: bone marrow-derived neutrophils

BMI: body mass index

BSA: bovine serum albumin

C1q: complement component 1q

C3: complement component 3

C5: complement component 5

C7: complement component 7

C9: complement component 9

cAMP: cyclic adenosine monophosphate

CCR2: C-C chemokine receptor type 2

CD: cluster of differentiation

CF: cystic fibrosis

CFRD: CF-related diabetes mellitus

CFTR: cystic fibrosis transmembrane conductance regulator

CFU: colony forming unit

CI: confidence interval

CR: complement receptor

CV: crystal violet

CXCL: C-X-C motive chemokine

CXCR1: chemokine (C-X-C motif) receptor 1

C-di-GMP: cyclic diguanylate

DAMP: damage-associated molecular pattern

DC: dendritic cell

dHL-60s: differentiated HL-60 cells (neutrophil-like cells)

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

EGTA: ethyleneglycoltetraacetic acid

ELISA: enzyme-linked immunosorbent assay

ENaC: amiloride sensitive epithelial sodium channel

EPIC: early Pseudomonas infection control

EPS: exopolysaccharides

ETI: elexaftor, tezacaftor, ivacaftor

ExoS: exoenzyme S

ExoT: exoenzyme T

ExoU: exoenzyme U

ExoY: exoenzyme Y

FACS: fluorescence-activated cell sorting

FBS: fetal bovine serum

FcR: Fc receptor

FEV_1: forced expiratory volume in the one second

G-CSF: granulocyte colony stimulating factor

G551D: glycine-to-aspartate non-synonymous substitution at position 551

GI: gastrointestinal

GM-CSF: granulocyte-macrophage colony stimulating factor

GTP: guanosine triphosphate

h: hours

HBSS: hanks' balanced salt solution

HOCl: hypochlorous acid

h.p.i.: hour post infection

ICC: intraclass correlation

IFN: interferon

Ig: immunoglobulin

IL: interleukin

IP-10: CXCL 10

i.p: intraperitoneal

IQR: interquartile range

KC: keratinocyte chemoattractant

LB: luria bertani

LDH: lactate dehydrogenase

LIF: leukemia inhibitory factor

LIX: CXCL 5

LOD: limit of detection

LPS: lipopolysaccharide

Ly6G: lymphocyte antigen 6 complex, locus G

mAb: monoclonal antibody

MALDI-TOF: matrix assisted laser desorption ionization-time of flight mass spectrometry

$MgCl_2$: magnesium chloride

MCP-1: monocyte chemoattractant protein 1

M-CSF: macrophage colony stimulating factor

MIC: minimal inhibitory concentration

MIG: CXCL 9

min: minutes

MIP-1α: macrophage inflammatory protein 1α

MOI: multiplicity of infection

MPO: myeloperoxidase

MRSA: methicillin-resistant *Staphylococcus aureus*

MSSA: methicillin-sensitive *Staphylococcus aureus*

MyD88: myeloid differentiation primary response gene 88

NADPH: nicotinamide adenine dinucleotide phosphate

NaCl: sodium chloride

NE: neutrophil elastase

NET: neutrophil extracellular trap

NF-κB: nuclear factor κB

NK: natural killer

NLRP: nod-like receptor caspase recruitment domain containing protein

NO: nitric oxide

NOD: nucleotide-binding oligomerization domain

OD: optical density

OPK: opsonophagocytic killing

OprF: outer membrane porin F

PA: *Pseudomonas aeruginosa*

PAMP: pathogen associated molecular pattern

PBS: phosphate buffered saline

p.i.: post infection

PIP3: phosphatidylinositol 3,4,5-triphosphate

PMA: phorbol myristate acetate

PRR: pathogen recognition receptor

PVDF: polyvinylidene fluoride

QS: quorum sensing

RANTES: chemokine ligand 5

RFI: relative fluorescence intensity

RI-MUHC: Research Institute of McGill University Health Centre

RNA: ribonucleic acid

ROS: reactive oxygen species

r.p.m: revolutions per minute

SA: *Staphylococcus aureus*

SD: standard deviation

SDS: sodium dodecyl sulfate

SEM: standard error of the mean

Spp.: species

SP: surfactant protein

T3SS: type 3 secretion system

T4P: type four pilus

TBS-T: Tris-Buffered Saline with Tween 20

TCA: trichloroacetic acid

Th: T helper cells

TLR: toll like receptor

TMB: 3, 3', 5, 5'-tetramethylbenzidine

TNF-α: tumor necrosis factor α

TRIF: TIR-domain-containing adapter-inducing interferon-β

U.S.: united states

VAP: ventilator-associated pneumonia

VBMM: Vogel-Bonner minimal medium

VEGF-A: vascular endothelial growth factor A

YEM: yeast extract media

WT: wild-type

Contribution to original knowledge

Chapter 1: Introduction

Sections **1.3.3** and **1.3.4** are partially adapted from:

Faure E, Kwong K, Nguyen D. Pseudomonas aeruginosa in Chronic Lung Infections: How to Adapt Within the Host? Frontiers in Immunology. **2018**; 9:1416

In sections 1.3.3 and 1.3.4, we have reviewed how PA utilizes multiple strategies to evade host defenses and modulate inflammatory responses during chronic infection. These interactions between PA and host are thus major determinants in the pathogenesis of chronic PA lung infections. These sections highlight bacterial factors that are present and absent during chronic CF infection, as well as how therapeutic treatments that target pathogen-specific antigens can improve PA eradication in people with CF.

Chapter 2: Failed Eradication Therapy of New-Onset *Pseudomonas aeruginosa* Infections in Children with Cystic Fibrosis Is Associated with Bacterial Resistance to Neutrophil Functions

(Originally published in Kwong K, Benedetti A, Yau Y, Waters V, Nguyen D. Failed Eradication Therapy of New-Onset *Pseudomonas aeruginosa* Infections in Children with Cystic Fibrosis Is Associated with Bacterial Resistance to Neutrophil Functions. Journal of Infectious Diseases. **2022**; 225(11):1886–1895.)

We characterized multiple bacterial phenotypes and examined neutrophil OPK with dHL-60 cells using a cohort of PA isolates collected from patients with new-onset PA infections to identify key determinants that may predict persistence. We found that strain-specific phenotypes such as loss of twitching motility and overproduction of alginate were correlated with impaired

neutrophil OPK. By using multivariable analysis, we observed that *in vitro* neutrophil OPK was a significant independent predictor of persistent infection. Altogether, our *in vitro* findings and multivariable analyses suggest that PA isolates exhibit strain-specific susceptibility to neutrophils may be a mechanism to target for the prevention of persistent infection.

Chapter 3: Evasion of neutrophil-mediated bacterial clearance in *Pseudomonas aeruginosa* isolates from new-onset infections in cystic fibrosis children (In preparation for submission)

We demonstrated that PA isolates exhibit strain-specific phenotypes such as loss of pilus-mediated twitching motility and wrinkly colony morphology (overproduction of Psl and Pel) from CF patients with failed eradication (Persistent isolates) evaded *in vitro* murine neutrophil OPK by inhibiting C3 complement deposition and bacterial adhesion to neutrophils compared to those with successful eradication (Eradicated isolates). This led to failed *in vivo* bacterial clearance that was independent of cytokine and chemokine responses. Furthermore, we showed that complementation of PilA in a Persistent isolate that lacked pilus-mediated twitching motility significantly restored the isolate's response to neutrophil OPK, indicating that understanding PA-neutrophil interactions will help us gain insights to further the development of therapeutics to improve PA eradication.

Chapter 4: Monoclonal antibodies targeting Psl and PcrV potentiate *in vitro* neutrophil opsonophagocytic killing and *in vivo* bacterial clearance of *Pseudomonas aeruginosa* isolates from cystic fibrosis children that failed tobramycin eradication therapy

(In preparation for submission)

We tested the efficacy of mAb (Psl0096, MEDI3902) treatment for their ability to potentiate neutrophil-mediated bacterial clearance against representative Persistent isolates that overproduced Psl and were PcrV positive. First, we showed that Psl0096 and MEDI3902 were equally effective at enhancing *in vitro* neutrophil OPK in response to a subset of PA isolates. We then observed that the potentiation of neutrophil intracellular killing of PA isolates by MEDI3902 was specific to PcrV expression. Finally, we demonstrated that *in vivo* bacterial burden was significantly reduced by MEDI3902 and conferred protection in mice infected with two representative Persistent isolates at 24 h p.i., suggesting that further optimization is required to achieve complete bacterial clearance using this promising mAb MEDI3902.

Contribution of authors

Chapter 1: Introduction

Sections **1.3.3** and **1.3.4** are partially adapted from:

Faure E, Kwong K, Nguyen D. Pseudomonas aeruginosa in Chronic Lung Infections: How to Adapt Within the Host? Frontiers in Immunology. **2018**; 9:1416

In the original publication, KK reviewed and contributed to sections on "Flagellin and Flagella Motility", "Secreted Proteases", "Exopolysaccharides (EPS)", "Biofilm Lifestyle" and "Genetic Adaptation During Chronic Infection". EF and DN wrote and edited the manuscript.

Chapter 2 Failed Eradication Therapy of New-Onset *Pseudomonas aeruginosa* Infections in Children with Cystic Fibrosis Is Associated with Bacterial Resistance to Neutrophil Functions

Kelly Kwong, Andrea Benedetti, Yvonne Yau, Valerie Waters, and Dao Nguyen

KK performed *in vitro* bacterial phenotypic assays and *in vitro* neutrophil OPK assays. AB assisted KK with statistical analyses. YY and VW developed and oversaw the SickKids early PA Eradication study and contributed PA clinical strains. DN and KK designed all the studies, wrote, and edited the manuscript.

Chapter 3 Evasion of neutrophil-mediated bacterial clearance in *Pseudomonas aeruginosa* isolates from new-onset infections in cystic fibrosis children

Kelly Kwong, Annie Beauchamp, Julien K. Malet, Ines Levade, Lucia Grana, Yvonne Yau, David Guttman, Patricia L. Howell, Valerie Waters and Dao Nguyen

KK cultured and characterized the phenotypes (Psl surface expression by ELISA, C3 complement deposition) of all bacterial isolates (unless specified), performed all *in vitro*

experiments such as primary murine neutrophil isolation, neutrophil OPK assays, bacterial adhesion assay, neutrophil CD63 surface expression, neutrophil intracellular ROS production, PilA complementation in Persistent isolate, processed mouse samples for cytokine profiling, flow cytometry and analyzed all experimental data. AB performed murine pulmonary intratracheal infections. KK assisted AB with mouse lung, BALF, and blood collection. JKM developed protocol for image analysis using ICY automated cell counting software. IL and LG performed comparative genome analysis. PLH contributed PA laboratory strains. YY and VW contributed PA clinical strains. DN assisted KK in the design of all the studies, oversaw analyses, wrote and edited the manuscript.

Chapter 4 Monoclonal antibodies targeting Psl and PcrV potentiate *in vitro* neutrophil opsonophagocytic killing and *in vivo* bacterial clearance of *Pseudomonas aeruginosa* isolates from cystic fibrosis children that failed tobramycin eradication therapy

Kelly Kwong, Annie Beauchamp, Chloe Pereira-Kelton, Yvonne Yau, Patricia L. Howell, Antonio DiGiandomenico, Valerie Waters and Dao Nguyen

KK performed *in vitro* neutrophil OPK assays with and without antibody treatment, Psl surface expression by ELISA, immunoblotting of PcrV, and analyzed all the experimental data. CPK assisted KK in testing *in vitro* neutrophil OPK assays with antibody treatments. A. Beauchamp performed murine pulmonary intratracheal infections. KK assisted AB with mouse lung and blood collection and processed lung samples. PLH contributed PA strains. AD contributed mAbs Psl0096, V2L2, MEDI3902 and R347 isotype control. YY and VW contributed PA clinical strains. DN assisted KK in the design of all the studies and data interpretation.

Chapter 1: Introduction

1.1 Cystic Fibrosis

1.1.1 History and epidemiology of cystic fibrosis (CF)

Cystic fibrosis (CF) is a multi-systemic autosomal-recessive genetic disease caused by mutations in Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). CF was initially recognized in infants with pancreatic insufficiency who failed to thrive in early childhood [1,2]. Among different ethnicities, the incidence of CF varies among Caucasian, First Nation, African, Asian, South Asian, and Hispanic populations. CF affects up to 4300 individuals in Canada, with a national incidence rate of 1 in every 3000 newborns, and Caucasians account for 93.2% of the Canadian CF population [3]. With improved management and treatments of CF, the life expectancy of people with CF has significantly increased over the past four decades, with a current median survival age of 53.1 in the US and 55.4 in Canada [3,4].

1.1.2 CFTR mutations and function

Defective CFTR (cystic fibrosis transmembrane conductance regulator) function is caused by mutations in the gene *cftr* on the long arm of chromosome 7 [5,6], with more than 2500 mutations identified since 1989 [4]. CFTR functions as an apical anion channel that transports chloride and bicarbonate ions across the surface of epithelial cells, and contains a ATP-binding regulatory domain [7,8]. CF mainly affects the respiratory and digestive systems. It also causes salty sweat due to impeded chloride absorption in the CF sweat glands, resulting in an increased concentration of NaCl on the skin's surface. Therefore, patients can be diagnosed with a sweat test [9].

CFTR alleles can be classified into 6 classes and disease severity varies depending on the functional consequences of the mutations, as summarized in Table 1.1. Among the 6 different classes of CFTR, class I and class II mutations result in the most severe CF phenotypes compared to others, due to a significantly reduced number of CFTR. In contrast, class IV and class V are considered less severe since CFTR undergoes proper biosynthesis and transport to the apical membrane, while class III and class VI can be varied in severity due to the protein's stability and very little functional CFTR is made [10,11]. Of note, the ΔF508 is the most common CFTR genotype and accounts for up to 88.4% of the top 10 most common CFTR mutations [3].

1.1.3 Extrapulmonary clinical manifestation of CF disease

CF affects multiple organs where CFTR is expressed, with manifestations in the gastrointestinal (GI) tract, vas deferens, pancreas, liver, sweat gland, and lung. The prevalence of sinus disease also increases in young CF adults and remains high throughout their life [3]. Defective CFTR in the GI tract results in poor growth. Exocrine pancreatic insufficiency leads to malabsorption, including of fat-soluble vitamins, while endocrine pancreatic insufficiency contributes to CF-related diabetes [12]. Moreover, hepatic steatosis, cholelithiasis, ductal stenosis, and focal biliary cirrhosis are also complications that can arise from the GI tract in people with CF [13–16]. Finally, 98% of males with CF are infertile due to obstruction of the vas deferens.

1.2 The pathogenesis of CF lung disease

Overview

Chronic lung disease is the major cause of morbidity and mortality in people with CF. Throughout the course of their lifetime, patients experience a gradual decline in pulmonary function, resulting in 80-95% of all deaths. Common chronic symptoms of CF lung disease are coughing, breathlessness, and sputum production. Additionally, CF patients also experience pulmonary exacerbations where their respiratory symptoms worsen acutely and often result in respiratory insufficiency, weight loss, and overall reduced well-being [17].

CF lung disease is characterized by a vicious cycle of mucus plugging, persistent infection, and unresolved inflammation leading to lung destruction in the form of bronchiectasis [18,19]. Many observational clinical studies, as well as *in vitro* and *in vivo* studies using CF disease models such as the CF pig have been performed to understand the pathogenesis of CF lung disease. Although CFTR dysfunction is responsible for CF lung disease, early events leading to the development of CF lung pathology remain to be fully understood. This section summarizes the following aspects related to the pathogenesis of CF lung disease: CFTR-related intrinsic and acquired defects on airway pathophysiology, the role of infection, and inflammation in the pathogenesis of CF lung.

1.2.1 Impact of CFTR dysfunction on airway defenses

Mucociliary clearance is one of the critical defense mechanisms of the lower respiratory tract against foreign pathogens. The mucociliary clearance system includes airway surface liquid (ASL), which is composed of a mucus layer contacting the air and covering a periciliary layer. It

is responsible for trapping bacteria, viruses, and other foreign particles entering the lower respiratory tract [20]. CFTR also regulates the activity of the epithelial sodium channel ENaC. CFTR dysfunction results in sodium absorption through ENaC [21]. Overexpression of ENaC in mice results in certain aspects of lung pathology similar to CF [22,23], including thinning and dehydration of ASL, leading to the secretion of hyper-viscous mucus, which becomes immobilized and tightly bound to the epithelial surface [24]. Dysfunctional CFTR also impairs the activity of secreted antimicrobial peptides. It has been shown that CFTR functions as a bicarbonate transporter that buffers the pH in the airways [25]. It is possible that abnormal bicarbonate transport leads to acidic pH, which may impair bacterial killing of highly pH-sensitive antimicrobial defensins. In a CF pig model, ASL is abnormally acidic due to the absence of bicarbonate secretion into the airway lumen compared to non-CF pigs [26], and various antimicrobial peptides such as β-defensin-3, LL-37, lactoferrin and lysozyme within the CF airways are compromised [27–30]. However, others have reported that antimicrobials in the airways of CF pigs are not impaired [31]. Whether these findings hold true in people with CF also remains unclear, as several studies also suggest that the pH of ASL in newborns with CF is similar to those of healthy individuals [32–34].

1.2.2 The acquisition and prevalence of different pathogens in CF lung disease

The airways of CF patients can be colonized by different microorganisms as early as infancy [3] (Figure 1.1), and infections often become chronic as patients age. Although impaired mucociliary clearance and host-mediated antimicrobials create opportunities for microbial colonization, it is still unclear why CF patients are more susceptible to some microorganisms than others.

Some of the classical CF pathogens are *Staphylococcus aureus* (SA), *Haemophilus influenzae, Pseudomonas aeruginosa* (PA), *Burkholderia spp,* and the prevalence of these pathogens varies with age. While *H. influenzae* and SA are the most prevalent pathogens that infect CF children, as patients transition into adulthood, PA becomes the predominant pathogen and infects up to 50% and 32% of CF patients in the US and Canada, respectively [3,4]. Other prevalent pathogens, such as *Burkholderia cepacia complex spp.* (with certain genomovars causing fatal acute pneumonia and associated with higher mortality than other pathogens), *Stenotrophomonas maltophilia, Achromobacter xylosoxidans*, non-tuberculosis *Mycobacteria* spp., and *Aspergillus fumigatus* (associated with increased pulmonary exacerbations) can also be found in older CF patients [35–37].

CF airway infections are widely recognized as polymicrobial, although the clinical significance and implications of these polymicrobial communities remain poorly understood. PA and SA co-infections have been of interest, with several studies studying these polymicrobial interactions in the context of CF lung infections and their impacts on the host [38–40]. More broadly, the CF lung microbiome is shaped by age, antibiotics, and CFTR treatments. Recently, few studies have observed that a lower microbial diversity is associated with poorer pulmonary function in older CF patients and a higher risk of receiving lung transplantation [41–43].

1.2.3 Inflammation in CF lung disease

Airway epithelial cells

Epithelial cells are typically the first cell type in the lower respiratory tract to come into contact with inhaled microbes. They are normally protected from bacterial invasion as a result of tight-junctions which maintain the epithelial layer structural integrity, mucociliary clearance, and

antimicrobial peptides produced by themselves or other immune cells [44]. Airway epithelial cells (AECs) express receptors such as toll-like receptors (TLRs) and pathogen recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) (e.g., peptidoglycans, lipopolysaccharides (LPS)) and danger-associated molecular patterns (DAMPs) (e.g., extracellular heat shock proteins, elastases). TLR 2 and 5 are located on the apical surface, nucleotide oligomerization domain (NOD)-like receptors are within the cytoplasm, and TLR 3, 7, and 9 are within the endosomal compartment [45]. Additionally, TLR 4 is expressed at the cell surface and within the endosome when it signals or comes in contact with an adapter protein TRIF [46]. Following recognition of different microbes, the downstream signaling pathways such as MyD88 and NF-κB are activated sequentially to initiate bacterial clearance via the production of proinflammatory cytokines and chemokines, such as IL-6 and IL-8 [47], which will recruit innate immune cells such as neutrophils. Moreover, lysozyme secretion by submucosal glands may also play a role in host airway defense against bacteria [11].

Airway epithelial cells in the CF lung have been linked to a hyper-inflammatory state. A body of literature suggests that increased expression of TLR 2 and TLR 5 correlates with elevated inflammatory responses due to infection and/ or CFTR-associated intrinsic defects [48,49]. Others have also shown that the NF-κB signaling pathway is constitutively activated due to CFTR defects [48], which heightens the secretion of proinflammatory cytokines and chemokines (IL-6, IL-8), leading to unresolved neutrophil recruitment.

Neutrophils

Neutrophils play a complex and incompletely understood role in the pathogenesis of chronic infection and lung disease in CF and are often considered a double-edged sword. Excess

neutrophils that persist in the airways are a main source of inflammation that contributes to the pathogenesis of CF lung disease [51,52]. Free neutrophil elastase (NE) activity detected in BALF is associated with early bronchiectasis in children with CF [53]. In addition, ineffective neutrophil-mediated antibacterial killing mechanisms such as reactive oxygen species (ROS), neutrophil extracellular traps (NETs), and degranulation activity also contribute to pulmonary inflammation and lung destruction [54–57]. CFTR has been detected in the phagolysosomal compartment of neutrophils after phagocytosis [58]. However, it has been difficult to determine whether defects in neutrophil-mediated bacterial clearance are caused by CFTR-associated intrinsic defects or by extrinsic factors from the CF inflammatory lung milieu since most studies have been done in *vitro* and animal models, and the functions of neutrophils can be shaped by multiple complex factors linked to CF lung disease and infections.

While excess neutrophilic inflammation is considered deleterious, neutrophils are also professional phagocytes critical for bacterial clearance. Bacterial entry into neutrophils is accomplished by active phagocytosis [59–61]. This engulfment process is mediated by bacteria-to-phagocyte contact, including receptors presented on neutrophils, ligand expression, and exposure on pathogens. Neutrophils express both phagocytic and non-phagocytic PRRs. TLRs and NOD-like receptors 1 and 2 are non-phagocytic PRRs that recognize PAMPs, including LPS, peptidoglycan, flagellin, or pilin [62,63]. Phagocytic PRRs, on the other hand, such as CR3, 4, CR1q, and FcR are highly expressed upon binding with complement mediators and stimulation with gram-positive or negative bacteria [64], while C-type lectin PRRs (e.g., Dectin-1) are essential for fungal recognition [65]. Opsonins such as complement and IgG are critical to facilitate effective opsonophagocytic killing (OPK) through the activation of the classical (complement mediators, e.g., C3, C5, C7, C9 binding of CRs), or alternative pathway (antibody-

binding of the FcR) [64,66]. Complements and IgG can be deposited onto the bacterial surface to initiate receptor engagement. This engagement process leads to the recruitment and phosphorylation of kinase signaling molecules, activation of cytoskeletal arrangement proteins Rho and Rac GTPases, and actin-related proteins that are essential for phagocytosis [67,68]. During phagocytosis, the cellular consumption of molecular oxygen O_2 is increased and further utilized by an NADPH-oxidase to generate ROS [69,70]. Reactive metabolite HOCl is highly bactericidal. Its formation requires myeloperoxidase (MPO), an enzyme that is part of the azurophil or primary granule of neutrophils [71]. Other ROS-independent antimicrobials, such as defensins and lactoferrin, are components of the primary granules [72], and are highly reactive against different microbes. Once the pathogen is trapped within the cell, it forms a membrane-enclosed phagosome and will be further processed through the fusion of different granules (primary, gelatinase, and specific granules) to form a mature phagolysosome where pathogens will be killed intracellularly [73]. Alternatively, reactive metabolites and potent antimicrobial products can also be released into the extracellular milieu, where pathogens will be killed extracellularly [74].

Macrophages and other immune cells

Maintenance of immunological and physiological homeostasis, such as efferocytosis of apoptotic and necrotic cells and recycling of surfactant molecules, are all critical functions of macrophages [75]. Dysfunctional CFTR also impacts macrophages to a degree that is similar to neutrophils [76]. Defective CFTR causes chloride conductance abnormalities [50,77,78], hyper-inflammatory responses due to increased secretion of TNFα, IL-1β, IL-6, and IL-8 [79], as well as impairment in phagocytosis, acidification, and ROS-mediated antibacterial functions [80,81].

Clinical studies have reported that CF macrophages in uninfected individuals are intrinsically hyper-responsive and elicit dysregulated inflammatory responses to infectious and non-infectious stimuli compared to non-CF macrophages. In a CFTR knockout murine model, the activity of different antibacterial molecules produced by CF macrophages is only suboptimal, possibly due to CFTR defects [76]. Interestingly, a study showed that ROS-mediated antibacterial functions were not impaired in primary human CF macrophages compared to non-CF macrophages [82]. Similar results were also reported by Keiser et al. using a CF ferret model [83]. Although this controversy has not been resolved, these findings indicate that differences observed in these studies may be attributed to the complex host environments.

Other immune cells, such as dendritic cells (DCs), lymphocytes, natural killer cells (NKs), and T-cells, are all critical in orchestrating the balance between innate and adaptive responses in non-CF, but little is known about their role in CF [84]. For example, DCs are antigen-presenting cells that can present bacterial antigens to activate T-cells, which further induce either Th1 or Th2 T-cell responses. However, CF DCs are less characterized due to the limited number of DCs in the CF lung and different subtypes of DCs in response to different cytokines. Furthermore, findings from others have shown that murine CF DCs elicit delayed differentiation following PA lung infection in mice [85]. Thus, its implication in CF remains to be determined [86].

1.3 *Pseudomonas aeruginosa* (PA) infection in CF

1.3.1 Natural history of PA infection

PA is a versatile bacterium and can be found in a wide range of environments, such as in soil, water pipes, sewage, or on the surface of sinks, toilets, and medical equipment. People who are immune-compromised, and those with genetic or acquired impaired host defenses (e.g., CF,

burn wound) are all susceptible to PA infections [87]. A recent study reported that up to 41% of CF individuals with new-onset PA infections shared strains with other CF patients from attending the same CF center, and that patient-to-patient transmission likely occurs in about one-third of patients with shared strains [88,89].

Children and young CF adults typically acquire new-onset PA infections as early as 6 months old, and infections can be spontaneously eradicated with or without the help of antibiotics. People with CF can be infected with PA strains that are phenotypically diverse [90]. Multiple studies have reported that PA isolates collected from children's sputum cultures exhibit up to 4 morphologically distinct colonies (also termed morphotypes), while others can also be infected with just 1 distinct morphotype [91,92] (Figure 1.2). As children transition into adulthood, up to 50% of individuals are chronically infected with PA. During chronic infection, although the recovery of a dominant PA strain becomes more frequent [90], PA can genotypically and phenotypically evolve and adapt to the host environment, allowing them to thrive and persist within the host for decades. Thus, chronic PA infections in the airways of CF are associated with lung function decline and worse clinical outcomes, and are typically impossible to eradicate even with aggressive antibiotic treatments.

1.3.2 Innate immune responses to PA in the CF airways

AECs in response to PA

AECs respond to PA infections in a MyD88-dependent manner to produce proinflammatory cytokines and chemokines such as IL-6 and IL-8, or KC in mice. Recognition of TLR 4 and 5 in response to PA LPS and flagellin are sufficient to induce these cytokine responses [47]. Each PA-specific PAMP can be recognized by its respective PRR (Table 1.2).

Studies have reported that MyD88 knockout mice fail to eradicate PA infections and show delayed and inadequate recruitment of neutrophils [93]. In addition, NF-κB, downstream signaling of MyD88 has also been implicated in aiding PA clearance. An *in vivo* study illustrated that mice treated with AdIkBdn (NF-κB inhibitor) elicited impaired neutrophil recruitment and bacterial burden [94]. Similarly, enhancing NF-κB by treating mice with AdRelA restored bacterial clearance [94].

Neutrophils in response to PA

Neutrophils and their ability for complement-mediated OPK are necessary for controlling and eradicating PA infection *in vivo*, as neutropenic mice are highly susceptible to even a very low dose of PA [60,95–97]. *In vivo* murine models of complement C3-knockout mice C3-/- and Fcγ-knockout mice Fcγ-/- demonstrated that both complement C3 and Fcγ are required for antibody-mediated recognition of neutrophils, especially in the context of PA infection [98]. The direct physical interaction between neutrophils and PA has been observed by Khatua et al. that sialic acids on the surface of neutrophils can be absorbed by PA, leading to inhibition of NE, ROS production, and NETs [99]. Immune mediators such as nitric oxide (NO) and ROS are critical for clearing PA. In a rat model of PA acute infection, inhalation of exogenous NO reduces bacterial burden at 24 h p.i. [100]. Likewise, NE is also essential in eliminating PA infection. Mice lacking NE resulted in increased lung bacterial burden compared to WT mice [101]. Interestingly, NE inhibitors such as Spi6 (in mice) or PI9 (in humans) [102,103] produced by the host lead to a transient increase in local extracellular NE activity in the lung (possibly NETs) and resulted in reduced PA bacterial burden in Spi6-/- mice compared to WT mice [102]. Furthermore, NE significantly reduces PA bacterial burden via direct degradation of OprF (PA

outer membrane porin) [101]. Others have also reported that OprF is a complement-binding acceptor molecule that promotes complement C3 binding and activation of complement-mediated OPK [104]. These findings suggest that altering the outer membrane protein of PA can modulate neutrophil-OprF-mediated killing.

Macrophages in response to PA

In addition to TLR binding of PA PAMPs, flagellin and T3SS are also recognized by NOD-like receptors, or NLRP 4, which activates caspase-1 and leads to the production of IL-1ß [105,106]. Although macrophages are generally involved in orchestrating inflammatory responses in the host to help eliminate bacterial infections and regulate cell death, their role in PA clearance is still unclear. Work by Hashimoto et al. showed that depletion of macrophages in mice elicited attenuated cytokine responses, but others reported no differences in mortality when compared to PA-infected WT mice [107,108], indicating the role of macrophages is not as critical as neutrophils in the context of PA clearance.

1.3.3 PA evasion of host immune responses in chronic CF infection

Overview

PA possesses multiple strategies to evade antibiotic and host-mediated antimicrobials. Biofilms and overproduction of exopolysaccharides (EPS) in the airway secretions, bacterial phenotypic and genetic adaption (e.g., loss of motility, mutations that confer loss of function or secretion of extracellular factors) to the host environment are mechanisms that can be exploited by PA to persist in the host for decades. Below, we discuss in detail how PA utilizes each mechanism to become a successful chronic infection pathogen.

Overproduction of Exopolysaccharide

Alginate overproduction

Alginate, when overproduced, confers a phenotype called mucoidy. This phenotype is relatively rare in environmental and new-onset infection PA isolates [91,92,109,110], but is commonly found in chronic CF infection isolates [111], and is associated with pulmonary exacerbations and lung disease progression [112,113]. Mucoidy inhibits host defenses and contributes to bacterial persistence through multiple mechanisms. To evade innate immune responses, mucoidy inhibits chemotaxis [114], shields the bacterium from activating complement-mediated neutrophil OPK, scavenges ROS, and thus confers resistance to host and antibiotic-mediated antimicrobials [115–119]. Mucoidy also diminishes host inflammatory responses. It has been shown that alginate overproduction represses flagellar biosynthesis due to the co-regulation of flagellin and alginate [120], and reduces TLR 5-dependent activation. In contrast, although the direct mechanism is not clear, mucoidy is linked with increases in bacterial lipoprotein expression [121], which activates TLR 2 in AECs [122], and it is associated with greater resistance to the anti-inflammatory effects of corticosteroids [123].

Psl and Pel overproduction

On the other hand, Psl and Pel are non-mucoid EPS. They are present at the surface and are structural components of biofilms, and thus have significant contributions to biofilm development. The overproduction of Psl and Pel leads to hyper-aggregation, leading to a phenotype called “wrinkly colony morphology”. This phenotype has been detected in CF clinical isolates and is associated with failed eradication in CF patients [91,124]. Moreover, Mishra et al. have shown that Psl impedes *in vitro* neutrophil OPK and *in vivo* bacterial clearance by

inhibiting complement deposition on the bacterial surface [125]. Interestingly, increased Psl production induces a neutrophilic response *in vitro* [126], but impairs bacterial eradication *in vivo* [127]. This may contribute to the pathogenesis and disease development in CF.

Similar to Psl, Pel is also critical to the biofilm matrix by initiating and maintaining cell-to-cell interaction. It cross-links extracellular DNA in the biofilm matrix, providing a scaffold for biofilms [128]. Since Pel is a cationic polysaccharide, it binds to aminoglycosides, resulting in increased tolerance and resistance to aminoglycoside antibiotics [129], and may thus confer resistance to inhaled tobramycin treatment. Although Pel has been implicated in the phagocytic killing by dHL-60s [130], compared to Psl, Pel's contribution to the host innate immunity is still largely unknown and under study.

Biofilm lifestyle

In contrast to the planktonic free-living bacteria, PA can also grow in biofilm form. Biofilms are formed by surface-adherent or self-aggregated bacteria encased within an extracellular matrix that impairs the diffusion of antimicrobial molecules such as antibiotics and immune cells. To understand the dynamic host-pathogen interplay in the development of biofilms *in vivo*, Thanabalasuriar et al. used a murine PA keratitis infection model and demonstrated that recruited neutrophils were triggered to form NETs by PA T3SS toxin release in order to reduce bacterial dissemination at the base of the biofilm [131]. Whether these findings are relevant to chronic CF lung infection is unclear since the latter appears to select against T3SS-expressing PA, and neutrophil recruitment to the cornea is likely quite different from the lung [132]. Our understanding of *in vivo* host responses to PA biofilms so far is limited by the lack of animal infection models that mimic human biofilm infections, especially in the chronic

stage of lung infection. Our insights are thus primarily drawn from *in vitro* studies that examine the response of various cell types to biofilm bacteria. Biofilms may be less immune-stimulatory than their planktonic form because bacterial factors are mostly embedded within the biofilm matrix, therefore hindering their recognition by the host. A few reports have shown that the expression of flagellin and T3SS is down-regulated [133,134], and the complement system is less activated during biofilm growth [135]. Innate immune cells such as phagocytes do not effectively eradicate PA in biofilm form and are typically followed by necrotic cell death, resulting in inflammation and collateral tissue damage [136–138].

Loss of pilin and pilus-mediated twitching motility

The pilus is a surface fiber-like appendage that is part of the Type 4 Pili (T4P) complex, and PilA is the major subunit among other minor pilins such as FilmU, PilV, PilW, PilX, and PilE [139–141]. PilA is essential for pilus production and serves as an adhesin to biotic and abiotic surfaces, as well as promotes biofilm formation *in vitro* [142]. Additionally, ATPase genes (*pilT, pilU, pilB*) that are required for pilin depolymerization, extension, and retraction are critical for twitching motility [143–146]. Starkey et al. have demonstrated that elevated levels of key secondary messenger C-di-GMP also repress the expression of global regulators and genes that link to motility, such as *rpoN*, *pilA,* and *fleQ* [147], therefore resulting in loss of twitching motility. Loss of twitching is a robust and reproducible *in vitro* phenotype associated with a persistent infection outcome following eradication treatment and pulmonary exacerbations in people with CF [92,148]. Up to 70% of PA isolates from CF individuals with chronic PA infections exhibit loss of twitching motility [149].

It is unclear whether host evasion is mediated by loss of pilus-mediated adhesin or twitching motility since most reports focus on comparing the WT and pilus-deficient mutants. For example, *pilT* or *pilU* mutant causes auto-aggregation (prolonged pili) and twitching deficiency [144,150]. AECs infected with a *pilT* or *pilU* mutant exhibit significantly lower cytotoxicity, suggesting that loss of twitching motility prevents stable host cell attachment [151]. Findings from the previous study are consistent with results reported by Basso et al. that loss of pilin (*ΔpilA*) and pilus-mediated twitching motility (*ΔpilT, ΔpilU)* reduced the direct bacterial-host-cell membrane contact and T3SS-dependent toxicity in infected epithelial cells [152]. Moreover, bone marrow-derived macrophages (BMDM) stimulated with *ΔpilA* elicit reduced host proinflammatory responses such as IL-1ß, IL-6 and IL-8 [153], indicating that loss of pilin evades inflammatory responses and is thus favored in chronic CF infection.

Loss of flagellum-mediated motility

The flagellar complex interacts with immune and non-immune cells through its structural components and motility function. Flagellin expression is under the complex regulation by several global transcriptional regulators [154–158]. However, flagellin expression is repressed in mucoid variants [147,159], during biofilm growth [160], and in response to host nutritional and inflammatory environment, as well in CF sputum, airway fluid [161], and NE [162]. Loss of flagellum-mediated swimming motility is frequently observed in up to 70% of clinical PA isolates collected from patients with poor pulmonary function [163,164]. Longitudinal studies of PA strains have shown evidence of convergent evolution and genetic mutations in regulatory genes such as *rpoN* and *fleQ,* which lead to the downregulation of flagellar expression and motility [132,165]. Moreover, PA isolates recovered from chronic CF lung infections exhibit loss

of flagellin and/ or flagellum-mediated swimming motility and fail to activate MyD88 and NLRC 4 [166], resulting in dampened proinflammatory cytokine or chemokine responses.

Loss of T3SS

Type 3 secretion system (T3SS) is a needle-like secretion apparatus found in Gram-negative bacteria and regulated by ExsA. The apparatus can activate the NLRC 4-inflammasome through NAIP recognition [167,168], leading to pyroptotic cell death and secretion of mature IL-1ß and IL-18. The T3SS injectosome protein PcrV is essential for the translocation of bacterial effector proteins across cellular membranes directly into the host cell cytoplasm and causes cytotoxicity or subversion of host defenses [169]. Moreover, PA produces four T3SS-dependent effectors, namely ExoS, ExoT, ExoY, and ExoU, and have negative impacts on the eukaryotic cytoskeleton, induce cytotoxicity, and impair neutrophil-mediated bacterial clearance (ExoS) [170–174]. Interestingly, loss of T3SS expression in PA seems to be favored in chronic infection, as most PA isolates from chronic infection are T3SS-negative [175–177]. However, many CF patients carry antibodies against T3SS effector proteins [178], suggesting the secretion of these effectors does occur, perhaps relatively early in the infection history. Loss of T3SS results in reduced inflammasome activation and lower pyroptotic cell death in macrophages and neutrophils [177]. In addition, ExoU-positive PA isolates are rarely detected in people with CF, indicating acute cytotoxicity is less compatible with chronic CF infection.

Other virulence factors

PA also secretes other virulence factors such as LPS, rhamnolipids, proteases, and pyocyanin that can damage the host. PA surfactants or rhamnolipid production can induce

neutrophil necrotic cell death [179]. Clinical studies have reported the presence of rhamnolipids in the sputum of CF patients with chronic PA infection [180]. LPS is composed of the lipid A and core oligosaccharides from the outer leaflet of the bacterial outer membrane and the O-antigen polysaccharide that interacts with the extracellular environment. The O-antigen consists of highly variable and immunogenic oligosaccharide repeats which elicit a strong humoral response [181]. To promote immune evasion in the CF lung, modification of the lipid A structure and loss of O-antigen are common among chronic CF infection [182], resulting in either enhanced immune stimulation or evasion, increased resistance to host antimicrobials peptides, and complement killing [183–185].

Furthermore, secreted proteases (LasA, LasB, AprA, and Protease IV) interact with a wide range of host molecules, alter host responses by degrading secreted cytokines and chemokines [186–189], immunoglobulins [190,191], antimicrobial peptides [192], as well as extracellular flagellin [193], thus dampening inflammatory responses. Loss of secreted protease activity is commonly seen with loss of function mutations in the gene encoding for LasR, the major quorum sensing transcriptional activator in PA. Quorum sensing is a bacterial communication system that regulates the expression of many exoproducts and virulence factors. Notably, LasR activates the expression of LasB, a metallo-protease that degrades elastin [194], phagocytic receptors [195,196], and disrupts epithelial tight-junctions [197]. LasB mutants are attenuated in virulence in experimental models of bacteremia [198], acute pneumonia [199], as well as burn wound model [200], leading to reduced host tissue destruction and invasion, dampened immune recognition, and increased accumulation of mediators and inflammation. Pyocyanin (green/blue pigmentation) on the contrary, stimulates IL-8 production [201] and neutrophil apoptosis [202], and impairs neutrophil-mediated antibacterial responses *in vivo*

directly [203]. Loss of pyocyanin and protease activities occur as part of the genetic adaptation of PA to the host environment and are associated with chronic and more advanced lung disease. Over one-third of CF patients with chronic PA infection harbor LasR quorum sensing and protease or pyocyanin-deficient variants [204–207], suggesting the host environment likely confers strong selective forces that shape host-pathogen interactions toward a state which promotes bacterial survival and persistence in the face of host defenses.

1.4 Current treatments for CF lung disease

Airway clearance, exercise, and mucolytic therapies

The management of CF lung disease is based on multiple aspects, including airway clearance and exercise, nutritional support, antibiotics, anti-inflammatory treatment, and CFTR modulator therapies. Airway clearance and exercise such as positive expiratory pressure, breathing modalities, and aerobic exercise are associated with decreased pulmonary exacerbations [208–210]. Likewise, mucolytic treatments such as dornase alfa reduces the viscosity of airway secretions and hypertonic saline, which increases mucociliary clearance, result in positive impacts on lung function [211,212].

Antibiotic regimens

Antibiotic treatments play a critical role in the management of bacterial infections in CF. The main goals of antibiotic treatments in CF patients with PA infections are: the treatment of acute pulmonary exacerbations, the management of chronic PA infection and the eradication of new-onset PA infections. Patients with pulmonary exacerbations and chronically infected with PA chronically are typically treated with anti-PA antibiotics, often combinations of oral (e.g.,

fluoroquinolones) and intravenous antibiotics (e.g., aminoglycosides, carbapenems or cephalosporins with anti-PA activity) [213,214]. Specifically, for those with chronic PA infection, long-term inhaled antibiotic therapies (e.g., tobramycin) are used to reduce PA burden, host inflammation, and the frequency of acute pulmonary exacerbations, as well as to maintain lung function. Inhaled antibiotics are a safe and attractive option by delivering high concentrations of antibiotics directly to the site infection while minimizing systemic exposure [215].

Since eradication of chronic PA infection is nearly impossible, much effort has been focused on targeting new-onset infection. Several studies have reported that early initiation of antibiotic treatments significantly delayed the onset of chronic CF infection [216,217]. Specifically, there is a small window of opportunity to eradicate PA infections in individuals with CF during the early stage of infection when PA is first detected [218] (Figure 1.2). The rate of PA eradication increases following 28 days of routine inhaled tobramycin treatment, with a median recurrence time of 26 months in those with new-onset PA infections [216]. Moreover, a study evaluated the efficacy of other antibiotic regimens in combination with tobramycin, such as ciprofloxacin, meropenem, and colistin, for long-term use in those with early PA infections. Treggiari et al. reported that different antibiotic regimens together with tobramycin demonstrated similar or noninferiority to inhaled tobramycin alone in the prevalence of PA positivity or rate of exacerbation [219,220]. Inhaled tobramycin is thus widely recommended in children with CF to eradicate new-onset PA infections.

Other newly developed antibiotic regimens have also been developed for patients with chronic PA infections. For example, fosfomycin/tobramycin inhalation is actively against Gram-negative, Gram-positive (e.g., SA, MRSA, MSSA) and anaerobic bacteria [221], and treatment

shows improvement in lung function in a Phase II study of CF patients infected with PA. Nebulization of levofloxacin has also demonstrated reduction in PA burden in patients chronically infected with PA [222], as well as liposomal amikacin is also effectively against most CF-relevant Gram-negative bacteria [223]. Yet, multi-center studies reported that infections were never completely eradicated in patients with chronic PA infections following treatments [224,225].

Anti-inflammatory therapies

Non-steroidal anti-inflammatory drugs and macrolide antibiotics have been studied and used in people with CF to reduce pulmonary exacerbations and inflammation [226,227]. Ibuprofen, for example, reduces the rate of lung function decline in CF children and adolescents. However, the intake has been limited since constant blood level monitoring is needed to optimize its effect [226,228]. Azithromycin has also been used in patients with chronic PA infections [227]. Although the exact mechanism is still unclear, clinical studies have reported a significant improvement in weight, lung function and reduced pulmonary exacerbations when azithromycin is administered alongside tobramycin in CF patients compared to placebo [229,230].

CFTR modulator therapies

In the last decade, CFTR modulators have been developed to correct and potentiate CFTR function, and have led to remarkable clinical improvement for people with CF. These currently available modulators are ivacaftor (potentiator), ivacaftor/lumacaftor (corrector/potentiator combination), and Trikafta, the combination of elexaftor, tezacaftor, and ivacaftor (ETI). The development of these therapies has advanced from targeting a specific rare

CFTR genotype to one or two copies of the typical ΔF508 CFTR mutation, resulting in up to 90% of people with CF benefiting from these therapies [4].

CFTR modulator therapies are administered in adults with mild, moderate, and severe diseases. Ivacaftor, for instance, has been used in patients with class III mutations as early as 4 months old, whereas ivacaftor/lumacaftor is given to patients with class I or class II ΔF508 mutation at the age of 2 years old, or the more advanced treatment Tezacaftor-ivacaftor is prescribed to children from 6 years old and up [231]. Although clinical trials reported an overall improved lung function and a marked reduction in PA load followed by ivacaftor/lumacaftor treatment in those with ΔF508/ΔF508 chronic CF infection, the infection was not eradicated [232,233]. This led to the development of additional CFTR corrector and potentiator compounds. In 2019, Trikafta/ETI, the most recent CFTR modulator therapy represented a major advance in the management of CF. Randomized clinical studies of ETI reported a significant improvement in lung function, respiratory symptoms, weight gain, and risk of exacerbations in people with one copy of ΔF508 [234,235]. However, these treatments may not be available to all CF patients, and the long-term effects on chronic airway infections remain to be determined.

Lung transplantation

Finally, lung transplantation is also an option for patients with end-stage lung disease. Patients undergoing lung transplantation have shown improvement in median survival. However, a dramatic decrease in lung transplantation has been observed since 2020 [3,4]. This is likely in part due to a significant improvement in health status since the recent introduction of Trikafta.

Other emerging therapies

Alternative therapies to improve infection outcomes are still needed because of the alarming rise in antimicrobial resistance, and increased drug tolerance in chronic infections due to the presence of certain strain-specific phenotypes or biofilm bacteria [236]. Anti-virulence therapies typically target bacterial virulence factors (such as QS signaling, biofilm EPS, T3SS and associated effectors), but only a few of these therapies have proceeded beyond pre-clinical studies [237]. These therapies face unique challenges due to the extraordinary bacterial phenotypic heterogeneity found in patients with early and chronic PA infections. For example, many PA isolates from patients in the chronic stage of infection do not express pilus, flagellum, and T3SS compared to those recovered from patients with new-onset PA infections. Therefore, anti-virulence therapies may need to be tailored to patients' infection states and microbiological status.

Interestingly, antibacterial antibodies can neutralize bacterial virulence factors, induce complement-mediated lysis and enhance OPK [238]. Antibody engineering and screening have accelerated antibody therapeutics due to technologies such as phage display libraries expressing fully human antibodies or traditional hybridoma to express a fully human antibody repertoire. However, only a few anti-PA antibodies have reached clinical trials. Polyclonal anti-PA antibodies (PsAer-IgY) [239] are currently in Phase 3 clinical trials (NCT01455675) to prevent recurrent PA infections in CF patients. Monoclonal antibodies that target the EPS alginate (AR-105, Aridis Pharmaceuticals) and Psl mAb Psl0096 [240], the T3SS needle protein PcrV mAb V2L2 [241]; KB001 [242], O11 serotype LPS (AR-101/KBPA101, Aridis Pharmaceuticals) [243], or combination mAb (bispecific anti-Psl/PcrV MEDI3902, MedImmune) [244] was

enrolled in phase two clinical trial for the prevention of acute PA pneumonia, but their utility in preventing or treating chronic PA infections in CF patients remains to be determined.

1.5 Thesis hypothesis and objectives

Risk factors for bacterial persistence following failed eradication treatment remain unknown in CF patients with new-onset PA infections. Surprisingly, a significant association is observed between strain-specific phenotypes recovered from CF patients and a persistent infection outcome after eradication treatment. Strain-specific phenotypes such as loss of pilus-mediated twitching motility and overproduction of EPS have been shown to reduce neutrophil-mediated bacterial clearance, but the bacterial mechanisms and their impacts on the host are largely unclear. In this thesis, we hypothesized that PA isolates' resistance to neutrophil antibacterial functions was associated with persistent infection in CF patients with new-onset PA infections and that targeting strain-specific bacterial determinants of persistent PA clinical isolates may restore neutrophil antibacterial functions to improve PA eradication.

The first objective, as presented in chapter 2, was to identify bacterial determinants and their associations with failed PA eradication treatment using a cohort of clinical Eradicated and Persistent PA isolates recovered from CF children with new-onset PA infections prior to inhaled tobramycin. We hypothesized that PA isolates' resistance to neutrophil antibacterial functions was associated with persistent infection.

The second objective, as presented in chapter 3, was to identify the bacterial determinants from representative Persistent isolates and mechanisms that contributed to resistance to neutrophil antibacterial functions *in vitro*, using primary murine neutrophils, and *in vivo*, using a murine pulmonary PA infection model. We hypothesized that strain-specific phenotypes such as

loss of pilus-mediated twitching motility and overproduction of EPS from representative Persistent isolates were resistant to primary murine neutrophil antibacterial functions and *in vivo* bacterial clearance compared to representative Eradicated isolates.

The final objective, as presented in chapter 4, was to investigate the effect of mAb that targets bacterial factors Psl and PcrV on the potentiation of neutrophil-mediated bacterial clearance *in vitro*, using *in vitro* neutrophil phagocytosis and intracellular bacterial killing assays with a subset of Persistent and Eradicated isolates, and *in vivo*, using a murine PA lung infection model. We hypothesized that mAb treatment may enhance neutrophil-mediated bacterial clearance *in vitro* and *in vivo* in PA isolates that produced high Psl and PcrV positive.

1.6 Figures

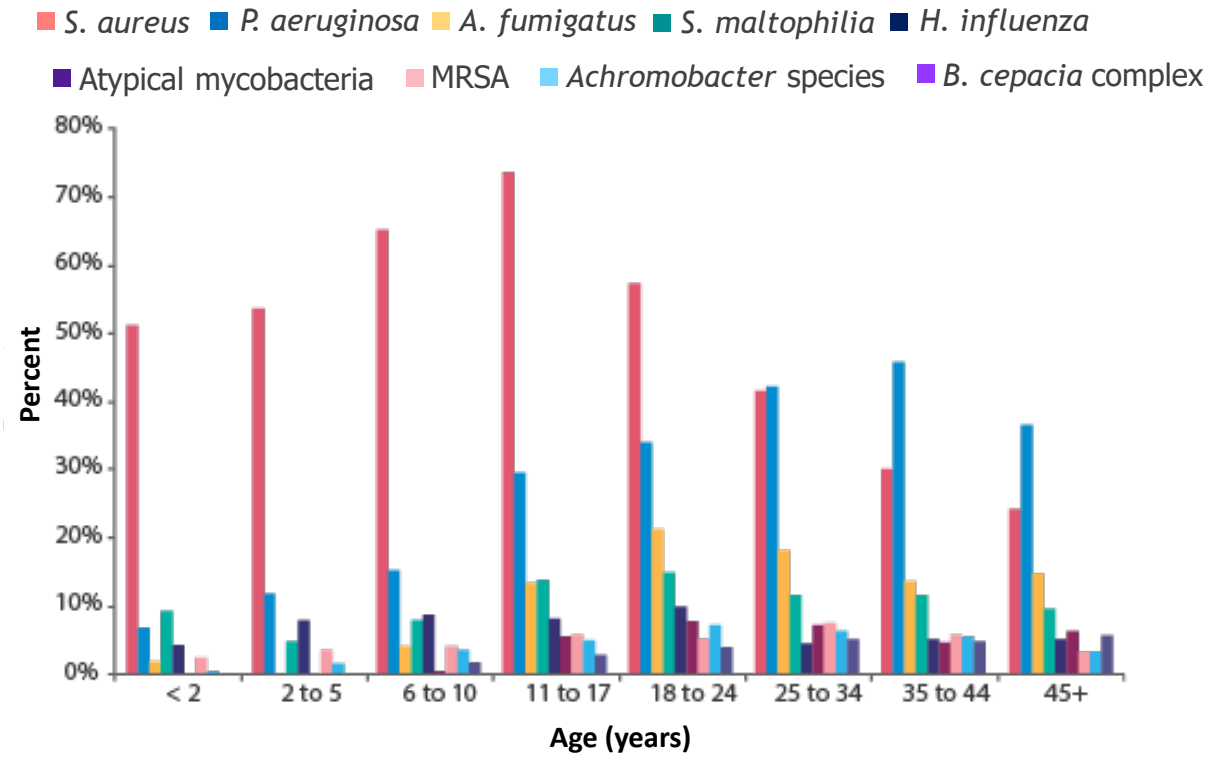

Figure 1.1 Age-specific prevalence of CF pathogens in CF patients, 2020 (adapted from CFC registry 2020 [3]).

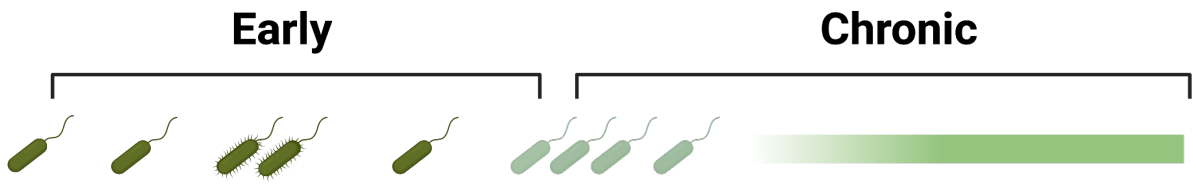

Figure 1.2 Schematic representation of the progression of PA infection in CF. People with CF are typically colonized by morphologically distinct PA isolates during the early infection stage. These individuals can remain PA-negative for several years until a repetitive recovery of a dominant PA isolate suggests a chronic infection is established.

1.7 Tables

Table 1.1 CFTR classification by functionality and consequence. Table adapted from [11].

CFTR Classification	Functional abnormality and consequence	Examples of mutations
Class I	-Major disruptions of insertions, deletions, and premature termination of CFTR biosynthesis -Decreased number	-G542X, 394ΔTT, 3905insT
Class II	-Misfolding of the CFTR protein -Premature degradation by ER and prevent localization to the plasma membrane -Decreased number	-ΔF508, A455E, N1303K
Class III	-Dysregulation of gating, thus lowering ATP binding and hydrolysis -Decreased function	-G551D, R117H
Class IV	-Defective chloride conductance -Decreased function	-R347H, R334W
Class V	-Unstable mRNA transcripts due to non-canonical and canonical splicing mutations -Decreased number	-621+1G>T, R117H+5T
Class VI	-Reduces surface stability or fast turn over -Decreased number	- S1455X, L1399X

Table 1.2 A list of PRRs in response to PA PAMPs. Table adapted from [245].

PRR	PAMP	References
TLR 2	Lipoproteins, alginate, C-terminal domain of ExoS, LPS, Flagellin, Slime-GLP	[246–251]
TLR 4	LPS, N-terminal domain of ExoS, alginate	[247–249]
TLR 5	Flagellin	[250]
TLR 9	DNA	[252]
NOD 1,2	Peptidoglycan	[63,253]
NLRC 4	Flagellin, T3SS needle	[167,254]
NLRC 3	Peptidoglycan, RNA	[255,256]

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Chapter 2: Failed eradication therapy of new-onset *Pseudomonas aeruginosa* infections in cystic fibrosis children is associated with bacterial resistance to neutrophil functions

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2.1 Abstract

Background. Antibiotics, such as inhaled tobramycin, are used to eradicate new-onset *Pseudomonas aeruginosa* (PA) infections in cystic fibrosis (CF) patients but frequently fail due to reasons poorly understood. We hypothesized that PA isolates' resistance to neutrophil antibacterial functions was associated with failed eradication in patients harboring those strains.

Methods. We analyzed all PA isolates from a cohort of 39 CF children with new-onset PA infections undergoing tobramycin eradication therapy, where N=30 patients had eradicated and N=9 patients had persistent infection. We characterized several bacterial phenotypes and measured the isolates' susceptibility to neutrophil antibacterial functions using *in vitro* assays of phagocytosis and intracellular bacterial killing.

Results. PA isolates from persistent infections were more resistant to neutrophil functions, with lower phagocytosis and intracellular bacterial killing compared to those from eradicated infections. In multivariable analyses, *in vitro* neutrophil responses were positively associated with twitching motility, and negatively with mucoidy. *In vitro* neutrophil phagocytosis was a predictor of persistent infection following tobramycin even after adjustment for clinical risk factors.

Conclusions. PA isolates from new-onset CF infection show strain-specific susceptibility to neutrophil antibacterial functions, and infection with PA isolates resistant to neutrophil phagocytosis is an independent risk factor for failed tobramycin eradication.

2.2 Introduction

Cystic fibrosis (CF) is a multisystem genetic disorder caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR dysfunction leads to impaired mucociliary clearance and airway mucus plugging which favor bacterial colonization and chronic infection [1]. Chronic infections with *Pseudomonas aeruginosa* (PA), the predominant opportunistic respiratory pathogen in CF, are associated with lung function decline and worse clinical outcomes [2]. To avoid progression to chronic infection, new-onset PA infections are routinely treated with antibiotics, most commonly inhaled tobramycin in North America [3,4]. Unfortunately, even with early interventions, antibiotic eradication therapy fails and PA infections persist in 28 to 40% of patients [3–7].

Several studies to date in different pediatric CF cohorts, namely in the U.S with the Early *Pseudomonas* Infection Control (EPIC) study [8,9], the Netherlands and Denmark [10], Australia [11] and our study cohort at the Hospital for Sick Children in Canada [12] have examined clinical and microbiological parameters to identify predictors of persistent infection following eradication therapy. Although these studies varied in design and sampling method for the recovery of PA, none found significant differences in the clinical characteristics (age, age at diagnosis, gender, CFTR genotypes, eradication treatments and lung function) between patients with successful eradication and those with persistent infection.

Interestingly, Mayer Hamblett et al. [9] (EPIC study) and Vidya et al. [12] (Hospital for Sick Children cohort study) also investigated phenotypic characteristics of the infecting PA isolates for their association with failed eradication therapy. Bacterial phenotypes commonly associated with chronic infections, namely mucoidy and lack of twitching motility, as well as wrinkly colonies surface and irregular edges, showed association with eradication failure [9,12].

Such bacterial phenotypes may impair the efficacy of antimicrobials [13,14], but also evade host defense mechanisms that are important for bacterial clearance [15,16]. Neutrophils are innate immune cells that carry out phagocytic and bacterial killing functions critical to PA eradication and enhance the efficacy of antibacterial therapy [17,18]. Multiple bacterial factors, such as surface molecules [19], motility appendages [20,21] and overproduction of exopolysaccharides (EPSs; alginate, Psl and Pel) [22–24], alter neutrophil-mediated antibacterial clearance. Bacterial characteristics of PA isolates at the time of new-onset infection may thus be determinants of neutrophil-PA interactions and be associated with outcomes of eradication therapy in CF patients.

Although many studies have investigated host-PA interactions in experimental *in vitro* and animal models of infection, few have linked these findings to clinical outcomes [10,25–28]. In this study, we characterized all PA isolates collected from new-onset infections of CF children from the Hospital for Sick Children cohort study [12] using *in vitro* assays for neutrophil phagocytosis and intracellular bacterial killing. We sought to identify the bacterial phenotypes that contributed most to *in vitro* resistance to neutrophil antibacterial functions, and to determine whether such resistance was associated with failed eradication therapy in CF patients.

2.3 Material and Methods

Cohort Design

Bacterial isolates and clinical data from a prospective cohort of CF children followed at the Hospital for Sick Children (Toronto, Canada) were used for this study [12]. Patients included in this study were (1) 5 to 18 years old, (2) able to produce sputum, and (3) diagnosed with new-onset PA infection during the period of 2011-2014, and were all treated with inhaled tobramycin twice per day for 28 days. All patients from this cohort were included except for those with PA

isolates with tobramycin minimum inhibitory concentration (MIC) >2 μg/mL to exclude tobramycin resistance as a cause of eradication failure and because the *in vitro* neutrophil assays can only be performed on aminoglycoside-susceptible isolates. New-onset PA infection was defined as a PA-positive sputum culture following at least 3 PA-negative sputum cultures in the previous 12 months. A sputum culture (posttreatment) was collected 1 week after the end of tobramycin therapy to determine the outcome of eradication therapy, based on previous definitions [3,5]. The infection was considered "Persistent" if the post-treatment sputum culture was positive for PA, and "Eradicated" if the posttreatment culture was negative [29]. This study was approved by the research ethics board (REB) at the Hospital for Sick Children.

PA Clinical Isolates

All PA clinical isolates were recovered from patients' sputum collected prior to the initiation of inhaled tobramycin as previously described [12]. In brief, sputum samples were homogenized with sputolysin and stored at -80 °C. PA isolates were recovered from frozen sputum, included multiple colony morphotypes when present, and were confirmed by matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) for species identification.

Phenotypic Characterization of PA Isolates

Phenotypic assays for twitching and swimming motility, biofilm formation by crystal violet (CV) assay, and mucoidy status were done as previously reported [12]. In brief, mucoidy was determined by colony morphology following growth on yeast extract media (YEM) agar for 24-48 hours. Swimming motility was determined by inoculating a single colony into 0.3% Luria-

Bertani (LB) agar and measuring the diameter (mm) of the zone of bacterial growth after overnight incubation at 37 °C. Twitching motility was determined by inoculating a single colony into 1% thin LB agar plate and measuring the diameter (mm) of the twitching zone following staining with 0.1% CV. Biofilm formation was measured by inoculating polystyrene 96-well plates with 100 µL of overnight bacteria cultures diluted 1:100 in LB medium. After static incubation overnight at 37 °C, the adherent biofilm biomass was stained with 0.1% CV, resolubilized with 95% ethanol and measured at optical density (OD) 600nm. The Congo red binding assay was used to assess exopolysaccharides (EPS)-mediated bacterial aggregation in liquid culture [30]. One hundred microliters of overnight PA cultures was diluted into 4 mL Vogel-Bonner minimal medium (VBMM) containing 40 µg/mL Congo red (Sigma catalog number C6767) and incubated for 18 hours at 37 °C with shaking at 250 rpm. Bacterial cells were then spun down and the absorbance of the supernatant was measured at OD at 490nm (OD_{490nm}). The Congo red binding was calculated as (OD_{490nm} [VBMM + Congo red control] - OD_{490nm} [sample]).

In vitro Neutrophil Phagocytosis and Intracellular Bacterial Killing Assays

A gentamicin protection assay was used to measure the phagocytic uptake of PA isolates by human neutrophil-like cells derived from immortalized HL-60 cells. In brief, HL-60 cells were cultured in Iscove's modified Dulbecco's medium (Wisent) and differentiated with 1.3% dimethyl sulfoxide and 2.3 µM all-trans retinoic acid for 3 days to generate neutrophil-like cells (dHL-60s) with >95% viability. Bacteria were grown in 5 mL LB miller medium overnight (18 hours) at 37 °C with shaking at 250 rpm, then spun down, washed twice with Hanks' balanced salt solution without calcium, magnesium and phenol red (Wisent), and diluted to 10^7 colony-

forming units (CFU)/mL. Two hundred fifty microliters of the diluted bacterial suspension was opsonized with 20% human serum (Millipore Sigma) and coincubated with $2.5x10^5$ dHL-60s at a multiplicity of infection (MOI) of 10 for 30 minutes at 37 °C. Following this incubation, 100 µg/mL gentamicin was added for another 30 minutes (T = 60 minutes) for the phagocytosis assay or 90 minutes (T = 120 minutes) for the intracellular bacterial killing assay. Cells were then washed twice, lysed with 0.1% Triton, and plated on LB agar medium for enumeration of viable intracellular bacteria by counting CFU after overnight incubation at 37 °C. The phagocytosis index was calculated as the number of internalized bacteria at T = 60 divided by total bacteria at T = 0. The intracellular bacterial killing was calculated as the number of internalized bacteria at T = 60 minus T = 120, then divided by total bacteria at T = 0. All experiments were done with at least 4 replicates in at least 2 independent experiments.

Statistical Analyses

Comparisons were performed using the Mann-Whitney nonparametric test or χ^2 test as indicated. Univariable Spearman correlation was first used to measure the association between each bacterial phenotype and in vitro neutrophil responses (phagocytosis or intracellular bacterial killing), and those with a *P*-value ≤ 0.2 were included in a subsequent multivariable model. A random effects model was used to determine the association between different bacterial phenotypes and in vitro neutrophil responses while accounting for clustering due to occurrence of multiple isolates per patient. A logistic regression model was used to determine the association between *in vitro* neutrophil responses and a persistent infection outcome. For the adjusted multivariable logistic regression model, clinical parameters or bacterial phenotypes were considered based on prior literature or selection using the Spearman correlation above, and

only 1 additional variable was tested in the model at a time given the power of our limited sample size. Statistical comparisons and regression analyses were performed using SPSS version 26 software (IBM SPSS, Chicago, Illinois). A *P* value of ≤ 0.05 was considered significant.

2.4 Results

Characteristics of Patients With Eradicated or Persistent PA Infections

From a total of 43 eligible patients, 39 patients were analyzed in this study, while 4 patients harboring 6 isolates with tobramycin MIC > 2 were excluded. The study thus included 30 patients with "eradicated" infections and 9 patients with "persistent" infections (i.e., failed tobramycin eradication therapy), resulting in 52 Eradicated PA isolates and 19 Persistent PA isolates. Several patients were infected with >1 morphologically distinct PA isolates (morphotypes), with a median of 2 (range, 1–3) morphotypes in patients with persistent infections, and a median of 1 (range, 1– 4) morphotype in patients with eradicated infections (Supplementary Figure 2.1). Consistent with our previous report [12], demographic and baseline clinical characteristics were similar between patients who failed eradication therapy and those who succeeded (Table 2.1).

PA isolates from patients with persistent infections are more resistant to neutrophil phagocytosis and intracellular bacterial killing compared to those from patients with eradicated infections

We compared the phagocytic uptake and intracellular bacterial killing by dHL-60s of PA isolates from the Eradicated group to those from the Persistent group. Since some patients were infected with >1 PA isolates, we analyzed our data by combining the neutrophil assay data for all

morphotypes in each patient. As shown in Figure 2.1, the average phagocytic index was > 2-fold lower (9.1% vs. 19.5%; $P = 0.0003$) in patients with persistent infections compared to those with eradicated infections. We measured the reduction in neutrophil intracellular bacterial load as a measure of intracellular bacterial killing and found it to be 2.5-fold lower (2.6% vs. 6.7%; $P = 0.018$) in the persistent infection group compared to the eradicated group. Notably, similar results were obtained when we used the maximal value (Supplementary Figures 2.2A and 2B) or the median (Supplementary Figures 2.2C and 2D) of combined neutrophil data from each patient. We also observed similar findings when all PA isolates were analyzed independently (Supplementary Figure 2.3) and noted that the phagocytic index was significantly correlated with intracellular bacterial killing ($r = 0.70$, $P < 0.0001$, Figure 2.2). These results thus indicated that patients who fail eradication therapy harbor PA isolates that are more resistant to neutrophil antibacterial functions than patients with successful eradication.

Loss of Twitching Motility and Mucoidy Are Associated With Impaired Neutrophil Antibacterial Functions

We first characterized the flagellum-mediated swimming motility, type IV pilus-mediated twitching motility, mucoidy (alginate overproduction), biofilm formation, and Congo red binding (Psl and Pel EPS-mediated bacterial aggregation) in all PA isolates (Table 2.2). In initial univariable analyses, we found that neutrophil phagocytosis was only significantly correlated with twitching motility ($r = 0.43$, $P < 0.001$) and not any other bacterial phenotype (Table 2.3). Neutrophil intracellular bacterial killing was significantly associated with twitching motility ($r = 0.43$, $P < 0.001$), mucoidy ($r = -0.26$, $P = 0.03$), and Congo red binding ($r = 0.32$, $P < 0.01$).

Next, since some patients have multiple morphologically distinct PA isolates, which could lead to clustering due to repeated measures, we calculated the intraclass correlation (ICC) for neutrophil phagocytosis and bacterial killing in our dataset. With an ICC value of 0.5, our data showed moderate clustering of neutrophil phagocytosis and bacterial killing measurements within each patient, that is, neutrophil responses elicited by PA isolates from the same patient are more similar to each other than those between different patients. To account for this data clustering, we used a random-effects model to determine the relationship between neutrophil phagocytosis or intracellular bacterial killing, and the 3 bacterial phenotypes identified in univariate analyses with a $P \leq 0.2$, namely twitching, mucoidy and Congo red binding (Table 2.4). We found that twitching motility (r = 0.27, $P = 0.02$) and mucoidy (r = -5.60, $P = 0.04$) were significantly associated with neutrophil phagocytosis, that is, a 1-mm increase in twitching was associated with a 0.27% increase in phagocytosis, and the presence of mucoidy was associated with an average 5.6% reduction in phagocytosis. However, Congo red binding (r = -13.94, $P = 0.44$) showed no association with phagocytosis. Twitching motility (r = 0.20, $P = 0.03$) and mucoidy (r = -4.53, $P = 0.03$) were also significantly associated with intracellular bacterial killing (Table 2.4).

Multivariate Analysis to Predict Persistent Infection Following Inhaled Tobramycin Therapy

We used a logistic regression model to assess whether the resistance of PA isolates to neutrophil phagocytosis is an independent predictor of failed eradication therapy after adjusting for other covariables. In patients infected with >1 PA morphotypes, we used the mean of neutrophil results as done for Figure 2.1. We observed a trend toward association between *in*

vitro intracellular bacterial killing and persistent infection, but this did not reach statistical significance. However, *in vitro* neutrophil phagocytosis was a significant predictor of persistent infection with an (odds ratio, 0.76 [95% confidence interval, .62–.94]; $P = 0.01$), indicating that every percentage increase in neutrophil phagocytosis was associated with a 24% reduction in the odds of a persistent infection outcome (Table 2.5). In addition, we further determined that *in vitro* neutrophil phagocytosis remained a significant predictor of failed eradication therapy even after adjustment for other clinical parameters (age, sex, age at diagnosis, forced expiratory volume in 1 second, body mass index, CFTR genotypes, pancreatic insufficiency, cystic fibrosis–related diabetes mellitus) and bacterial phenotypes (twitching, mucoidy) (Table 2.5).

2.5 Discussion

Although several studies have previously reported on the phenotypic characteristics of PA isolates in patients undergoing eradication therapy [9–12,31], our study is the first to examine the association between bacterial–neutrophil interactions (i.e., phagocytosis and intracellular killing) and outcomes of PA antimicrobial eradication therapy in patients with CF. Our study showed that PA isolates from patients who failed tobramycin eradication therapy were more resistant to neutrophil phagocytosis and intracellular bacterial killing than those from patients with successful eradication. Furthermore, loss of twitching motility and mucoidy were associated with resistance to neutrophil antibacterial functions.

Among the few studies that have characterized bacterial phenotypes in patients undergoing eradication therapy, Douglas et al. also identified a high proportion of mucoidy among isolates from new-onset infections (18.2%), and 3 out of 6 isolates from patients who failed eradication were mucoid [11]. Interestingly, Mayer-Hamblett et al. tested 22 bacterial

phenotypes and reported that wrinkly colony surface and irregular colony edges morphologies were associated with increased eradication failure in the 194 participants from the EPIC trial; they concluded that eradication failure in CF children was associated with PA phenotypes typical of chronic infection [9] and host adaptation [32,33]. Whether the occurrence of such "chronic phenotypes" reflect a pre-existing but undiagnosed infection [34], acquisition of shared strains, or patient-to-patient transmission [35] remains unknown. A recent study of our cohort by Stapleton et al. reported that 41% of CF patients with new-onset PA infection shared strains with other patients based on whole genome sequencing of their PA isolates, and patient-to-patient transmission was potentially involved in a third of patients with shared strains [36], a proportion similar to results reported by Marvig et al [37].

Numerous PA phenotypes modulate host-pathogen interactions important to bacterial clearance, but few studies have examined whether such interactions are associated with infection outcomes in patients with CF. Tramper-Stranders et al. found no differences in bacterial motility, protease, or pyocyanin production between Persistent and Eradicated isolates, but did not observe that Persistent isolates caused greater cytotoxicity in IB3-1CF bronchial cells compared to the Eradicated isolates [10]. In a study that examined bronchoalveolar lavage fluid (BALF) at the time of new-onset PA infection in a cohort of 26 children with CF, Douglas et al. reported a trend toward higher neutrophil counts, neutrophil elastase, and IL-1ß in the BALF of patients who failed eradication therapy [11].

Neutrophils are the primary phagocytic cells recruited to eradicate PA in the lung [16,17], yet appear ineffective at effectively eliminating PA in chronic stages of infections [38]. In addition to the inflammatory milieu of the CF lung, neutrophil functions are also modulated by the CF-adapted PA phenotypes commonly encountered in chronic infections. Several studies

have examined how phagocytic responses differ upon stimulation or infection with CF-adapted clinical isolates. Mahenthiralingam et al. originally observed that chronic infection PA isolates commonly lacked swimming and twitching motility, were mucoidy, and were resistant to nonopsonic phagocytosis by murine macrophages, compared to their clonally related isolates recovered from new-onset PA infection [39]. Two more recent studies compared neutrophil extracellular trap (NET) formation in response to pairs of clonally related PA isolates from early and chronic CF infections, and found that CF-adapted isolates elicited significantly less NET formation [40,41]. However, none of these studies investigated whether the neutrophil responses to early infection PA isolates were predictive of subsequent clinical outcomes.

Neutrophil phagocytosis and intracellular killing of bacteria are complex processes that involve interactions with numerous bacterial surface molecules and motility appendages [42]. For example, complement deposition, opsonization, and reactive oxygen species-mediated killing are hindered by the overexpression of EPSs such as alginate in mucoid isolates, and Psl and Pel in wrinkly colony isolates [24,43,44]. Furthermore, pili act as ligands for nonopsonic phagocytosis [45], and nonmotile mucoid PA are resistant to nonopsonic phagocytosis [22]. The association of loss of twitching motility and mucoidy with impaired neutrophil-mediated bacterial clearance in our PA clinical isolates is thus mechanistically plausible and could increase the risk of persistent infection in CF patients. Future studies with larger collections and more comprehensive phenotyping of PA isolates may identify other bacterial characteristics associated with impaired neutrophil antibacterial functions.

In a recent study of a subset of our cohort, Beaudoin et al. observed higher levels of the exopolysaccharide Psl in biofilm grown Persistent PA isolates [31]. The wrinkly colony morphology described by Mayer-Hamblett et al. to be associated with eradication failure is also

typically caused by the overproduction of exopolysaccharides Psl and Pel [9,13]. Since the overproduction of Psl and/or Pel reduces complement deposition, confers resistance to neutrophil antibacterial functions [24], and promotes bacterial persistence in mouse infection models [46], these observations raised the possibility that high Psl and/or Pel expression may be associated with eradication failure through their effects on neutrophil functions. However, using the Congo red aggregation assay [30], we found no differences between Persistent and Eradicated isolates, nor any association with differential in vitro neutrophil responses.

Our study has several limitations. Chronic PA infections of the CF lung are genetically and phenotypically highly diverse, and occur in the context of polymicrobial communities. Multiple PA sub-lineages often co-exist [47,48], and Stapleton et al. reported that mixed strains were found in 16% of new-onset PA infections [36]. Since we only tested morphologically distinct PA clones recovered from each single sputum sample, we may have overlooked some of the phenotypic diversity. However, when multiple PA morphotypes were present, we analyzed all neutrophil assay results, using either the maximal, the median, or the mean value for each patient and found similar results, suggesting that the *in vitro* neutrophil phenotypes were robust measures. Additionally, the status of persistent infection was defined based on a positive sputum culture after the completion of tobramycin treatment, without confirmation by whole genome sequencing to exclude the possibility of a new PA infection. We chose to test neutrophil responses using the HL-60 cell lines in order to obtain robust and reproducible measurements of neutrophilic functions in response to a large number of PA isolates and wide range of bacterial phenotypes. However, we recognize that our results have not been validated in primary human neutrophils, and our experimental system does not account for acquired or intrinsic neutrophil defects associated with the CF lung milieu or CFTR defect [49,50]. Finally, the multivariable

analyses were limited by the small sample size, particularly for patients with persistent infections. Our study sample size only had sufficient power for 1 additional variable to be included in the logistic regression analyses, leading us to examine the effect of different clinical parameters and bacterial phenotypes in a sequential manner.

Our study suggests that decreased neutrophil phagocytosis of PA is an independent predictor of failed tobramycin eradication. Whether *in vitro* neutrophil assays could be used in a clinical setting remains unknown, and further studies are required to address the potential polyclonal nature of new-onset PA infections and to validate our results in an independent cohort. Nonetheless, our results provide biological insights into why eradication therapy might fail in CF patients. Although the mechanisms underlying the failure of tobramycin eradication are likely multifactorial, our study supports the notion that strain-specific PA-neutrophil interactions are important determinants of the outcome of inhaled tobramycin eradication treatment. These results thus highlight the possibility that novel nonantibiotic therapies that target PA-neutrophil interactions and enhance neutrophil-mediated antibacterial functions should be considered to improve the outcome of PA eradication in CF.

2.6 Figures

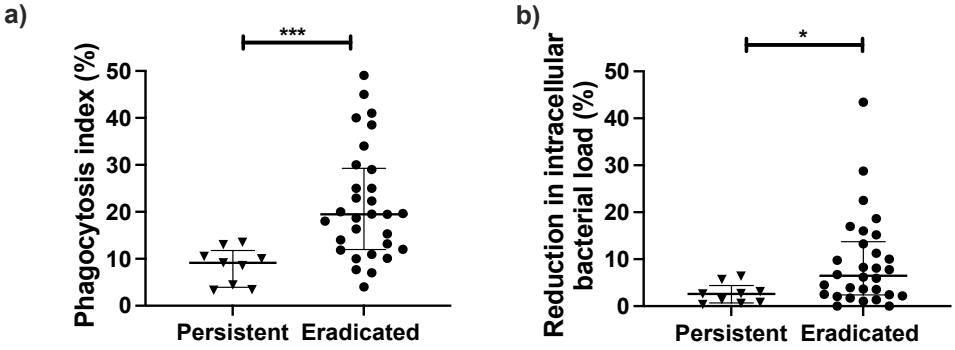


Figure 2.1 PA isolates from patients with persistent infections exhibit lower neutrophil antibacterial functions compared to those from patients with eradicated infection. a) Neutrophil phagocytosis and b) intracellular bacterial killing of the Persistent (N = 9 patients) group and the Eradicated (N = 30 patients) group. The data was analyzed per patient by averaging the neutrophil results of all PA isolates from each patient. Results are shown as median and IQR. Statistical comparisons were performed using Mann-Whitney test (*$P < 0.05$, ***$P < 0.001$).

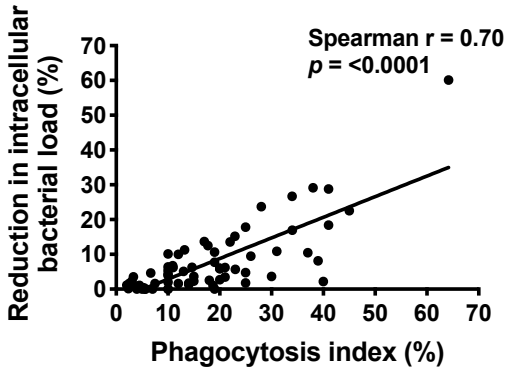


Figure 2.2 Neutrophil phagocytosis is significantly associated with intracellular bacterial killing. The association was calculated by spearman correlation co-efficient.

2.7 Tables

Table 2.1 Baseline Characteristics of Study Patients by Persistent or Eradicated Status

Characteristic	Persistent (n = 9)	Eradicated (n = 30)	*P* Value
Age, y, median (range)	10 (6.3–17.1)	11.3 (6.5–17.5)	.52
Female sex, No. (%)	4 (44)	18 (60)	.41
Age at diagnosis, No. (%)			
<2 y	8 (89)	21 (70)	.08
>2 y	1 (11)	9 (30)	
FEV_1 % predicted, median (range)	85.8 (55.4–120.5)	88.8 (39.4–126.8)	.52
Genotypes, No. (%)			
Homozygous ΔF508	4 (44)	16 (53)	.83
Heterozygous ΔF508	3 (33)	10 (33)	
Other	2 (22)	4 (13)	
Pancreatic insufficiency, No. (%)	8 (89)	29 (97)	.28
CFRD, No. (%)	1 (11)	2 (6.7)	.91
BMI *z* score, median (range)	–0.55 (–1.63 to 1.21)	–0.07 (–1.44 to 2.44)	.51

Table 2.1 Data were previously published in Vidya et al. [12] and modified accordingly to the current study population. Abbreviations: BMI: body mass index; CFRD: CF-related diabetes mellitus; FEV_1% pred: forced expiratory volume in 1 second.

Table 2.2 Comparison of Bacterial Phenotypes in Persistent Versus Eradicated *Pseudomonas aeruginosa* Isolates

PA Phenotypes	Persistent PA (n = 19)	Eradicated PA (n = 52)	*P* Value
Biofilm production (OD_{595nm}), median (IQR)	0.21 (0.15–0.24)	0.21 (0.10–0.32)	.97
Twitching, mm, median (IQR)	18.0 (3.7–20.8)	26.9 (19.3–36.7)	<.01
Swimming, mm, median (IQR)	11.2 (0–17)	13.9 (10.1–16.0)	.33
Mucoidy, No. (%)	12 (63)	18 (35)	.03
Congo red binding (OD_{495nm}), median (IQR)	–0.25 (–0.32 to –0.21)	–0.23 (–0.31 to –0.15)	.14

Statistical comparisons were performed using Mann-Whitney or Chi-square test.

Abbreviations: IQR, interquartile range; OD, optical density; PA, *Pseudomonas aeruginosa*

Table 2.3 Correlation Between Bacterial Phenotypes and *In Vitro* Neutrophil Antibacterial Responses in Univariable Analysis

	Phagocytosis		Intracellular Bacterial Killing	
PA Phenotypes	Coefficient	*P* Value	Coefficient	*P* Value
Twitching	0.43	<.001	0.43	<.001
Swimming	0.07	.55	0.05	.69
Mucoidy	–0.19	.09	–0.26	.03
Congo red binding	0.18	.13	0.32	<.01

The association between each bacterial phenotype and neutrophil phagocytosis or intracellular bacterial killing was calculated using the Spearman correlation coefficient.

Abbreviations: PA, Pseudomonas aeruginosa.

Table 2.4 Association Between Bacterial Phenotypes and *In Vitro* Neutrophil Antibacterial Responses in Multivariable Analysis

	Phagocytosis			Intracellular Bacterial Killing		
PA Phenotype	Coefficient[a]	(95% CI)	*P* Value	Coefficient[a]	(95% CI)	*P* Value
Twitching	0.27	(.04–.51)	.02	0.20	(.02–.38)	.03
Mucoidy	–5.60	(–11.04 to –.14)	.04	–4.53	(–8.63 to –.42)	.03
Congo red binding	–13.94	(–49.81 to 21.11)	.44	–8.06	(–35.31 to 19.17)	.56

Abbreviations: Cl, confidence interval; PA, *Pseudomonas aeruginosa*

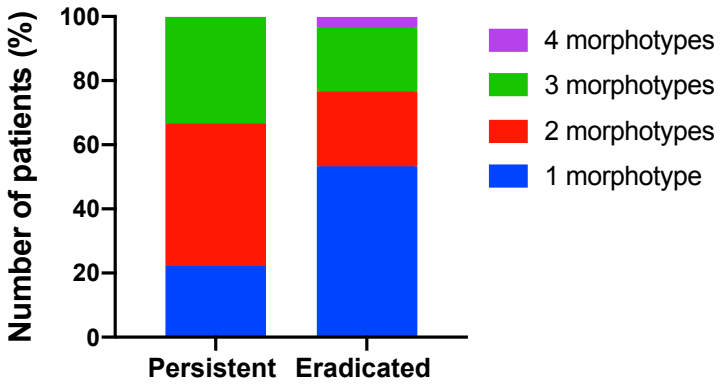
[a]The regression coefficient was calculated using a random-effects model fitted on all on all isolates (n = 19 Persistent and n = 52 Eradicated isolates).

Table 2.5 Logistic Regression Model Suggest That Impaired *In vitro* Neutrophil Phagocytosis of *Pseudomonas aeruginosa* Isolates Is Associated With Failed Eradication Therapy in Cystic Fibrosis Patients

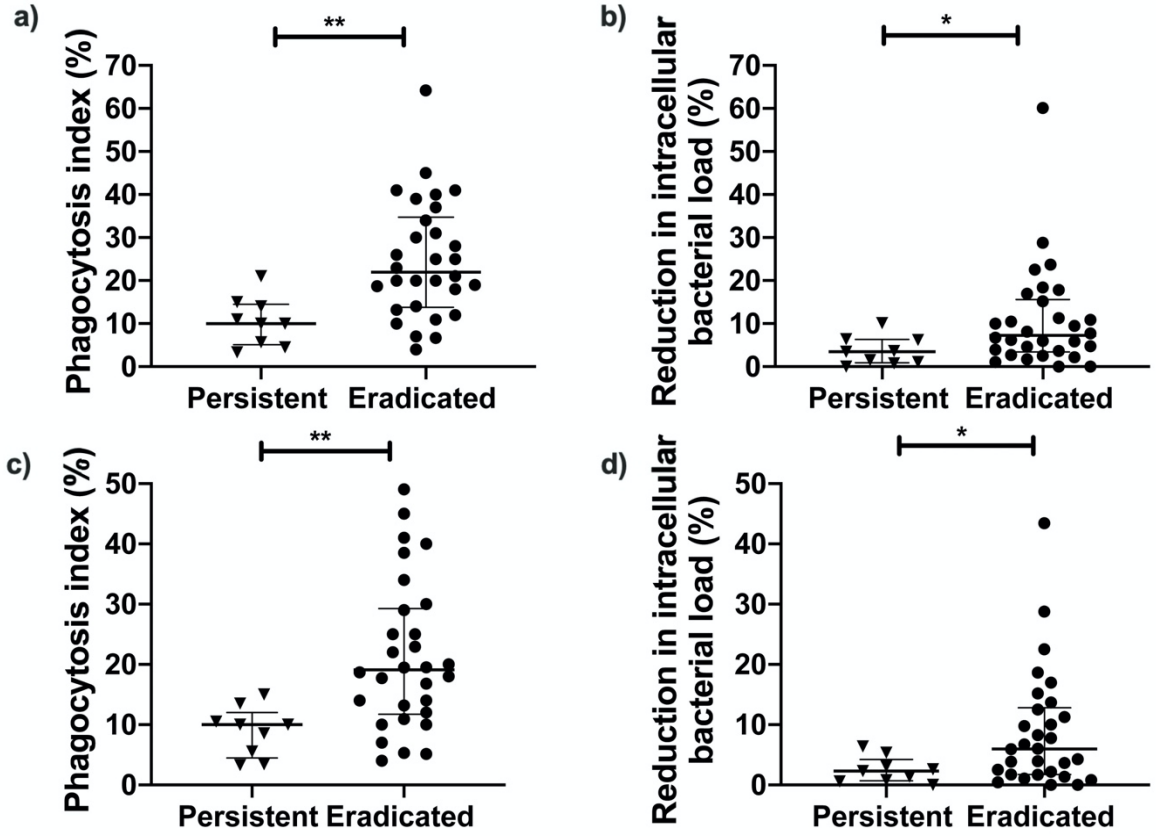
Unadjusted Model							
		OR		(95% CI)		*P* Value	
In vitro neutrophil phagocytosis		0.76		(.62–.94)		.01	
In vitro neutrophil intracellular bacterial killing		0.76		(.66–1.01)		.06	
Adjusted Model[a]							
	OR	(95% CI)	*P* Value	Variable[a]	OR	(95% CI)	*P* Value
In vitro neutrophil phagocytosis	0.76	(.62–.94)	.01	Age	0.91	(.67–1.25)	.57
	0.76	(.62–.94)	.01	Female sex	1.27	(.19–8.58)	.81
	0.76	(.60–.95)	.02	Age at diagnosis >2 y	0.85	(.06–12.43)	.91
	0.73	(.57–.95)	.02	Heterozygous	0.76	(.06–9.05)	.83
				Other	2.01	(.13–33.8)	.61
	0.77	(.62–.95)	.02	BMI	0.82	(.54–1.23)	.33
	0.71	(.56–.92)	<.01	Pancreatic insufficiency	0.02	(.00–3.23)	.14
	0.76	(.62–.94)	.01	CFRD	0.67	(.01–36.72)	.84
	0.76	(.61–.94)	.01	FEV_1 %, predicted	1	(.96–1.05)	.94
	0.77	(.63–.95)	.01	Mucoidy	0.39	(.05–2.74)	.34
	0.79	(.63–.90)	.04	Twitching	0.96	(.87–1.06)	.38

Abbreviations: BMI: body mass index; CFRD: CF-related diabetes mellitus; CI: confidence interval; FEV_1% pred: forced expiratory volume in 1 second: OR: odds ratio. In the adjusted model, each additional clinical parameter or bacterial phenotype was included 1 at a time. A *P* value of 0.05, with OR evaluated using a 2-sided 0.05 level of significance.

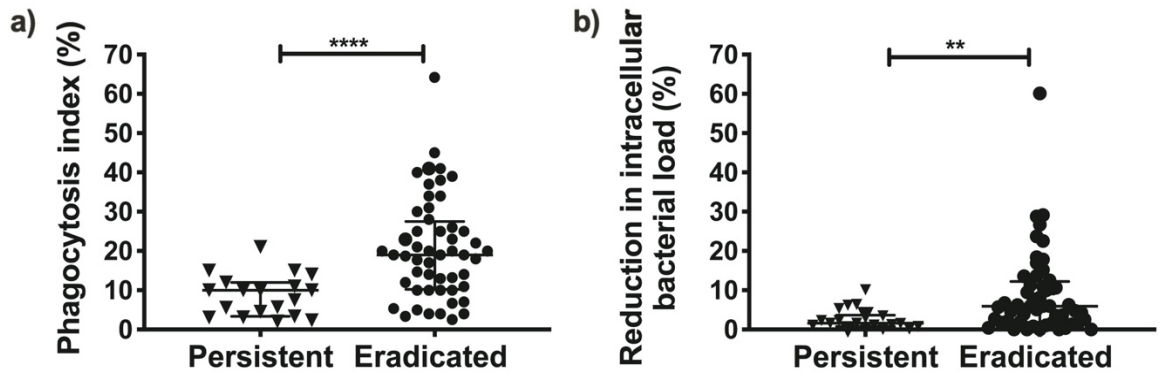
2.8 Supplementary Figures



Supplementary Figure 2.1 Distribution of PA morphotypes among persistent (N = 9) and eradicated (N = 30) patients.



Supplementary Figure 2.2 PA isolates from patients with persistent infections exhibit lower neutrophil phagocytosis and intracellular bacterial killing compared to those from patients with eradicated infection. Results were analyzed per patient (N=9 Persistent patients and N=30 Eradicated patients) by using the maximal value (a and b) or the median (c and d) of the neutrophil results of all PA isolates from each patient. Results are shown as median and IQR. Statistical comparisons were performed using Mann-Whitney test (*$P < 0.05$, **$P < 0.01$).



Supplementary Figure 2.3 Persistent isolates elicit a significantly lower a) neutrophil phagocytosis and b) intracellular bacterial killing of the persistent (n = 19 isolates) and the eradicated (n = 52 isolates) PA isolates. Results are shown as median and IQR. Statistical comparisons were performed using Mann-Whitney test (***P* < 0.01, *****P* < 0.0001).

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Preface to chapter 3

In chapter 2, we showed that clinical PA isolates collected from CF children who failed eradication treatment (Persistent isolates) were resistant to *in vitro* neutrophil OPK compared to those from patients who succeeded in eradication treatment (Eradicated isolates). In addition, we observed that *in vitro* neutrophil OPK was a significant independent predictor of persistent infection. Moreover, our study is consistent with results from previous clinical studies, that PA isolates collected from patients with strain-specific phenotypes such as loss of pilus-mediated twitching motility and wrinkly colony morphology were associated with failed eradication and elicited reduced *in vitro* neutrophil OPK. Although the failure of PA eradication is likely multifactorial, little is known about how Persistent isolates with strain-specific phenotypes from early CF infection evade the host innate immune responses and promote bacterial persistence. Understanding the bacterial mechanisms in mediating resistance to neutrophil OPK is critical for eradicating and preventing PA infections. In the following chapter, using *in vitro* models with primary murine neutrophils and *in vivo* murine PA lung infection model, we investigated the mechanisms that Persistent isolates exploited to evade *in vitro* neutrophil-mediated bacterial clearance, leading to eventual failure of eradication *in vivo*.

Chapter 3: Evasion of neutrophil-mediated bacterial clearance in *Pseudomonas aeruginosa* isolates from new-onset infections in cystic fibrosis children

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3.1 Introduction

Cystic fibrosis (CF) is a genetic multi-system disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, with lung disease being the most significant cause of symptoms and death. CF lung disease is characterized by a vicious cycle of persistent infection and sustained inflammation leading to a progressive decline in lung function [1,2]. *Pseudomonas aeruginosa* (PA), a gram-negative opportunistic pathogen, causes chronic airway infections in up to 60% of adults with CF and is associated with worse clinical outcomes [3]. Antibiotics, such as inhaled tobramycin, are routinely used to treat new-onset PA infections in an attempt to eradicate PA infections at an early stage and prevent progression to chronic infections [4–7]. Unfortunately, eradication therapies fail in up to 40% of patients even in the absence of antibiotic resistance, resulting in PA infections that persist [8]. The reasons for failed PA eradication remain unclear as no significant clinical predictors have been found among patients with persistent infections compared to those who succeeded in eradication [9–11].

Neutrophils are primary innate immune cells that are critical for PA eradication and can enhance eradication therapy. Phagocytosis and intracellular bacterial killing, ROS-dependent and independent killing are hallmarks of neutrophil antimicrobial functions [12], but numerous PA factors can modulate this complex process and impede host-mediated clearance [13,14]. Mayer Hamblett et al. [15] and Vidya et al. [8] previously characterized the phenotypes of PA isolates infecting CF children from two early PA eradication clinical studies (EPIC and Hospital for Sick Children cohort study), and showed that strain-specific phenotypes such as mucoidy, lack of twitching motility and wrinkly colony morphology (overproduction of Psl and Pel EPS) were associated with persistent infection following eradication therapy. These findings thus led us to characterize all new-onset PA isolates collected from CF children [8] using *in vitro* neutrophil

opsonophagocytic killing (OPK) assays to examine the link between specific PA phenotypes, their resistance to neutrophil antibacterial functions, and outcomes of eradication therapy in CF patients [16]. We showed that infection with PA isolates resistant to *in vitro* neutrophil phagocytosis was a significant independent predictor of failed eradication therapy. More specifically, PA isolates from persistent infections (referred to as Persistent isolates) were more resistant to neutrophil-mediated phagocytosis and intracellular bacterial killing compared to those from eradicated infections (termed Eradicated isolates), and twitching-deficiency and mucoidy were the bacterial phenotypes most strongly associated with impaired *in vitro* neutrophil antibacterial functions [16].

In this study, we sought to understand how Persistent isolates evade neutrophil-mediated bacterial clearance. We chose representative Persistent and Eradicated isolates and investigated the bacterial-neutrophil interactions *in vitro* and their implications for bacterial clearance *in vivo.* Using primary murine serum and neutrophils, we demonstrated that Persistent isolates were resistant to complement binding on the bacterial surface, displayed reduced bacterial adhesion to neutrophils, elicited lower OPK and degranulation compared to Eradicated isolates. Furthermore, mice infected with Persistent isolates exhibited a significantly higher lung bacterial burden and failed *in vivo* clearance than those infected with Eradicated isolates. Additionally, leukocyte and neutrophil recruitment, as well as the host inflammatory cytokines and chemokines were examined in Persistent and Eradicated isolate infections, yet these results were not completely responsible for failed *in vivo* bacterial clearance caused by Persistent isolates. Interestingly, *pilA* overexpression in a Persistent isolate restored the isolate's susceptibility to neutrophils. Our data indicate that impaired *in vitro* neutrophil functions elicited by Persistent isolates may be

mechanisms that explain failed *in vivo* clearance, suggesting therapeutic strategies that enhance neutrophil-mediated bacterial clearance may improve PA eradication of CF patients.

3.2 Materials and Methods

Study design and PA isolates

PA clinical isolates were recovered from patients' sputum collected before the initiation of inhaled tobramycin as part of the Early PA Eradication Study and CF Sputum Biobank at the Hospital for Sick Children (REB#1000019444) as previously described [8]. New-onset PA infection was defined as a PA-positive sputum culture with at least 3 PA-negative sputum cultures in the previous 12 months, and a sputum culture was collected one-week posttreatment to determine the outcome of eradication therapy [17,18]. PA infection was considered "persistent" if the post-treatment sputum culture was positive, and "eradicated" if the post-treatment sputum culture was negative. For this study, we selected two pairs of representative clinical PA isolates from persistent and eradicated infections, referred to as Persistent and Eradicated isolates respectively, based on their susceptibility to *in vitro* neutrophil phagocytosis and intracellular bacterial killing as previously determined [16].

Bacterial culture conditions and phenotypic characterization

To generate bacterial suspensions for all assays described below, bacterial isolates were grown in 5 mL Luria-Bertain (LB) broth (Thermo Scientific) overnight at 37 °C with shaking at 200 rpm, spun down for 5 min at 12000 rpm, washed once with sterile PBS and resuspended in buffer to the indicated OD_{600nm} as specified in each assay. Assays for twitching and swimming motility, biofilm formation, Congo red binding of bacterial aggregation, and mucoidy status were

performed as previously described [16]. Briefly, twitching motility was determined by inoculating a single colony into 1% thin LB agar plate for 2 days at 37 °C and measuring the diameter of the twitching zone after staining with 0.1% crystal violet. Swimming motility was determined by inoculating a single colony into 0.3% LB agar and measuring the diameter of the zone of bacterial growth following overnight incubation at 37 °C. Biofilm formation was measured by inoculating 96-well polystyrene plates with 100 μL of diluted overnight bacterial cultures in LB medium, and incubating overnight under the static condition at 37 °C. The adherent biofilm biomass was stained with 0.1% crystal violet, resolubilized with 95% ethanol, and measured for absorbance at OD_{600nm}. Mucoidy status was determined by colony morphology following growth on yeast extract mannitol (YEM) agar for 24-48 h. Lastly, exopolysaccharide (EPS)-mediated bacterial aggregation was assessed with the colorimetric Congo red binding assay and rugose colony morphology on Congo red agar plate [19].

Psl surface expression

Psl expression was measured by ELISA as done in [20] with minor modifications. Bacteria grown overnight and resuspended in PBS (OD_{580nm} 0.2) were used to coat Nunc MaxiSorp™ plates (Thermo Scientific 44-2404-21). Following overnight incubation at 4°C, the plates were centrifuged at 7000 rpm for 5 min, and the supernatants were discarded. Primary anti-Psl antibody Psl0096 (AstraZeneca) was added (1:12000) to PBS containing 1% BSA and 0.1% Tween-20 for 1.5 h at room temperature (RT), followed by 1:25000 donkey anti-human HRP-conjugated secondary antibody (Jackson Immuno Research Laboratories 709-035-149) for 30 min and detection with TMB SureBlue peroxidase substrate (BD Biosciences 555214) was measured at OD_{450nm}.

Congo red binding of Psl and Pel-mediated aggregation on solid medium

Congo red binding of Psl and Pel on solid medium was performed as previously described [19]. Briefly, 2 μL of overnight bacterial culture was spotted on the surface of Vogel-Bonner minimal medium agar containing Congo red for 18 h at 37 °C. Wrinkly colony morphology was assessed by ranking in a blinded fashion by 2 individuals.

C3 complement deposition

C3 binding to bacterial surfaces was measured by flow cytometry. Bacterial suspensions (10^9 cells/mL in sterile PBS) were opsonized with 20% pooled mouse serum (C57BL/6 mice, Charles River Laboratories) for 1 h at 37 °C. Samples were then washed with PBS, stained with 1:500 primary C3 polyclonal antibody (Thermo Scientific PA5-21349) for 1 h on ice, followed by staining with 1:500 goat anti-mouse Alexa Fluor 647 secondary antibody (Thermo Scientific A-21244) for another 1 h. 10000 events were acquired for each sample using the Fortessa flow cytometer (BD Biosciences) with gating on the AF647+ population and using non-opsonized bacteria as negative controls after gating to exclude debris and doublets.

Primary murine neutrophil isolation

For all *in vitro* neutrophil assays, bone marrow-derived neutrophils (BMDN) were isolated from C57BL/6 male mice (8-10 weeks, Charles River Laboratories) using STEMCELL EasySep™ mouse neutrophil negative selection kit (STEMCELL 19762) according to the manufacturer's instructions.

Neutrophil opsonophagocytosis and intracellular bacteria killing

Assays to measure *in vitro* phagocytosis and intracellular bacterial killing were performed as previously described using BMDN [16]. Bacterial suspensions (10^7 cells/mL in Hank's balanced salt solution without calcium, magnesium, and phenol red (HBSS) (Wisent 311-511-CL)) were opsonized with 10% pooled mouse serum, followed by incubation with 2.5 x 10^5 neutrophils at a multiplicity of infection (MOI) of 10 for 30 min at 37 °C. Gentamicin 100 μg/mL was then added for 30 min (T = 60 min for phagocytosis) or 1.5 h (T = 120 min for intracellular bacterial killing) to kill extracellular PA. BMDN were then washed with HBSS, lysed with 0.1% Triton X-100 and plated on LB agar to measure viable intracellular bacteria by colony-forming unit (CFU) counts. For our calculations, the input bacterial is the total bacteria at T = 0 min (initial inoculum). Phagocytosis index was calculated as the number of internalized bacteria at T = 60 min per $5x10^5$ input bacteria. The intracellular bacterial killing was calculated as the relative reduction in intracellular bacterial burden, namely [the number of internalized bacteria at 60 min minus the number of internalized bacteria at 120 min] per $5x10^5$ input bacteria.

Bacterial adhesion to neutrophils

Bacterial suspensions were prepared at 1.2 x 10^7 cells/mL with HBSS. 2.5 x 10^5 BMDN per well were seeded on a microscopic multi-chamber slide (Labtek 154534) followed by incubation with Cytochalasin B (Sigma C6762) for 30 min at 37 °C to inhibit phagocytosis. BMDN were then incubated with PA at MOI 20 in the presence of 10% pooled mouse serum for 1 h at 37 °C. Following infection, BMDN were washed with PBS and blocked with 10% BSA overnight at 4 °C. Primary anti-PA antibody (1:1000) (Abcam ab68538) was used to stain for extracellular PA at 4 °C overnight followed by staining with goat anti-mouse Alexa Fluor 647

secondary antibody (Thermo Scientific A-21244) (1:500) for 1 h at RT. All samples (infected and uninfected BMDNs) were also stained with DAPI (Thermo Scientific D1306) in the presence of 1% Triton-X100 for 1 min at RT and washed with PBS three times prior to confocal microscopy imaging.

BMDN were visualized by confocal microscopy (Zeiss LSM 700) using a 63x objective. Approximately 3 to 4 field of views per experiment from 2 independent experiments were acquired, and 200 cells were analyzed using the Icy automated cell quantification software [21]. The proportion of bacteria associated with neutrophils was calculated as the number of neutrophils with RFI higher than uninfected neutrophils over the total number of neutrophils.

Neutrophil CD63 surface expression

We measured surface CD63 expression on BMDN as a marker of neutrophil degranulation following infection with PA. Bacterial suspensions (10^7 CFU/mL in HBSS) were opsonized with 10% pooled mouse serum and incubated with 5 x 10^5 neutrophils (MOI 10) for 2 h at 37 °C. Neutrophils were then washed, fixed, and stained with viability marker eFluor 780 (1:1000) (Thermo Scientific 65-0865-14) and followed by staining with 1:100 of APC-conjugated anti-murine CD11b, BV510-conjugated anti-murine Ly6G and PECy7-conjugated anti-murine CD63 (Biolegend). For each sample, 100,000 events were acquired by Fortessa flow cytometer (BD Biosciences). Gating was performed to exclude debris and doublets, live CD63 positive neutrophils were identified as eFluor 780^-, CD11b-APC^+, Ly6G-$BV510^+$, and CD63-$PECy7^+$.

Neutrophil intracellular oxidative burst

We used the Luminol assay to measure reactive oxygen species (ROS) production in neutrophils as previously described [22]. Bacterial suspension ($5x10^8$ cells/mL in HBSS with calcium and magnesium) were opsonized with 10% pooled mouse serum for 30 min at 37 °C, then washed, resuspended in HBSS (with calcium and magnesium) and incubated with neutrophils (MOI 50) in the presence of 50 μM of Luminol (Sigma 123072), 2000 U of catalase (Sigma C-3155), and 50 U of superoxide dismutase (Sigma S-7571) in white microtiter plates (PerkinElmer) at 37 °C. ROS generated by neutrophils were measured by luminescence every 5 min using a Tecan microplate reader (Infinite 200, Lifesciences). The area under the concentration-time curve (AUC) was calculated to determine the relative ROS generated by neutrophils over the time course of 2 h. Results were normalized to ROS induced by phorbol myristate acetate (PMA) to account for variation between experiments.

***pilA* complementation in Persistent isolate**

Plac-*pilA* was first constructed by overexpressing *pilA* under a synthetic *lac* promoter in a miniTN7-based vector using the Gibson assembly system (NEB). Genetic complementation of *pilA* was then generated by chromosomal insertion of Plac-*pilA* construct into 565P. Sequencing of the construct and *in vitro* bacterial twitching assay were done to validate the complementation of *pilA* in 565P. The primers used to construct the Plac-*pilA* were: Forward 5'-ATA GAT CTA. AAC TAT GAC AAT-3' and reverse 5'-AGC CTT TTT GAG CTT TCA TGC TTA ATT TCT CCT CTT TAA TT-3' (fragment 1); forward 5'-CGA AAG GTT GTG ATA ACT AAG AAT TCG ACG AGC CTG CTT TT-3'and reverse 5'-ATG GTA AGC CCT CCC GTA TCG-3'

(fragment 2); forward 5'-ATA CGG GAG GGC TTA CCA TC-3' and reverse 5'-ATT GTC ATA GTT TAG ATC TAT-3' (fragment 3).

Murine PA pulmonary infection

A murine PA pulmonary infection model was used to compare the *in vivo* clearance of different PA isolates. Specific pathogen-free C57BL/6 male mice (8-10 weeks, Charles River Laboratories) were anesthetized and infected with $1x10^7$ CFU/mouse using a non-surgical endotracheal method [23]. At designated time points post-infection (p.i.) (6, 48, 96 h p.i.), mice were euthanized, blood was collected by cardiac puncture, and the lung vessels were perfused with ice-cold PBS. Serum was collected from whole blood using Z-Gel microtubes (Sarstedt) and stored at -80 °C until analysis.

The bronchoalveolar lavage fluid (BALF) was collected by injecting and aspirating 4 x 500 μL of ice-cold PBS through an endotracheal catheter, and cells were pelleted down (1500 rpm, 10 min). Aliquots of the BALF cell-free supernatant were stored at -80 °C with protease inhibitor cocktails (Thermo Scientific 78441) until analysis. Following the lavage, lungs were removed, placed in 10 mL ice-cold PBS, and minced. To quantify the PA bacterial burden, 1 mL of the minced lung was homogenized with 0.2 mm stainless steel beads using a bullet blender (Next Advance). Lung homogenates were serially diluted and plated on pseudomonas isolation agar (PIA) for CFU counts. For cytokine and flow cytometry analyses, the remaining minced lung sample was split into two parts. One part was homogenized in the bullet blender, pelleted down 12000 rpm for 5 min, and aliquots of cell-free supernatant were stored at -80 °C with protease inhibitor until analysis. The other part of the minced lung sample was digested with collagenase (Sigma C5138) and filtered through a 100 μm cell strainer. Following red blood cell

lysis, cells were spun down, washed, resuspended in PBS, and proceeded with flow cytometry analysis.

All animal experiments were carried out in accordance with the Canadian Council on Animals Care and with approval from the Animal Care Committee of the Research Institute of the McGill University Health Centre (AUP #7586).

Cytokine and chemokine measurements

Serum, protease-inhibitor treated BALF and lung homogenate supernatant samples were analyzed for a panel of mouse cytokine/ chemokine using Luminex 31-Plex Discovery Assay Array (MD31) with the following biomarkers: Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, VEGF-A (Eve Technologies).

Flow cytometry analysis of BALF and whole lung homogenates

Two million cells from whole lung single-cell suspensions or BALF were fixed and stained with eFluor780 fixable viability dye (1:1000) (Thermo Scientific 65-0865-14), then surface stained with fluorescently-conjugated murine innate immune cell markers that target neutrophils (CD45-PE, CD11b-APC, Ly6G-BV510), monocytes (CD45-PE, CD11b-APC, Ly6C-BV785), macrophages (CD45-PE, CD11b-APC, $Ly6G^{lo}$-BV510, $F4/80^{hi}$-Pacific blue) and dendritic cells (CD45-PE, CD11b-APC, $Ly6G^{lo}$-BV510, CD11C-BV711). 100,000 events were analyzed using the Fortessa (BD Biosciences) with gating on live CD45+ cells after excluding

debris and doublets. Unless specified, all antibodies used in this experiment were from Biolegend and were diluted to a final concentration of 1:100.

Whole genome sequencing and analysis

All PA isolates were sequenced using Illumina NextSeq and analyzed as described in [24]. Sequencing reads were previously deposited in NCBI (PRJNA556419). Following de-novo assembly, sequencing read quality assessment and adaptor trimming, targeted comparative genome analysis was then performed on the two pairs of representative Persistent (565P and 505P) and Eradicated (513E, 558E) isolates.

Data and Statistical analyses

Flow cytometry data were analyzed using FlowJo Software, version 10.8.1 (Ashland, OR, USA). All results are shown as mean ± SEM or median ± IQR as indicated. Statistical analyses were done using Prism 9 software (Graphpad). Comparisons were performed using ANOVA Dunn's test or unpaired two-tailed student's t-test as indicated. A P value of < 0.05 was considered statistically significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$

3.3 Results

A collection of clinical PA isolates from the Early PA Eradication cohort study at the Hospital for Sick Children was previously assayed for various bacterial phenotypes [8,25], and susceptibility to neutrophil phagocytosis and intracellular bacterial killing [16]. In our recent study by Kwong et al., we found that PA isolates from persistent infections were resistant to neutrophil phagocytosis and intracellular bacterial killing compared to PA isolates from

eradicated infections, and among non-mucoid PA isolates, loss-of twitching motility was the only bacterial phenotype significantly associated with resistance to neutrophil antibacterial functions [8,16]. To further investigate these bacterial-neutrophil interactions and their implications to bacterial clearance *in vivo*, we selected two pairs of representative PA isolates among the Eradicated and Persistent isolates, based on their susceptibility to neutrophil OPK in our original dHL-60 cells *in vitro* assays ([16], Figure 3.1). The bacterial characteristics of these strains that are relevant to CF infections are shown in Table 3.1. The Persistent (565P and 505P) and eradicated isolates (513E and 558E) that showed dominant *in vitro* bacterial phenotypes were therefore chosen as representatives of each group. Notably, Persistent isolates overproduced Psl and Pel (wrinkly colony morphology and high Congo red binding) and were deficient in pilus-mediated twitching motility, while Eradicated isolates did not overproduce Psl and Pel and had functional twitching motility. All the isolates were non-mucoid.

We first validated the susceptibility of our representative PA isolates to phagocytosis and intracellular bacterial killing in BMDN. As expected, Persistent isolates 565P and 505P were more resistant to phagocytic uptake and intracellular bacterial killing compared to Eradicated isolates 513E and 558E (Figures 3.2a, b). To further dissect the mechanisms that contribute to the PA strain-specific interactions with neutrophils, we then compared the Persistent and Eradicated isolates in a series of *in vitro* assays.

Complement-dependent opsonization is important for bacterial clearance, particularly for neutrophil-mediated antibacterial functions [26]. Surface molecules such as the EPS Psl can interfere with complement deposition on the PA bacterial surface [27]. We thus measured the surface binding of C3, the most abundant complement component that binds to the PA surface [27], and observed a significantly lower C3 surface binding on Persistent isolates 565P and 505P

(42.6% and 50%) compared to the Eradicated isolates 513E and 558E (72.1% and 75.7%) (Figure 3.2c).

Surface adhesion to neutrophils is a critical initial step to initiate phagocytic bacterial uptake, and this process can be modulated by strain-specific bacterial characteristics such as complement deposition, bacterial motility, and surface appendages [27,28]. We thus compared the ability of Persistent and Eradicated isolates to bind neutrophils in a bacterial adhesion assay using BMDN. As shown in Figure 3.2d, a lower proportion of PA-adherent BMDN was observed in infection with Persistent isolates (565P and 505P) compared to infection with Eradicated isolates (513E and 558E) (42.5% and 30.5% vs. 82.5% and 56.5%, respectively), suggesting that the Persistent isolates had reduced adhesion to neutrophils.

To further examine whether the resistance of Persistent isolates to neutrophil intracellular bacterial killing is attributable to impaired oxidative or non-oxidative killing mechanisms, we assessed the intracellular ROS production and degranulation of BMDN infected with different PA isolates. Isolate 505P induced a significantly lower ROS production than 513E and 558E, while 565P did not (Figure 3.2e), suggesting that impaired ROS production was not sufficient to explain impaired intracellular killing in both Persistent isolates. Next, we assessed neutrophil degranulation as a proxy for non-oxidative antimicrobial killing activity by measuring neutrophil surface CD63 expression in BMDN infected with different PA isolates. As shown in Figure 3.2f, the proportion of CD63+ neutrophils was lower among 565P and 505P-infected neutrophils compared to those infected with 513E and 558E. These results suggest that impaired neutrophil degranulation in response to 565P and 505P may have contributed to the reduced killing of these Persistent isolates compared to Eradicated isolates. Our findings thus raise the possibility that

Persistent isolates may also be resistant to the direct bacterial killing mechanisms mediated by other neutrophil granules such as primary and secondary granules.

Loss of twitching motility is likely an important contributor to OPK resistance. This phenotype was significantly associated with resistance to neutrophil OPK *in vitro* in our previous study of 71 CF clinical isolates, and 26% (5/19) of Persistent isolates were non-twitching [16]. This led us to examine the contribution of twitching deficiency in the OPK resistance of 565P and 505P. We first performed whole genome sequencing in all 4 PA isolates to identify the genetic mutation(s) potentially responsible for the twitching defect. We analyzed the sequence of the following genes known to be involved directly or indirectly in type 4 pilus (T4P) biosynthesis and twitching motility: *pilA, pilB, fimV, pilT, pilU, pilS, pilR, RpoN, RetS, fliC, algT/U, tadD, tadB, tadA, rcpC, rsm, flp* [29]. We also examined genes indirectly involved in surface attachment and motility, namely EPS production and global regulators: *mucA, mucD, algK, algE, algG, pslA, pslB, pslH, pslI, pslL, pelA, pelB, pelD, pelF, pelG, cdrA, wspA, wspC, wspE* [30]. We found that *pilA* was absent in 565P, while 505P encoded a *pilA* variant, a functional allele previously found in pediatric CF isolates [31]. No other non-synonymous genetic variants were identified in any of the other genes.

Since the 565P isolate lacked *pilA*, we asked whether overexpressing *pilA* would functionally complement twitching motility and improve neutrophil antibacterial functions. While the twitching motility in strain 565P Plac-*pilA* was modest (Figure 3.3a), this was sufficient to restore significant neutrophil phagocytosis, intracellular bacterial killing, and degranulation (Figures 3.3b-d). Together, these results suggested that twitching deficiency was an important evasion mechanism of neutrophil antibacterial functions in 565P. Attempts to genetically manipulate and transform the 505P with the Plac-*pilA* construct were unsuccessful.

In order to determine whether *in vivo* host clearance of 565P and 505P was also impaired compared to 513E and 558E, we used a murine pulmonary infection model. We quantified the lung bacterial burden at 6, 48 and 96 h p.i and observed no differences in bacterial burden between the different isolates at 6 h p.i. While we observed a decrease in bacterial burden for all isolates over time, bacterial clearance was significantly greater with 513E and 558E compared to 565P and 505P (Figure 3.4a) at 48 h p.i. (median CFU counts $5x10^3$ (513E) and $1.1x10^5$ (558E) compared to $1x10^5$ (565P) and $4.8x10^5$ (505P)). By 96 h p.i., 513E and 558E were completely cleared in all mice, while 565P and 505P infected mice still had a median of $1.4x10^4$ and $1.1x10^4$ CFU/ mouse respectively and none had completely cleared their infection (Figures 3.4a and b).

To assess whether impaired bacterial clearance of Persistent isolates was due to differential leukocyte recruitment, we measured the immune cell counts in the lung homogenates of mice infected with the different PA isolates at 6, 48, and 96 h p.i. and found no significant differences in the total leukocyte counts (Figure 3.5a), neutrophil counts (Figure 3.5b) nor the percentage of neutrophils (Figure 3.5c) across the different infection groups. Our data thus far indicates that bacterial persistence in mice infected with 565P or 505P could not be explained by any defect in immune cell recruitment.

To further examine whether the host cytokine and chemokine responses differed between infections with different PA isolates, we measured a 31-plex panel of murine cytokines and chemokines involved in inflammatory and immune responses in serum, lungs, and BALF. Eight key analytes were induced upon PA infection at 6 h p.i., namely the mediators of innate immune cell activation, proliferation, and migration important to bacterial clearance (GM-CSF, G-CSF, MCP-1, IL-6, KC, MIP-1α, IL-1β and TNF-α) [32–36] (Figures 3.6a-c), consistent with the existing literature [37]. Among these analytes, we noted a significantly lower IL-1β, IL-6 and

MCP-1 in serum and BALF samples of 505P-infected mice compared to all other ones at 6 h p.i. However, these differences were only observed in 505P-infected mice and were not accompanied by reduced immune cell counts, indicating that they were unlikely to drive the *in vivo* clearance defect observed with both 565P and 505P infections. The other 23 analytes were also analyzed, but their concentrations were below their respective detection limits [38] (Data not shown). Together, these cytokine profiles do not explain the impaired *in vivo* clearance of both Persistent isolates 565P and 505P.

3.4 Discussion

The mechanisms underlying PA-neutrophil interactions in CF persistent infection remain incompletely understood. Our study showed that Persistent isolates 565P and 505P were resistant to several *in vitro* neutrophil antibacterial functions (bacterial adhesion to neutrophils, OPK, and CD63 degranulation) compared to Eradicated isolates 513E and 558E. In contrast to Eradicated isolates, the Persistent isolates also failed to be cleared *in vivo,* but this persistence could not be attributed to impaired cytokine responses or immune cell recruitment. In the non-twitching 565P isolate, restoring its pilus-mediated twitching motility significantly increased its susceptibility to *in vitro* neutrophil OPK and CD63 degranulation activity. Altogether, our findings suggest that strain-specific bacterial phenotypes observed in PA CF clinical isolates, particularly loss of pilus-mediated motility, are an important determinant of bacterial-neutrophil interactions that drive bacterial clearance, and resistance to neutrophil-mediated antibacterial functions is a key contributor of bacterial persistence *in vivo*.

Others have previously examined strain-specific PA-neutrophil interactions using clinical CF isolates and focusing on various neutrophil functions implicated in bacterial clearance. For

example, two studies assessed *in vitro* neutrophil extracellular trap (NET) formation in response to paired clonally related PA isolates from early-stage and chronic CF infections and reported relatively lower NETs elicited by chronic PA isolates compared to their clonally related early isolates [39, 40]. Mahenthiralingam et al. originally reported that ~40% of PA isolates from CF infection lacked swimming (flagellar-mediated) motility and were more resistant to OPK by mouse macrophages than motile isolates, but neutrophil phagocytosis was not tested. While they attempted to detect pilus expression, they did not assess twitching (pilus-mediated) motility in these isolates [41].

PA produces three EPS, namely alginate, Psl and Pel, and EPS overproduction contributes to resistance to antibiotics and neutrophil-mediated antimicrobial mechanisms through several different mechanisms [42]. Overproduction of alginate results in a phenotype called mucoidy, which is common among chronic CF infection isolates [43,44], but relatively rare in environmental and new-onset CF infection isolates [45,46]. Although mucoidy was among the bacterial phenotypes associated with failed eradication therapy [16], and with impaired *in vitro* neutrophil antibacterial functions in our previous study [16], we chose to study representative Persistent and Eradicated isolates that were non-mucoid. Since alginate overproduction is known to inhibit neutrophil elastase activity, NETs, and ROS-mediated antibacterial functions [47,48], the study of non-mucoid isolates allowed us to examine the contribution of other bacterial factors without the confounding effect of alginate overproduction.

Psl and Pel EPS are both cell surface-associated and secreted, and their overproduction leads to a wrinkly colony morphology due to increased levels of the intracellular signaling molecule cyclic di-GMP (C-di-GMP) [30]. Both of our Persistent isolates displayed a wrinkly

colony morphology (albeit at a greater degree in 565P), as well as higher Congo red binding, which serves as a surrogate measurement for Psl and Pel production [19], than Eradicated isolates. While our Psl measurement by ELISA did not suggest differences between Persistent and Eradicated isolates, Morris et al. demonstrated that Persistent isolates 565P and 505P expressed high Psl when grown as mature biofilms and measured with immunofluorescence by confocal microscopy using the same antibody [25]. In addition to their role as a constituent of the biofilm matrix, Pel and Psl are important surface molecules that modulate innate immune responses. Psl is involved in cell-substrate bacterial attachment to biotic and abiotic surfaces [49–51], and may thus contribute to the observed reduced neutrophil adhesion in Persistent isolates. Consistent with Mishra et al. who reported that Psl interferes with complement-mediated neutrophil OPK [27], we also found a lower deposition of complement C3 on the surface of Persistent bacteria compared to Eradicated isolates. The PA outer membrane porin OprF also serves as an acceptor molecule to enhance complement binding, and loss of OprF reduces complement fixation [52]. While we did not examine OprF expression in our PA isolates, since chronic CF infections seem to select against OprF-expressing PA [53,54], it is possible that Persistent isolates exhibit minimal or no OprF [55-56], thus altering complement deposition on 565P and 505P.

We observed impaired clearance of Persistent isolates *in vivo*, which is the end result of numerous host-pathogen interactions and responses. Using a genetically engineered PA mutant *wspF* which overproduces Psl and displays the wrinkly phenotype, Pestrek et al. showed that the *wspF* mutant failed to be eradicated in a mouse acute pulmonary infection model compared to its wild-type parental strain [35]. Notably, the Psl-overproducing *wspF* mutant induced a vigorous neutrophilic inflammatory response *in vitro* and *in vivo*, consistent with observations by Rybtke

et al. [57] and ours that neutrophil recruitment is not deficient. Interestingly, we did observe a trend towards higher concentrations of IL-1β, KC, IL-6, MCP-1 in serum and BALF from 565P-infected mice compared to 505P-infected mice at 6 h p.i. It is plausible that the EPS production seen in 565P increases attachment to host cells and TLR-dependent activation [35]. Nevertheless, these cytokine differences did not result in detectable differences in immune cell recruitment nor bacterial clearance. Moreover, Baker et al. reported that degradation of Pel increased neutrophil killing of PA biofilms [58], suggesting that Pel may also hinder neutrophil-mediated antibacterial functions. Although the details of this interaction remain to be elucidated, we however did not measure Pel expression as no established methodology exists.

Bacterial surface appendage type four pili (TFP) promotes biofilm formation, colonization to abiotic and biotic surfaces, and facilitates direct bacterial-host cell attachment [59,60]. PA type 4 pilin production is encoded by *pilA*, and its expression is essential for PilA, a major pilin subunit that acts as an adhesin [61]. PilA transcription is regulated by RpoN, but can be altered by PilB or PilT ATPases, and the two-component system PilSR, as well as the secondary global regulator C-di-GMP [29,30,62]. Genes that are associated with bacterial motilities (twitching, swimming, swarming) can be impacted by increased levels of C-di-GMP via direct or indirect mechanisms [30]. In *Salmonella enterica*, phagocytic function requires the adhesin *fimA*, a major type 1 fimbria that closely resembles T4P found in PA [63]. In PA, it is still not known whether the T4P adhesin (pilus tip) and/or pilus-mediated twitching motility is required to initiate phagocytic uptake. The PilT and PilU retraction ATPases are important for surface attachment to epithelial cells, leading to cytotoxicity [64], and the establishment of corneal infections [65]. Others have also demonstrated that phagocytic uptake of non-motile bacteria is dependent upon the activation of PIP3/AKT pathway [28,66], which regulates actin

polymerization and cytoskeletal arrangements [67,68]. It is likely that both adhesion and twitching motility contribute to opsonic and non-opsonic phagocytic uptake, and studies using *pilB*, *piT/U,* and *pilA* mutants could help further dissect their respective roles. Our results showed that overexpression of *pilA* led to a significant improvement in twitching and restored 565P's susceptibility to neutrophil antibacterial functions. We speculate that T4P-mediated twitching motility increases bacteria-host cell proximity, while the tip of PilA acts as an adhesin to stabilize the interaction between PA and neutrophils to initiate phagocytic uptake.

While non-twitching Persistent isolate 565P lacked a *pilA* gene, 505P encoded for a group I *pilA* allele, described as a distinct allele most commonly found among CF clinical isolates, particularly those recovered from CF children [31,69]. Among the five pilin alleles described in PA, the group I pilin may encode for a shorter pilin associated with a lower hydrophobicity and twitching motility compared to others [31,70]. This genetic variation may contribute to the variability of twitching motility among clinical isolates. Furthermore, Tan et al. showed that group I pilin from strain PA1244 conferred a fitness survival in a mixed murine pulmonary infection model compared to pilin-deficient PA1244, and that the survival advantage was attributable to its resistance to pulmonary surfactant protein A (SPA)-mediated opsonic phagocytosis [71].

There are several limitations to our study. First, PA phenotypes such as Psl and Pel overproduction and loss of pilus-mediated twitching motility are typical of chronic CF infections. This raises the possibility that the Persistent isolates recovered from our patient cohort, despite being defined as "new-onset" infections, may in fact arise from chronic infections not detected previously. Second, the persistent infection outcome following tobramycin eradication therapy was defined by a PA-positive sputum culture 1 week after the end of treatment. A comparison of

PA isolates recovered before and after tobramycin treatment by whole genome sequencing or genotyping would provide confidence on the true persistent nature of these infections in CF patients. Third, we limited our studies to four representative clinical PA isolates. Even though they display prototypical phenotypes of our larger cohort collection, the limited sampling may have introduced a bias. Fourth, our *in vitro* and *in vivo* models do not account for intrinsic neutrophil defects associated with CFTR dysfunction or acquired defects secondary to the inflammatory milieu of the CF airway. Primary murine CF neutrophils or mouse models could be used to examine the contribution of a CF host. Finally, we recognize that our *in vivo* infection model does not capture the dynamic neutrophil-mediated antibacterial functions. Models such as *in vivo* phagocytosis [72], or intravital microscopy would strengthen our study of PA-neutrophil interactions [37].

In summary, Persistent isolates display strain-specific phenotypes (higher Psl and Pel production and lack of twitching motility) and can evade neutrophil-mediated bacterial clearance through several mechanisms, notably resistance to OPK. Enhancing PA-neutrophil interactions could be a therapeutic strategy to improve the eradication of new-onset PA infections.

3.5 Figures

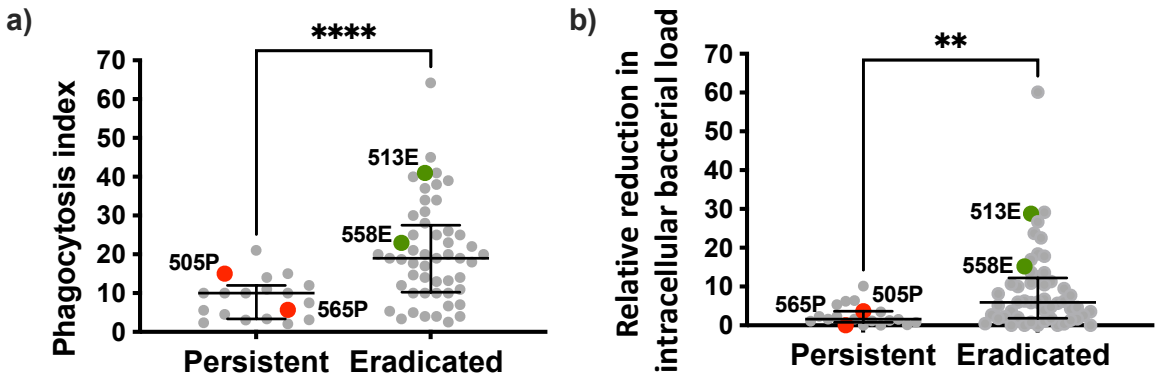


Figure 3.1 PA clinical isolates from patients with persistent infections are resistant to *in vitro* a) phagocytosis and b) Intracellular bacterial killing compared to those from patients with eradicated infections. This figure includes data adapted from Kwong et al. 2022. Assays were performed with immortalized human neutrophil-like dHL-60 cells. Representative Persistent and Eradicated isolates are highlighted. Results are shown as median ± IQR (n ≥ 4 biological replicates from n ≥ 2 independent experiments). $^{**}P < 0.01$, $^{****}P < 0.0001$ using the Mann-Whitney test.

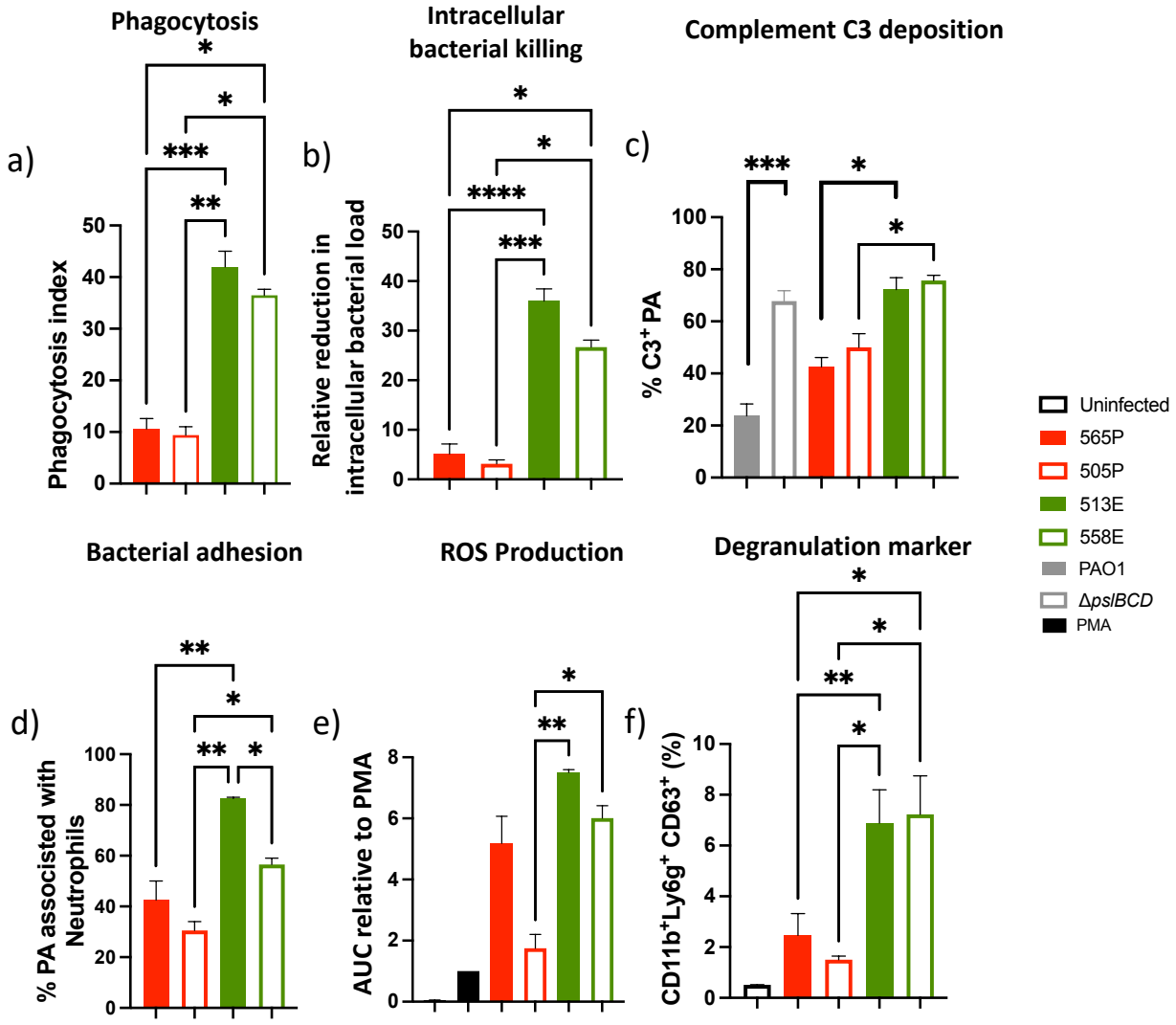


Figure 3.2 Persistent isolates are resistant to primary murine neutrophil antibacterial functions compared to Eradicated isolates *in vitro.* a) Phagocytosis; b) Intracellular bacterial killing; c) C3 complement deposition on PA cells; d) Bacterial adhesion to neutrophils; e) ROS production; f) CD63+ neutrophils in response to PA isolates. Results are shown as mean ± SEM (n ≥ 6 biological replicates from n ≥ 2 independent experiments). **P* < 0.05, ***P* <0.01, ****P* < 0.001, *****P* < 0.0001 using the ANOVA Dunn's test.

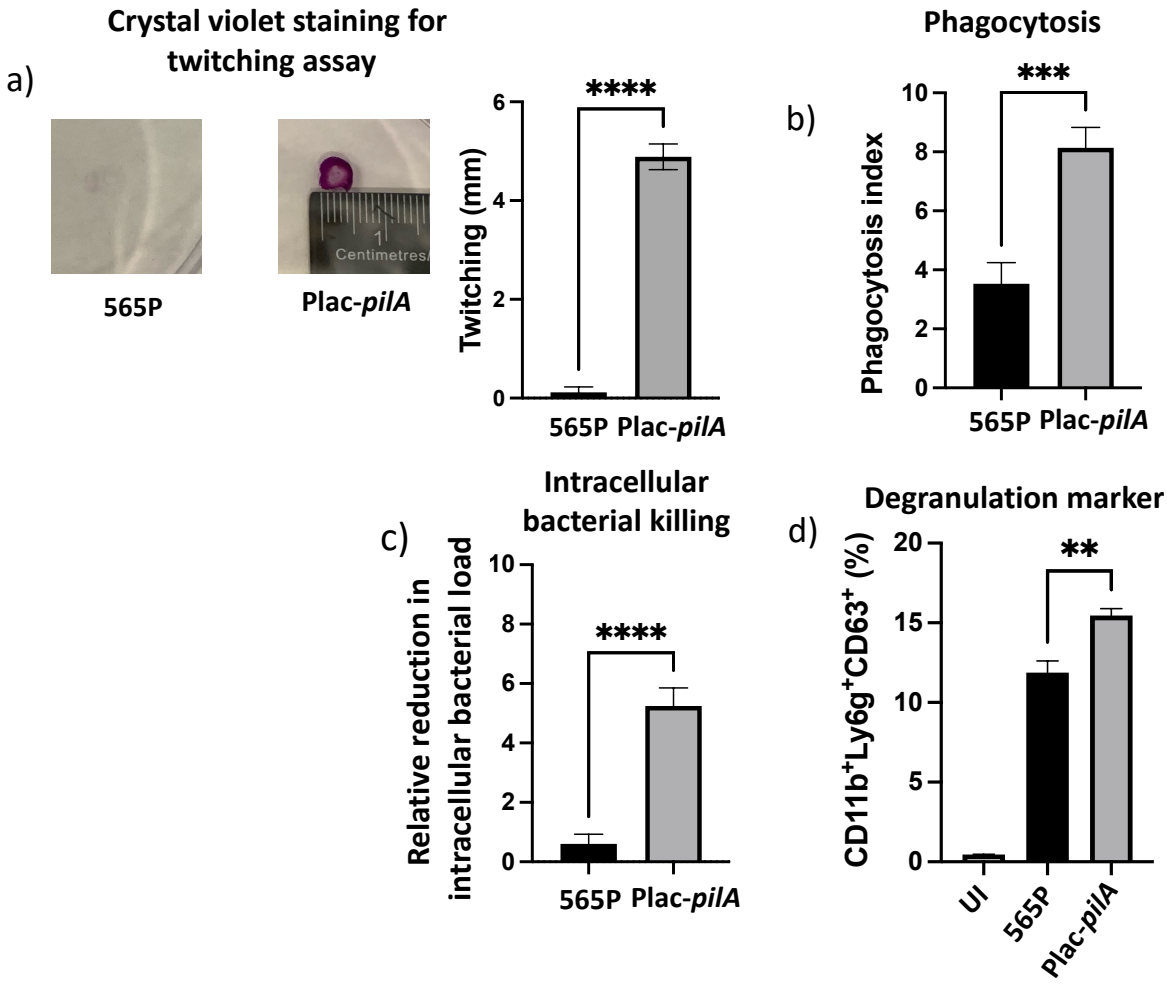


Figure 3.3 Overexpression of *pilA* restored twitching motility in 565P and its susceptibility to neutrophil antibacterial functions. a) Twitching motility of 565P and Plac-*pilA* with crystal violet staining of the twitching zone, b) Neutrophil phagocytosis, c) Intracellular bacterial killing, and d) Degranulation in response to *pilA* overexpressing 565P Plac-*pilA* and its parental 565P. Results are shown as mean ± SEM (n≥ 6 replicates from ≥ 2 independent experiments). ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 using Dunnet's test.

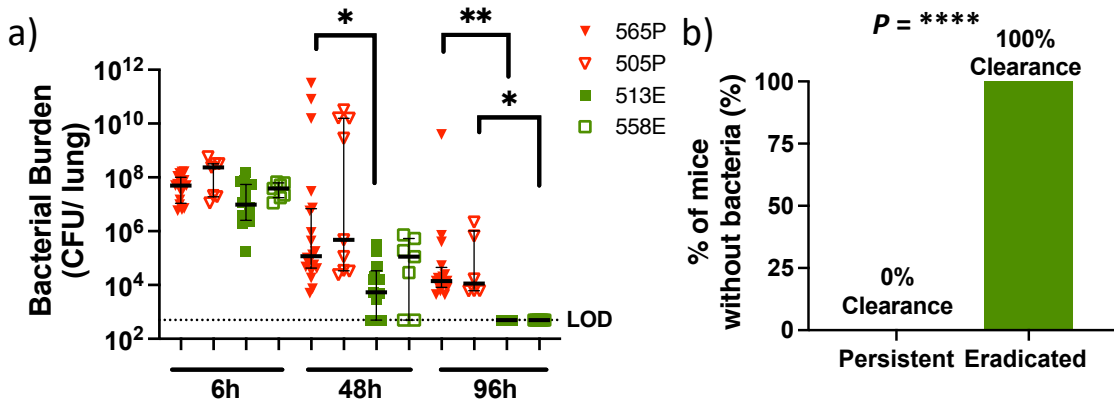


Figure 3.4 Bacterial clearance of Persistent isolates 565P and 505P is impaired in a murine lung infection model. a) Bacterial burden recovered from whole lung homogenates at 6, 48, and 96 h p.i. Results are shown as median. N ≥ 6 mice per strain pooled from ≥ 2 independent experiments. LOD: limit of detection. b) The proportion of mice with complete bacterial clearance at 96 h p.i. *$P < 0.05$, **$P < 0.01$, **** $P < 0.0001$ using ANOVA Dunn's test for a) and Fisher's exact test for b).

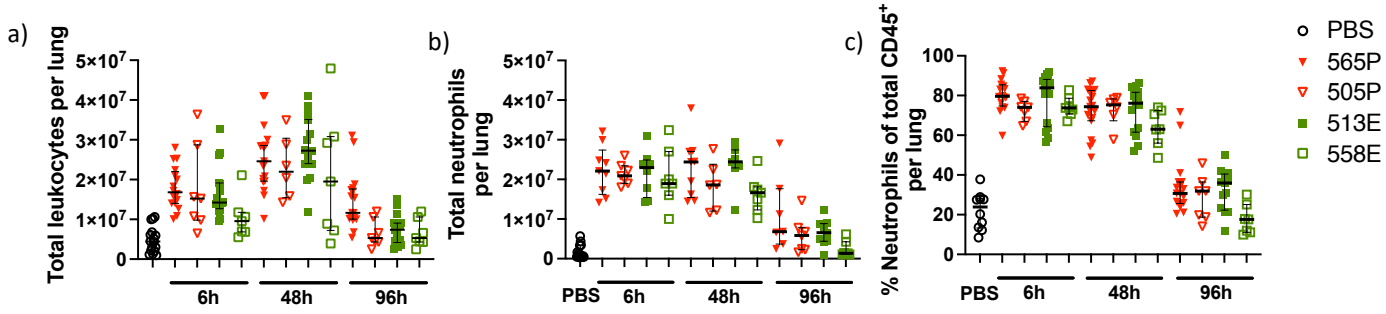
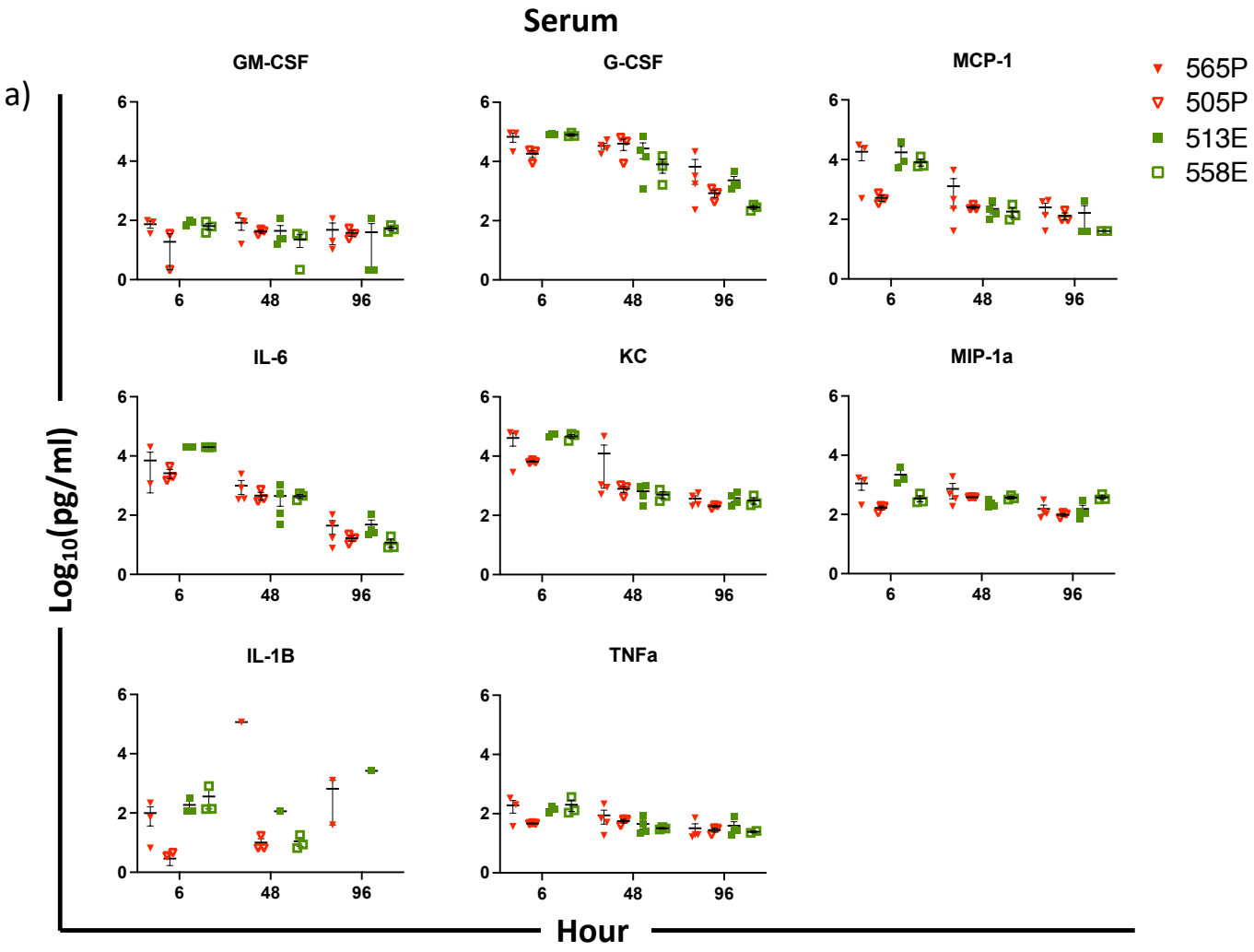
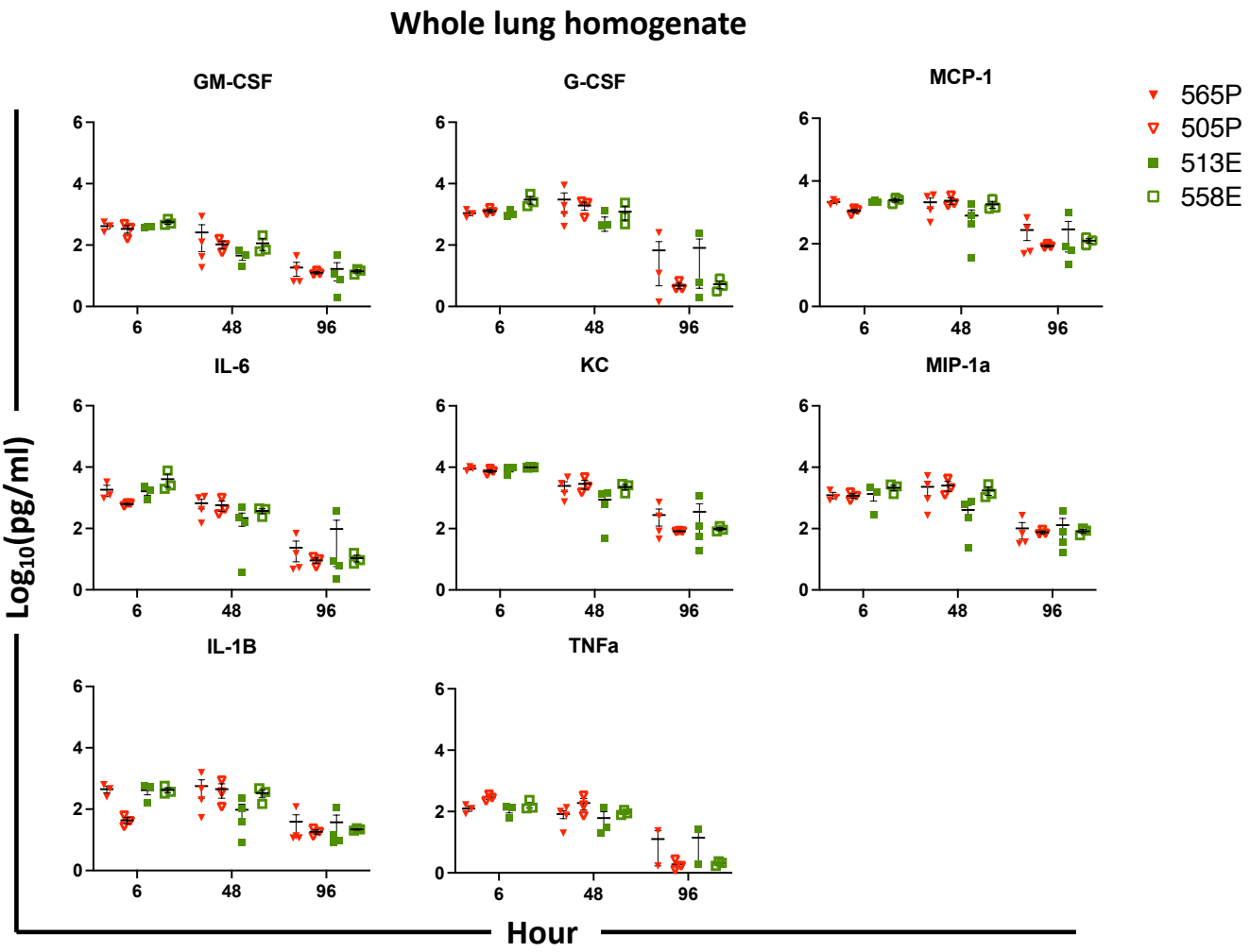


Figure 3.5 Leukocyte recruitment to the lung in Persistent vs. Eradicated isolate infections. a) Proportion of neutrophils, b) Number of neutrophils, and c) Leukocytes in whole lung homogenates at 6, 48, and 96 h p.i. Results are shown as median. N ≥ 7 mice per strain from ≥ 2 independent experiments. No statistical differences were detected between the groups using ANOVA Dunn's test.


a)
Serum
GM-CSF
G-CSF
MCP-1
IL-6
KC
MIP-1a
IL-1B
TNFa
565P
505P
513E
558E
Log10(pg/ml)
Hour
0
2
4
6
6
48
96

b)


Whole lung homogenate
GM-CSF
G-CSF
MCP-1
IL-6
KC
MIP-1a
IL-1B
TNFa
565P
505P
513E
558E
Log10(pg/ml)
Hour
0
2
4
6
48
96

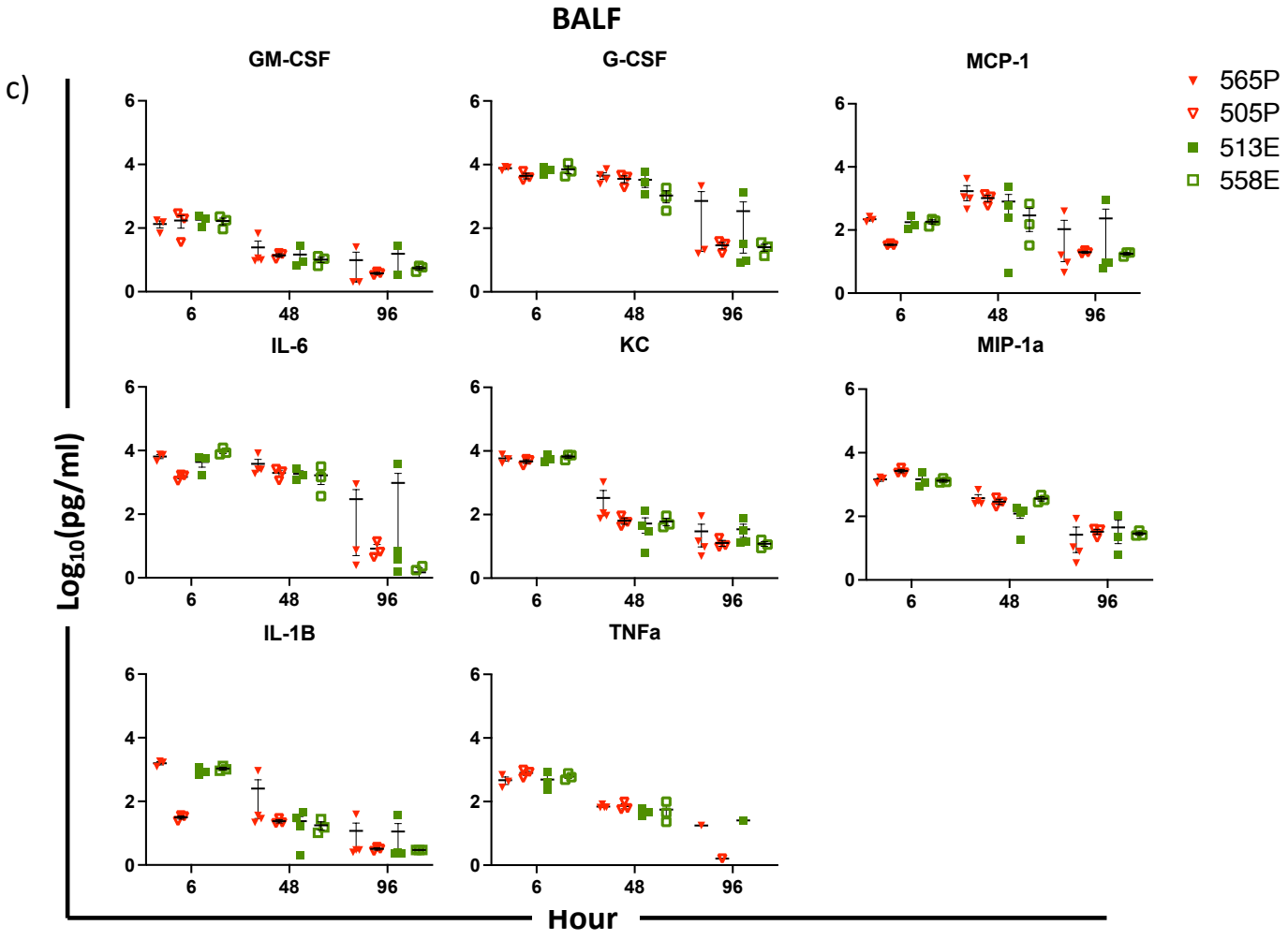


Figure 3.6 Systemic and lung cytokine responses in mice infected with 565P, 505P, 513E, and 558E isolates. a) Cytokines and chemokines detected in serum, b) Whole lung homogenates and c) the BALF at 6, 48, and 96 h p.i. Results are shown as mean ± SEM (N = 3 mice per time point and isolates) using ANOVA Dunn's test.

3.6 Table

Table 3.1 Phenotypic characteristics of PA isolates.

Isolates	Eradication status	Twitching motility (mm)	Swimming motility (mm)	Biofilm formation (OD_{600nm})	Mucoidy	Congo Red binding	Wrinkly colony morphology	Psl production (OD_{450nm})
565P	Persistent	0	0	0.12	-	-0.38	+++	1.8
505P	Persistent	1.5	7.83	0.24	-	-0.32	+	1.9
513E	Eradicated	23	12.67	0.18	-	-0.16	-	0.9
558E	Eradicated	26.5	12	0.21	-	-0.07	-	1.7

Representative Persistent and Eradicated PA isolates were selected according to their susceptibility to *in vitro* antibacterial functions by dHL-60 cells, twitching motility, and EPS production. Among these measurements, pilus-mediated twitching motility (P = **), wrinkly colony morphology (P = *), and Psl production (P = **) were significantly different in a subset of well-characterized Persistent isolates (n = 7) compared to Eradicated isolates (n = 8). **P < 0.01 and *P < 0.05. [8,16,25].

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Preface to chapter 4

To evade innate immune bacterial clearance mediated by neutrophils, representative Persistent isolates with strain-specific phenotypes (loss of pilus-mediated twitching motility and wrinkly colony morphology) inhibited C3 complement fixation and reduced bacterial adhesion to neutrophils, thus resulting in resistance to *in vitro* neutrophil-mediated OPK and failed bacterial clearance *in vivo* compared to representative Eradicated isolates. In addition, we assessed the host inflammatory responses systemically. As a result, leukocyte and neutrophil recruitment, and cytokine responses did not explain failed bacterial clearance in mice infected with Persistent isolates. Moreover, overexpression of PilA in a Persistent isolate that lacked pilus restored the isolate's susceptibility to neutrophil OPK, suggesting that targeting bacterial factors that enhance PA-neutrophil interactions could be a therapeutic treatment to improve clinical outcomes of patients with CF. Toward this goal, in chapter 4, we explored and tested a promising antibacterial monoclonal antibody treatment (monospecific mAb Psl0096 that targets Psl and bispecific mAb MEDI3902 that targets Psl and PcrV) for their ability to potentiate neutrophil-mediated bacterial clearance using a subset of representative Persistent and Eradicated isolates both *in vitro* and *in vivo*.

Chapter 4: Monoclonal antibodies targeting Psl and PcrV potentiate *in vitro* neutrophil opsonophagocytic killing and *in vivo* bacterial clearance of *Pseudomonas aeruginosa* isolates from cystic fibrosis children that failed tobramycin eradication therapy

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4.1 Introduction

Cystic Fibrosis (CF) is a multi-system genetic disease caused by defects in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. However, CF lung disease is the major cause of morbidity and mortality, and it is characterized by impaired mucociliary clearance, accompanied by persistent bacterial infection and sustained inflammation leading to tissue damage and progressive decline in lung function [1]. *Pseudomonas aeruginosa* (PA) is the predominant opportunistic pathogen that infects adult CF patients. Although people with CF typically acquire PA as early as infancy, PA infections often progress to chronic infections associated with worse clinical outcomes [2]. To delay or prevent chronic infection, much effort has been focused on eradicating new-onset PA infection, with a small window for therapeutic intervention when PA is first detected [3,4]. While early interventions with antibiotic eradication therapies, such as inhaled tobramycin, have significantly improved the success of PA eradication [5–7], treatments still fail in up to 40% of CF patients. Therefore, novel therapies are still needed to improve PA eradication in CF patients.

Neutrophils carry out phagocytosis, intracellular bacterial killing, and are the primary innate immune cells responsible for eradicating PA. Even with antibiotic treatments, neutrophil-mediated antibacterial functions are critical for PA clearance [8,9]. Using a collection of clinical isolates recovered from the early PA eradication cohort study of CF children diagnosed with new-onset PA infection who underwent inhaled tobramycin eradication treatment [10], we recently compared PA isolates from patients who failed eradication (Persistent isolates) to those from patients who succeeded (Eradicated isolates) and showed that PA isolates' resistance to *in vitro* neutrophil phagocytosis was a significant independent predictor of failed eradication treatment outcome [11].

PA can evade and compromise innate immune defenses using different strategies [12]. Notably, surface molecules, such as exopolysaccharides (EPS), modulate PA-neutrophil interactions. PA produces three EPS, namely alginate, Psl, and Pel. The presence of mucoidy (overproduction of alginate) is associated with chronic infection and worse clinical outcomes in CF. In contrast, Psl and Pel are non-mucoid EPS. Both are significant biofilm matrix components and are involved in the biofilm development [13,14]. Pel initiates and maintains cell-to-cell interaction and likely increases resistance to aminoglycosides [15–17]. Although Pel interferes with the neutrophil-mediated bacterial killing of biofilms [18], the exact mechanisms remain poorly understood. Psl is also involved in early cell-cell and cell-substrate bacterial attachment to biotic and abiotic surfaces [19,20], helps form bacterial aggregates during biofilm maturation, and has been detected in sputum from CF patients [13,21]. Moreover, Morris et al. showed that PA isolates from patients who failed eradication therapy exhibited increased Psl production when grown in mature biofilms compared to PA isolates from patients who succeeded in tobramycin eradication therapy. Furthermore, Psl has also been shown to impede *in vitro* neutrophil phagocytic uptake and *in vivo* clearance by inhibiting complement fixation [22]. This suggests that Psl is a key determinant associated with persistent infection and could be targeted to improve PA eradication treatment [21].

Other virulence factors that significantly contribute to the evasion of neutrophil antibacterial functions are PA Type Three Secretion System (T3SS) and its effectors. The PA T3S injectisome is a needle-like apparatus and a major determinant of virulence. The injectisome protein PcrV impairs neutrophil-mediated antimicrobial functions by inhibiting the acidification process [23] and is essential for the translocation of effectors (ExoS, ExoT, ExoU, ExoY) into the host cell [24]. Additionally, effectors secreted via the injectisome reduce neutrophil oxidative

and non-oxidative antimicrobials such as reactive oxygen species (ROS) production, phagocytosis, modulate host cell endocytosis, and degranulation [23,25–27].

Anti-PA antibodies are pathogen-specific antibacterial antibodies that neutralize virulence factors and enhance neutrophil-mediated bacterial clearance. Monoclonal antibodies targeting either Psl alone (Psl0096) or in combination with PcrV (MEDI3902) have been developed at AstraZeneca (previously MedImmune) using human antibody phage display libraries [28–30]. Psl0096 and MEDI3902 target the same Psl epitope, while MEDI3902 is a bispecific mAb that targets both Psl and T3SS PcrV. Although Psl0096 enhances *in vitro* opsonophagocytic killing (OPK) against non-CF clinical PA [28], the potentiation of *in vivo* bacterial clearance mediated by Psl0096 was inferior to MEDI3902 in a murine PA infection model [31]. MEDI3902 was previously tested in a randomized phase II clinical trial to prevent nosocomial ventilator-associated pneumonia (VAP) in mechanically ventilated critically ill patients. Although the results from the phase II clinical trial were not favorable since it did not reduce PA incidence in patients with VAP [32], its use in people with CF may still hold potential for the prevention or treatment of PA infections.

Interestingly, studies also reported that MEDI3902 significantly augmented neutrophil-mediated bacterial clearance *in vitro* and *in vivo* when combined with antibiotics such as tobramycin and meropenem [29,33,34]. Recently, Alves et al. showed in a rabbit acute pneumonia model that prophylactic treatment with MEDI3902 24 h before infection resulted in a 4-fold reduction in the lung bacterial burden compared to IgG control-treated rabbits [35]. Moreover, the bacterial burden in the lung, spleen, liver, and kidney was significantly reduced when MEDI3902 was used with meropenem compared to MEDI3902, meropenem, or IgG treatment alone [34]. These studies provided promising insights to use MEDI3902

therapeutically in our unique cohort of CF children to prevent and treat new-onset PA infections and improve PA eradication.

In this study, we sought to examine the ability of the monospecific anti-Psl mAb Psl0096 and the bispecific anti-Psl/PcrV mAb MEDI3902 to potentiate *in vitro* neutrophil-mediated OPK and *in vivo* bacterial clearance of clinical PA isolates from CF new-onset infections. From our early PA eradication study collection [10,21], we characterized representative Persistent and Eradicated PA isolates for their Psl and PcrV expression. We demonstrated that MEDI3902 and Psl0096 were equally effective at potentiating *in vitro* neutrophil OPK of both Persistent and Eradicated isolates. Furthermore, we observed that the potentiation of neutrophil killing against PA isolates by MEDI3902 treatment was correlated to each isolate's strain-specific PcrV expression. Finally, MEDI3902 significantly reduced *in vivo* bacterial burden of two representative Persistent isolates at 24 h p.i.

4.2 Material and Methods

Study design and PA isolates

All PA clinical strains were isolated from the sputum of patients diagnosed with new-onset PA infection and were collected prior to inhaled tobramycin treatment as part of the Early PA Eradication Study and CF Sputum Biobank at the Hospital for Sick Children (REB#1000019444) as previously described [10]. New-onset PA infection was defined as a PA-positive sputum culture with at least 3 PA-negative sputum cultures in the previous 12 months. A sputum culture was collected 1 week posttreatment to determine the outcome of inhaled tobramycin therapy [36,37]. PA isolates were referred to as Persistent or Eradicated based on sputum culture positive or negative result post-tobramycin treatment.

Bacterial culture conditions and phenotypic characterization

All bacterial isolates (unless otherwise specified) were grown planktonically in 5 mL Luria-Bertani (LB) broth overnight at 37 °C with shaking at 200 r.p.m, spun down for 5 min at 12000 r.p.m, washed with PBS, and diluted to the indicated OD_{600nm} as specified in each assay. In addition*, in vitro* bacterial phenotypic assays such as biofilm formation, Congo red binding for bacterial aggregation, and mucoidy status were performed as previously described [11].

Psl surface expression by ELISA

Psl expression was measured by ELISA as done in [28] with minor modifications. Bacterial cultures grown planktonically overnight were spun down, resuspended in PBS (OD_{580nm} 0.2) and used to coat Nunc MaxiSorp^{TM} plates (Thermo Scientific 44-2404-21). Following overnight incubation at 4 °C, supernatants were discarded after centrifugation at 7000 r.p.m for 5 min. Primary anti-Psl antibody Psl0096 (1:12000) (AstraZeneca) was added to the staining buffer (PBS with 1% BSA and 0.1% Tween-20) and incubated for 1.5 h at room temperature (RT), followed by 1:25000 donkey anti-human HRP-conjugated secondary antibody (Jackson Immuno Research Laboratories 709-035-149) for 30 min and detection with TMB SureBlue peroxidase substrate (BD Biosciences 555214) was measured at OD_{450nm}.

Psl surface expression by flow cytometry

Surface-bound Psl was measured by flow cytometry as previously described [28]. Bacteria grown planktonically overnight were washed with PBS once and resuspended in FACS Buffer (0.1% Tween-20 and 1% BSA) to OD_{650nm} 0.4. $2x10^7$ bacterial suspensions in 50 μL were

mixed with 25 μL of 1:50 FITC-conjugated Psl0096 mAb (AstraZeneca) for 1 h at 4 °C. Following incubation, samples were washed, resuspended with FACS buffer, and 10000 events were acquired on the Accuri C6 flow cytometer (BD) and analyzed using Flowjo. Samples stained with FITC-conjugated IgG control were used to gate out the FITC-Psl negative cell population.

PcrV and ExoS expression by immunoblot

Overnight bacterial cultures were grown in LB, washed with PBS once, and resuspended to an OD_{600nm} 0.2 in LB with or without supplementation with 5 mM EGTA, 20 mM $MgCl_2$ (T3SS-inducing conditions). Bacteria were then incubated for ~3-4 h at 37 °C with shaking at 200 r.p.m until the OD_{600nm} reached 1.0. Secreted proteins from culture supernatants were precipitated with trichloroacetic acid (TCA) and quantified with the Pierce bicinchoninic acid (BCA) protein assay kit following the manufacturer's protocol. 3 μg protein was loaded on a 10% SDS PAGE gel and transferred onto a PVDF membrane for PcrV detection. The membranes were blocked for 30 min in Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 10% milk at RT. PcrV was detected by immunoblotting using 1:420 primary anti-PcrV mAb (AstraZeneca V2L2) at 4 ºC overnight, followed by 1:5000 secondary cross-Adsorbed Donkey anti-Human antibody DyLight 680 (Thermo Scientific PISA510130). Similarly, ExoS was detected using 1: 5000 primary anti-exoS polyclonal antibody (Dr. Arne Rietsch, Case Western Reserve University) at 4 ºC and followed by 1:5000 secondary cross-Adsorbed anti-rabbit DyLight 800 (Thermo Scientific SA5-35571). PcrV expression was assessed independently by 2 readers ranking the band intensity from 0 (absent) to 4 (highest) in a

blinded fashion, using the *ΔpcrV* mutant and the T3SS overexpressing PA strain CHA as a negative and positive control, respectively.

In vitro neutrophil assays for phagocytosis and intracellular bacterial killing

In vitro opsonophagocytosis and intracellular bacterial killing were performed as previously described with minimal modification [11]. Briefly, bacterial suspensions (10^7 cells/mL in Hank's balanced salt solution) were opsonized with 10% baby rabbit serum (Cedarlane Labs CL3441) in the presence of 0.1 μM MEDI3902, Psl0096, or R347 IgG control (AstraZeneca). 2.5 x 10^5 differentiated immortalized human HL-60 cells (dHL-60) were incubated with opsonized bacteria at a multiplicity of infection (MOI) of 10 for 30 min at 37 °C. Gentamicin 100 μg/mL was then added to kill extracellular PA for an additional 30 min to measure phagocytosis (T=60 min time point) or an additional 1.5 h (T=120 min time point) to measure intracellular bacterial killing. Infected dHL-60 cells were then washed, lysed with 0.1% Triton X-100, serially diluted, and plated on LB agar to quantify viable intracellular bacteria. The phagocytosis index was calculated as the number of PA internalized at 60 min, namely the number of intracellular PA at [T = 60 min] per $5x10^5$ total bacteria at T= 0 h (initial input inoculum). The intracellular bacterial killing was calculated as the relative reduction in intracellular bacterial burden, namely the number of intracellular PA at [T = 60 min] minus the number of intracellular PA at [T = 120 min] per $5x10^5$ input bacteria. The fold increase in OPK relative to its IgG treatment was calculated as mAb-treated OPK divided by IgG-treated OPK.

In vivo murine PA lung infection model

Specific pathogen-free C57BL/6 male mice (8-10 weeks) from Charles River Laboratories were prophylactically treated with 15 mg/kg MEDI3902, or 11.4 mg/kg IgG (R347) isotype control via the intraperitoneal (i.p) route 24 h prior to infection, as previously done [28]. Mice were infected with $1x10^7$ CFU/mouse via a non-surgical endotracheal route, sacrificed at 24 h and 96 h p.i., and the pulmonary vessels were perfused with ice-cold PBS to clear intravascular blood. The lungs were then removed, placed in ice-cold PBS, minced, and homogenized in a bullet blender (Next Advance) containing 0.2 mm stainless steel beads (Beads lysis kit, Next Advance). Whole lung homogenates were then serially diluted and plated on Pseudomonas isolation agar (Sigma-Aldrich 17208-500G) to quantify viable lung bacterial burden by CFU counts. All animal experiments were carried out in accordance with the Canadian Council on Animals Care and with approval from the Animal Care Committee of the Research Institute of the McGill University Health Centre (AUP #7586).

Confocal imaging of PA biofilms

Psl binding of PA biofilms was previously done and analyzed [21]. In brief, representative PA clinical isolates were grown in 8-chambered microscopic slides containing LB for 2 days at 37 °C. After removing suspension cells, a fluorescently-labeled anti-Psl antibody was added for 90 min. After 2x washing with LB, fresh LB was added back to the wells prior to imaging with confocal laser scanning microscopy.

Cytotoxicity assay

Lactate dehydrogenase (LDH) released by damaged mammalian cells into the supernatant following PA-airway epithelial cell infections was measured according to the manufacturer's instructions as previously described [38]. LDH concentrations were measured at $OD492_{nm,}$ and cytotoxicity was expressed at the ratio of LDH release (supernatant) to total LDH (supernatant and cell lysate).

Statistical analysis

All results are shown as median ± IQR unless otherwise indicated. Statistical analyses were done using Prism 9 software (Graphpad). Comparisons were performed using ANOVA Dunn's test, Mann Whitney U test, or χ^2 test as indicated. A P value of ≤ 0.05 was considered statistically significant. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$.

4.3 Results

In this study, we used a subset of well-characterized representative Persistent (n=7) and Eradicated (n=8) clinical PA isolates, which were previously assayed for several *in vitro* bacterial phenotypes [39]. They were selected as representative Persistent and Eradicated isolates based on their similarity in mucoid status, protease production, swimming and twitching motility, biofilm Crystal Violet assay, and tobramycin minimum inhibitory concentration [39]. We also quantified EPS (Psl and Pel)-mediated bacterial aggregation with the Congo Red binding assay, PcrV expression by immunoblotting. We estimated extracellular Psl expression using several complementary methods, namely ELISA, flow cytometry, and confocal microscopy. The PA isolate phenotypic characteristics are presented in Tables 4.1 and S4.1. All isolates were PcrV

positive (Figure 4.1, Table 4.1), without significant differences between the Persistent and Eradicated groups (Figure 4.1b). We also did not detect any differences between the two groups in Congo red binding or Psl expression by ELISA and flow cytometry (Table 4.1).

To test the ability of anti-Psl and anti-Psl/PcrV mAbs to potentiate neutrophil-mediated phagocytosis and intracellular bacterial killing of these PA clinical isolates, we used *in vitro* assays with dHL-60 cells in the presence of the bispecific anti-Psl/PcrV MEDI3902, the monospecific anti-Psl Psl0096, or IgG isotype control. In an initial analysis, we found that MEDI3902 significantly enhanced neutrophil phagocytosis and intracellular bacterial killing of Persistent isolates but not Eradicated isolates (Figure 4.2). There was a trend towards increased neutrophil OPK with Psl0096, but this did not reach statistical significance in any PA groups. To account for strain-to-strain differences in baseline OPK obtained with the IgG control when assessing the effect of MEDI3902 and Psl0096, we then calculated the fold increase in neutrophil OPK of mAb-treated bacteria relative to IgG control-treated ones. MEDI3902 and Psl0096 had equivalent neutrophil potentiating effects of Persistent and Eradicated isolates. Using a cut-off of a $\geq$ 2-fold increase in OPK to be biologically meaningful, Psl0096 and MEDI3902 elicited a $\geq$ 2-fold increase in OPK in 86% and 100% of Persistent isolates, respectively, while only 65% of Eradicated isolates responded to Psl0096 or MEDI3902 treatment (Figures 4.3a-d). However, results were similar when all PA isolates were pooled (Figures 4.3e, f).

Since there was significant strain-to-strain variability in the *in vitro* effect of MEDI3902, we asked whether the magnitude of the effect was correlated with PA isolates' PcrV and/or Psl expression levels. Interestingly, we found a significant dose-dependent effect where potentiation of neutrophil intracellular bacterial killing increased with PA isolates' increasing PcrV expression ($r = 0.7$ $P = 0.009$, Figure 4.4b). In contrast, no significant correlations were observed

between Psl or PcrV expression and the potentiation of neutrophil phagocytosis by MEDI3902 (Figures 4.4a, c-d) or Psl0096 treatment (Supplementary figure S4.1). This suggests that each isolate's PcrV expression may determine the potentiation of intracellular bacterial killing by MEDI3902.

Our group recently demonstrated that isolates 565P and 505P failed *in vivo* bacterial clearance and persisted at 96 h p.i. in a murine pulmonary infection model. We therefore sought to determine whether MEDI3902 enhanced the clearance of these infections *in vivo*. Both 565P and 505P isolates had phenotypic characteristics representative of the Persistent group (Table 4.1), including Psl and PcrV expression (Supplementary table 4.1). Using the same murine pulmonary infection model as above, we treated mice with MEDI3902 or IgG control 24 h prior to infection. As shown in Figure 4.5, MEDI3902-treat mice had a significantly lower lung bacterial burden at 24 h p.i. in 565P ($8.3x10^3$ vs. $2.5x10^5$ CFU/ lung) and 505P ($1.8x10^4$ vs. $4x10^5$ CFU/ lung) infections compared to their respective IgG control-treated group. At 96 h p.i., a similar bacterial burden (viable CFU below the limit of detection (LOD)) was detected in 565P-infected mice in MEDI3902 compared to the IgG control treatment group (Figure 4.5a). However, a trend towards a lower bacterial burden was recovered in 505-infected mice by MEDI3902 compared to IgG control treatment (LOD vs. $1.5x10^4$ CFU/ lung) at 96 h p.i., but not statistically significant (Figure 4.5b). By estimating the proportion of mice with complete bacterial clearance (CFU below the LOD), MEDI3902 increased the rate of clearance at 24 h (47 % vs. 0%, P = **, Supplementary figure S4.2a) but not 96 h p.i. (80 vs. 67%, Supplementary figure S4.2c) in 565P-infected mice compared to IgG control. For 505P infected mice, no complete clearance was observed at 24 h p.i. in any treatment group (Supplementary figure S4.2b), but MEDI3902 increased the rate of clearance at 96 h p.i (67% vs. 8%, P = **,

Supplementary figure S4.2d). Our findings thus far suggest that MEDI3902 significantly reduces the bacterial burden in 565P and 505P-infected mice compared to the IgG isotype control. However, the magnitude and timing of its effect varied according to the PA isolate. MEDI3902 ameliorated bacterial clearance at 24 h p.i. for 565P infection but only by 96 h p.i. for 505P infection. This observation may be linked to a higher PcrV expression in 565P than in 505P, which is expected to be more responsive to MEDI3902 treatment than 505P.

In these experiments, we also noted that the IgG control treatment seemed superior to our historical no treatment (untreated infection) group at 96 h p.i. This led us to compare the bacterial burden and proportion of mice with complete bacterial clearance in those groups (Supplementary figure S4.3). We observed that 0% of the historically untreated 565P-infected mice had complete clearance at 96 h p.i. compared to 67% of the IgG-treated mice (P = ***, Supplementary Figure S4.4a) with a median bacterial burden of $1.4x10^4$ CFU/ lung compared to viable burden that was below the limit of detection (Supplementary figure S4.3). Similarly, we found a comparable trend for 505P infection, with 0% and 9% of mice showing complete bacterial clearance in the historically untreated compared to IgG-control treated mice (Supplementary figure S4.4b), resulting in a median bacterial burden of $1.1x10^4$ vs. $1.5x10^4$ CFU/ lung (Supplementary figure S4.3). These findings indirectly implicate that non-specific IgG isotype controls alone may confer some protection in mice compared to no treatment. However, since the historical untreated and IgG-treated mice were not infected at the same time, biological variability between independent infections may also contribute to differences seen in total bacterial burden between the two groups.

A previous study showed MEDI3902 improved *in vitro* and *in vivo* clearance and reduced cytotoxicity of a highly cytotoxic keratitis clinical isolate 6077 compared to its effector-deficient

mutant [29]. The authors suggested that MEDI3902 was efficacious against isolates that were more cytotoxic. Since 565P is more responsive to MEDI3902 *in vivo* at 24 h p.i., we hypothesized that 565P might secrete more PcrV and ExoS, and was more cytotoxic than 505P. As shown in Figure 4.1 and Supplementary figure S4.5a, the expression of PcrV and ExoS was higher in 565P than in 505P. Additionally, cytotoxicity was also measured by LDH release at 24 h p.i. using a model of airway epithelial cells infected with 565P or 505P. Consistent with PcrV and ExoS secretion levels, 565P induced greater cytotoxicity than 505P (Supplementary figure S4.5b). Our findings thus indirectly support the idea that strain-specific PcrV and ExoS secretion may contribute to the relative magnitude of the effect of MEDI3902 *in vitro* and *in vivo*.

4.4 Discussion

While Psl0096 and MEDI3902 have previously been shown to enhance neutrophil OPK against non-CF PA isolates [28–31], this is the first study to examine the effect of mAb treatment at potentiating neutrophil-mediated bacterial clearance *in vitro* and *in vivo* using CF clinical PA isolates. Our results showed that Psl0096 and MEDI3902 were equally effective in potentiating *in vitro* OPK of Persistent and Eradicated isolates. Moreover, we demonstrated that prophylactic treatment of MEDI3902 enhanced *in vivo* bacterial clearance in a murine lung infection model with Persistent isolates 565P and 505P, albeit with different degrees of efficacy depending on the PA isolate. The efficacy of MEDI3902 may vary depending on the infection strain, with a correlation to strain-specific PcrV expression. Our results also suggest that MEDI3902 regimens could be optimized to achieve better bacterial clearance and further developed as a therapeutic strategy to improve PA eradication in new-onset CF infection.

Thanabalasuriar et al. previously showed that the anti-Psl component of the bispecific MEDI3902 augmented the recognition of PA to increase phagocytic uptake, while its anti-PcrV component potentiated intracellular bacterial killing, presumably through its anticytotoxic activity which enabled acidification of endosomal compartments [31]. These findings are congruent with our observed correlation between isolate-specific PcrV levels and the degree to which MEDI3902 potentiates intracellular bacterial killing. Together, these findings support the notion that MEDI3902's effect on neutrophil-mediated intracellular bacterial killing is PcrV-dependent. We recognize that PcrV levels measured in our experimental system were artificially induced with EGTA to generate a low calcium medium and may not reflect PcrV secretion in our *in vitro* or *in vivo* infection model. We also note that, in addition to PcrV, effectors such as ExoS may also modulate the effect of MEDI3902. The secretion of ExoS requires the T3S injectisome protein PcrV, and injection of ExoS into neutrophils can inhibit antibacterial mechanisms such as ROS [40], OPK [26], and degranulation [27].

Zegans et al. previously showed PA keratitis clinical isolates expressed Psl (as measured by ELISA with Psl0096) and were susceptible to anti-Psl Psl0096 mAb-mediated OPK [41], but did not distinguish whether this mAb effect was specific to phagocytosis or intracellular bacterial killing, or was correlated with strain-specific Psl levels. Morris et al. previously characterized the Psl levels in the Persistent and Eradicated isolates from our study and found increased Psl levels in the Persistent isolates compared to the Eradicated ones when Psl was measured in biofilms by confocal microscopy quantification of anti-Psl mAb binding. However, they observed no differences when Psl was measured by dot-blot or ELISA using the same anti-Psl mAb [21], suggesting that strain-specific Psl expression may vary between biofilm and planktonic growth. Since bacteria used for neutrophil assays were grown planktonically, we thus assessed Psl levels

in planktonic bacteria by ELISA and flow cytometry. Despite this, we found no significant correlation between the magnitude of MEDI3902 potentiation of phagocytosis and the PA strain-specific Psl levels by whichever method was used. These results may indicate that there is truly no association, or that we were unable to identify an existing association due to the small sample size or experimental methods. For example, processing steps such as washing or sonication might disrupt surface Psl and affect its detection by Psl0096. Therefore, it is plausible that the amount of surface Psl measured by ELISA or flow cytometry does not reflect Psl expression during neutrophil infection assays. Moreover, it is important to note that Pel production was not measured in these isolates, and certain isolates may produce more Pel than Psl. Since Pel is also linked to the resistance of neutrophil antibacterial functions [18], MEDI39302 and Psl0096 may not be effective against Pel-dominant isolates.

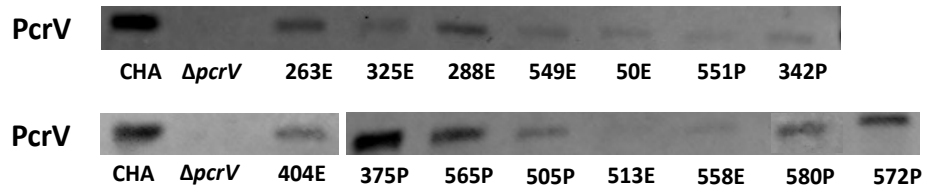
Next, the *in vivo* prophylactic model could be optimized to test whether 565P and 505P infections can be completely eradicated upon MEDI3902 treatment. We demonstrated that MEDI3902, used prophylactically, reduced the bacterial clearance *in vivo* at 24 h p.i., but its effect by 96 h p.i. was more modest than IgG control. Since IgG can also act as an opsonin to facilitate adequate neutrophil-mediated bacterial clearance [42], it is possible that IgG could have underestimated the effect on the MEDI3902 treatment group (Supplementary figure S4.3). While promising, MEDI3902 treatment could be further optimized with different regimens, such as administering a booster dose of MEDI3902 after infection or testing MEDI3902 in combination with anti-PA antibiotics to achieve greater bacterial clearance. Moreover, some of the limitations of a prophylactic treatment include repeated treatments are needed in individuals to maintain its effect, and it is impractical to administer MEDI3902 to CF patients regardless of whether

patients are PA positive or negative. Thus, developing MEDI3902 as a treatment against established PA infections is more cost-efficient and preferable.

Although new highly effective CFTR modulator therapies have reduced the incidence of new-onset PA infections in CF patients [43], they are not available to all CF patients. Novel approaches to improve PA eradication remain needed, and MEDI3002 may be an interesting approach to be further investigated.

4.5 Figures

1a)



1b)

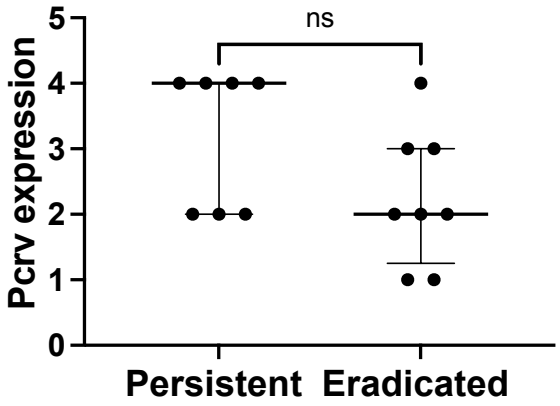


Figure 4.1 Secreted PcrV expression in clinical PA isolates. a) Immunoblots of PcrV, and b) PcrV expression of Persistent compared to Eradicated isolates. PcrV assessment was done by ranking the band intensity in a blinded fashion, as mentioned in the methods. Results are shown as median ± IQR; N = 2 replicates from 2 independent experiments (representative blot shown). Statistical comparisons were performed using Mann-Whitney test. Secreted PcrV was measured by immunoblot in culture supernatants from bacteria grown in T3SS-inducing media. 3 μg protein per isolate was loaded into a 10% SDS PAGE gel. PcrV overexpressing clinical PA strain CHA and PcrV-deficient mutant *ΔpcrV* were used as a positive and negative control, respectively.

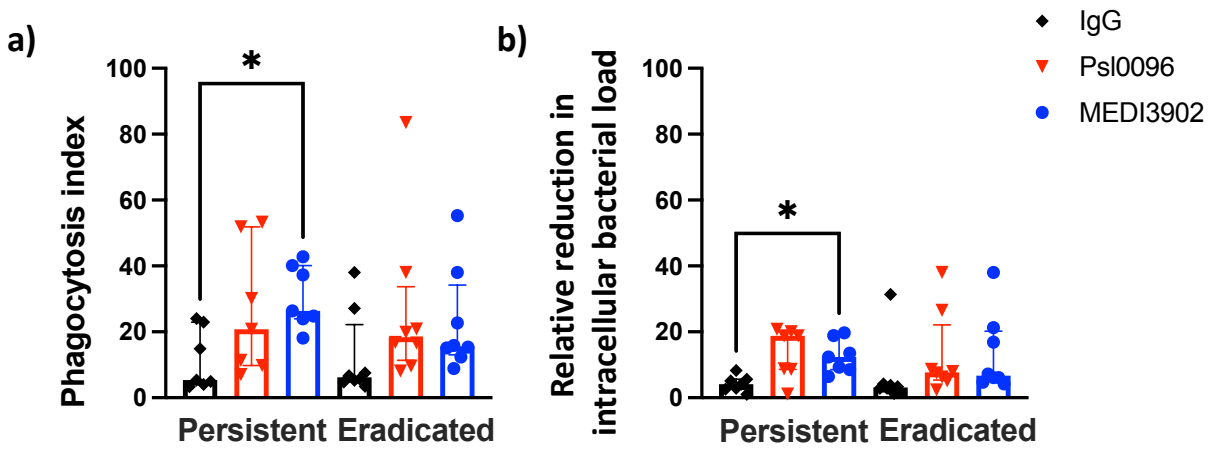


Figure 4.2 MEDI3902 enhances *in vitro* neutrophil OPK of CF clinical PA isolates.

Persistent and Eradicated isolates were tested for neutrophil a) Phagocytosis and b) Intracellular bacterial killing upon treatment with Psl0096, MEDI3902, or IgG isotype control. Results are shown as median ± IQR; N ≥ 6 replicates from ≥ 2 independent experiments; statistical comparisons were performed using Dunn's test.

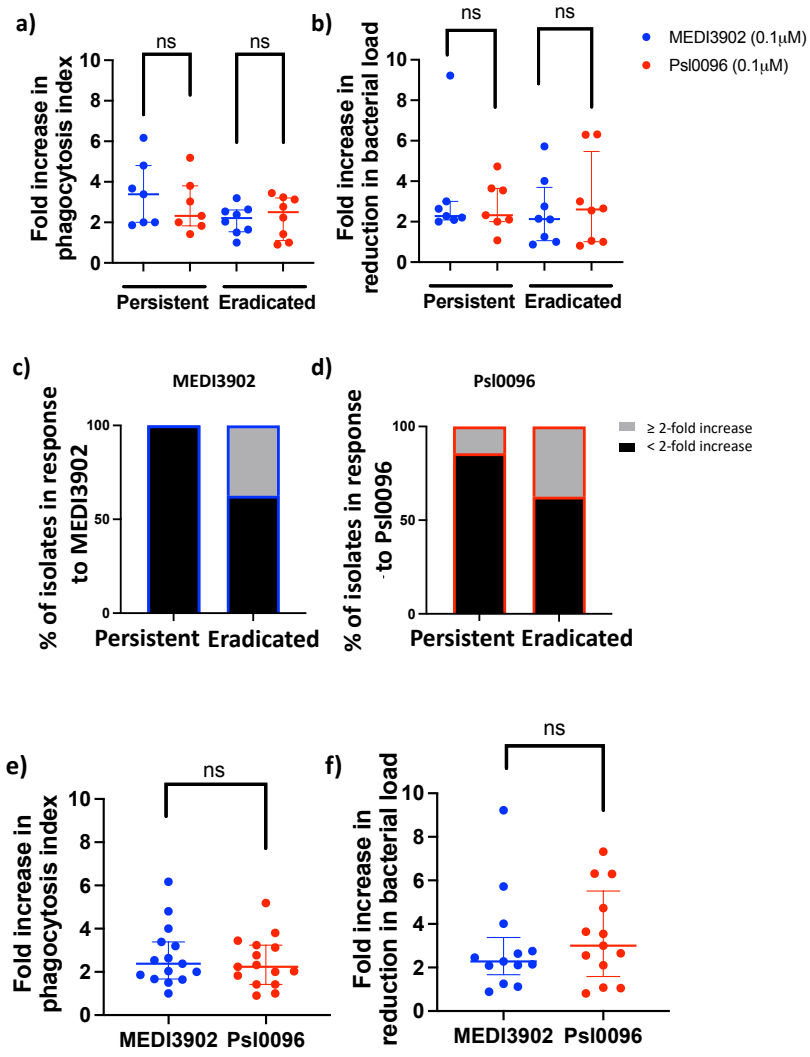


Figure 4.3 MEDI3902 and Psl0096 are equally effective in increasing *in vitro* neutrophil OPK of Persistent and Eradicated isolates. a) and e) Neutrophil phagocytosis; b) and f) Intracellular bacterial killing of clinical PA isolates by either Psl0096 or MEDI3902 treatment. Results are shown as median ± IQR; statistical comparisons were performed using Mann-Whitney test. The potentiation of neutrophil OPK in response to Persistent and Eradicated isolates by c) MEDI3902 or d) Psl0096 relative to IgG control treatment. The fold increase in OPK was calculated as mAb-treated OPK divided by IgG-treated OPK, with ≥ 2 fold-increase

considered as effective. Statistical comparisons were performed using χ^2 test. Experiments were performed with ≥ 6 replicates in ≥ 2 independent experiments.

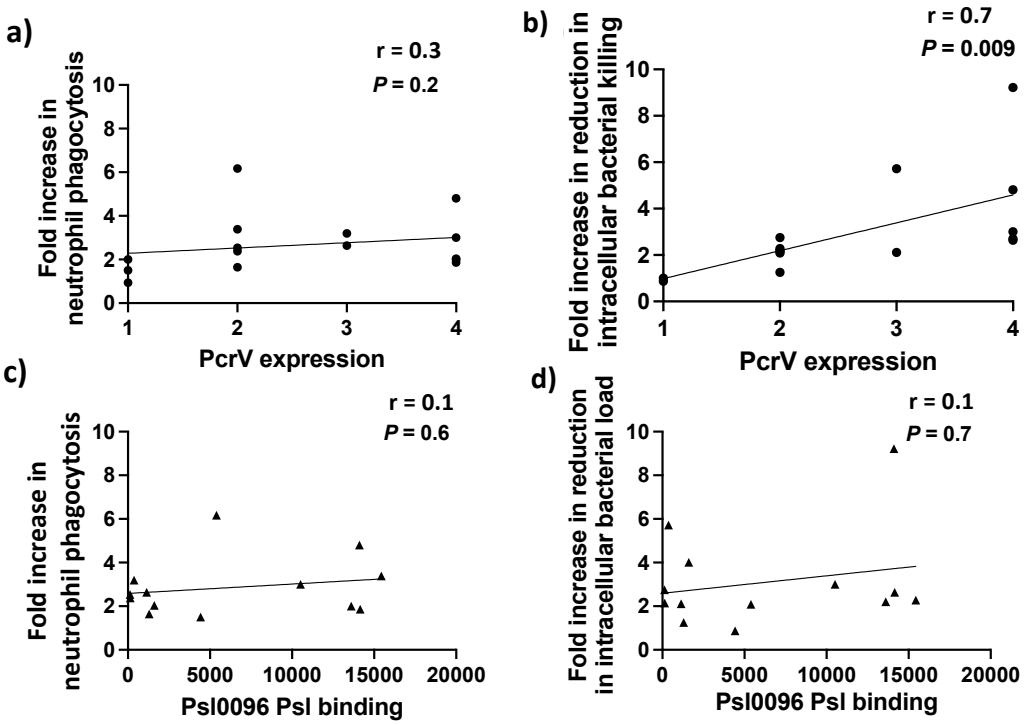


Figure 4.4 Correlations between neutrophil OPK potentiation by MEDI3902 and Psl or PcrV expression across clinical PA isolates. Correlation of a, c) Phagocytosis; b, d) Intracellular bacterial killing and PcrV expression or Psl binding (by confocal) of clinical isolates by the treatment of MEDI3902. Each dot represents a clinical isolate (n = 15). Correlations were calculated by the Spearman correlation coefficient (r). A *P*-value < 0.05 was considered significant.

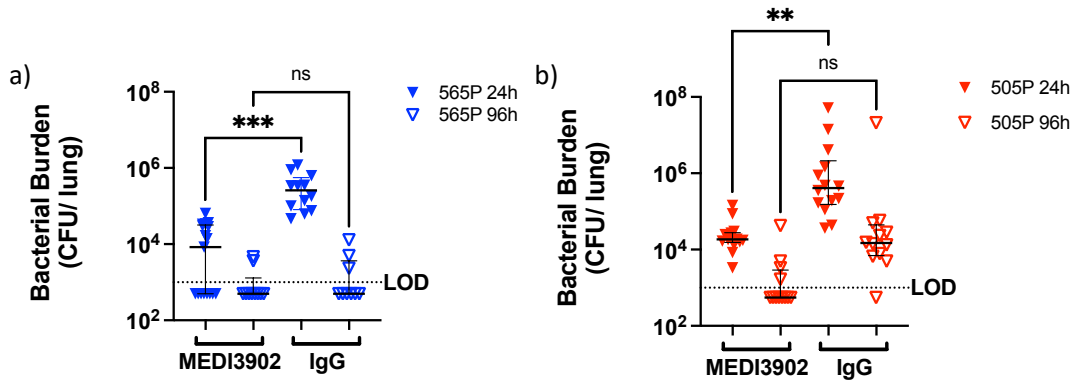


Figure 4.5 The effect of MEDI3902 on bacterial burden in mice infected with Persistent isolates. Bacterial burden was recovered at 24 h p.i. and 96 h p.i. from whole lung homogenates in a) 565P or b) 505P-infected mice prophylactically treated with MEDI3902 or IgG control. Results are shown as median ± IQR; statistical comparisons were performed using Dunn's test. N ≥ 9 mice per group from ≥ 2 independent experiments. LOD (Limit of detection).

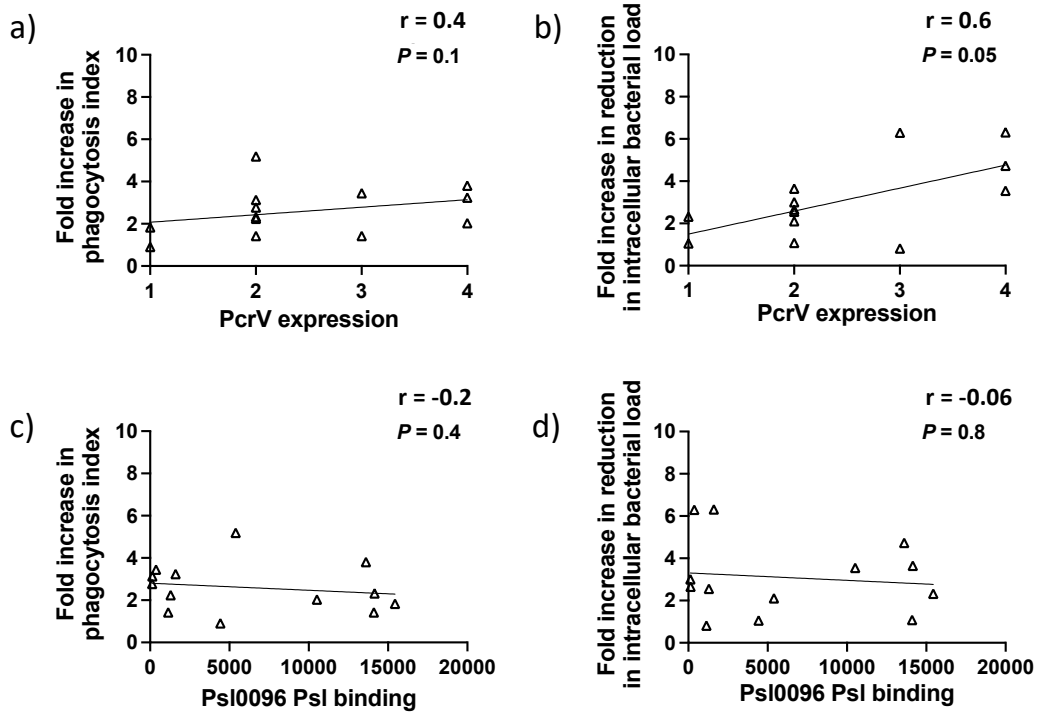
4.6 Tables

Table 4.1 *In vitro* bacterial phenotypic characteristics in clinical Persistent and Eradicated isolates.

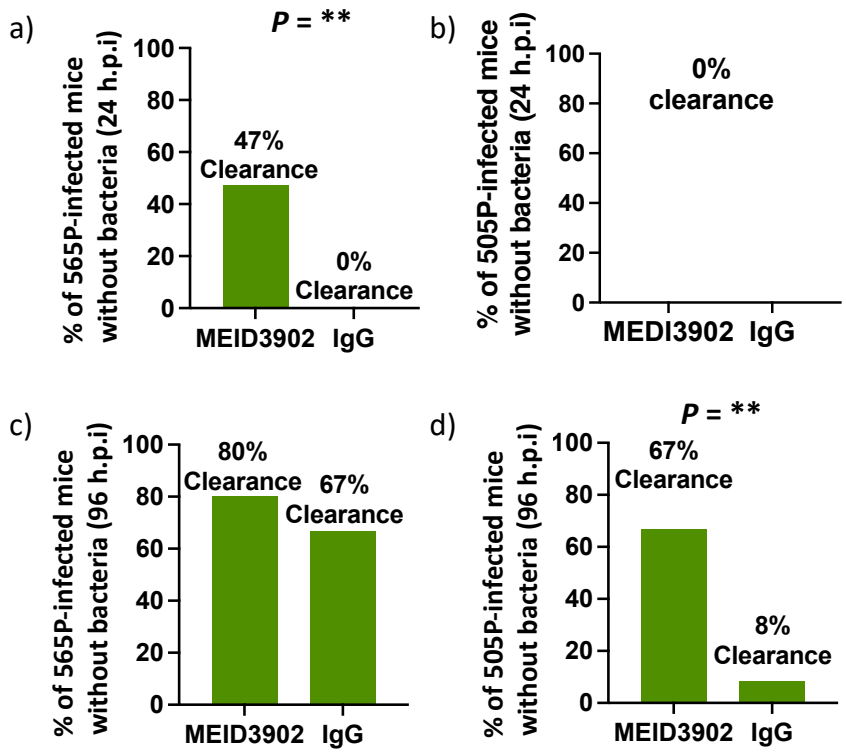
PA phenotypes	Persistent N = 7	Eradicated N = 7	*P*-Value
Biofilm formation (OD_{600nm}), median (IQR)	0.21 (0.12-0.24)	0.20 (0.09-0.29)	0.97
Congo Red binding (OD_{495nm}), median (IQR)	-0.30 (-0.32- -0.24)	-0.22 (-0.3- -0.09)	0.07
Psl binding by ELISA (OD_{450nm}), median (IQR)	1.81 (1.71-2.09)	2.11 (1.91-2.31)	0.15
Psl binding by confocal (μm^3/100000 μm^3 biofilm), median (IQR)	13857 (10512-14151)	1141 (200.5-1530)	0.0003
Psl binding by flow cytometer (MFI), median (IQR)	1631176 (1139842-2046835)	1626362 (1358672-1943694)	0.98
PcrV expression (by ranking) median (IQR)	4 (2-4)	2 (1.25-3)	0.16

*Table adapted from results (Biofilm formation, Congo red binding, Psl binding by confocal) published in [11] and [21]. Statistical comparisons were performed using Mann-Whitney or χ^2 test. Abbreviations: IQR, interquartile range; OD, optical density.

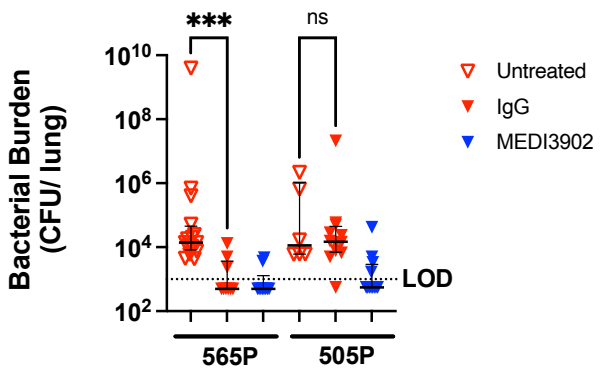
4.7 Supplementary figures



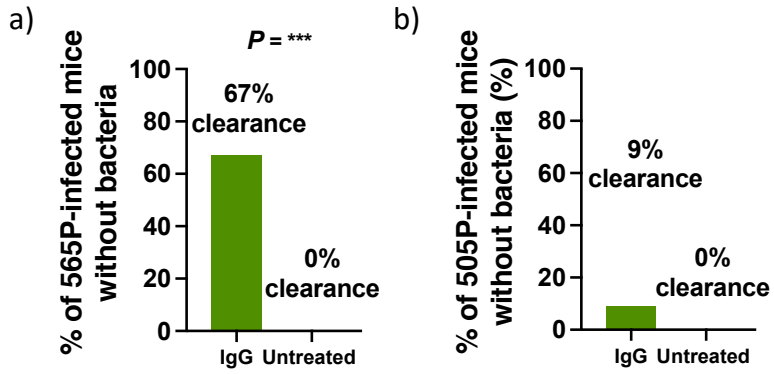
Supplementary figure S4.1 Correlations between neutrophil OPK potentiation by Psl0096 and Psl or PcrV expression across clinical PA isolates. Correlation of a, c) Phagocytosis; b, d) Intracellular bacterial killing and PcrV expression or Psl binding of clinical PA isolates by the treatment of Psl0096. Each dot represents a clinical isolate (n = 13). Correlations were calculated by the Spearman correlation coefficient (r). A *P*-value < 0.05 was considered significant.



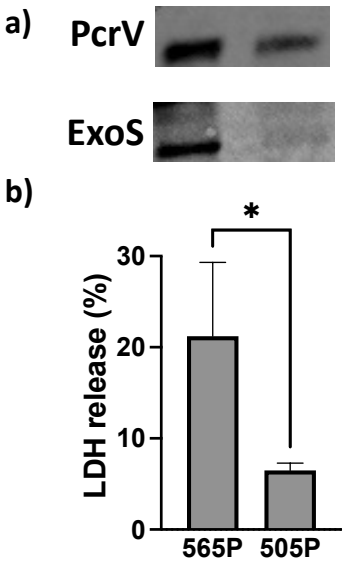
Supplementary figure S4.2 The effect of MEDI3902 in mice infected with Persistent isolates at 24 and 96 h p.i. The proportion of 565P-infected mice by MEDI3902 vs. IgG treatment at a) 24 and c) 96 h p.i. The proportion of 505P-infected mice by MEDI3902 vs. IgG treatment at b) 24 and d) 96 h p.i. Statistical comparisons were performed using χ^2 test. N ≥ 9 mice per group from ≥ 2 independent experiments.



Supplementary figure S4.3 *In vivo* bacterial burden recovered from 565P or 505P-infected mice with IgG, MEDI3902 treatment or untreated at 96 h p.i. Results are shown as median ± IQR; N ≥ 9 mice per group from ≥ 2 independent experiments; statistical comparisons were performed using Mann-Whitney test. LOD (Limit of detection).



Supplementary figure S4.4 The effect of IgG control in mice infected with Persistent isolates at 96 h p.i. A greater proportion of mice in IgG treatment group cleared a) 565P infection, b) 505P infection at 96 h p.i. compared to no treatment (untreated). Statistical comparisons were performed using χ^2 test. N ≥ 9 mice per group from ≥ 2 independent experiments.



Supplementary figure S4.5 PcrV and ExoS secretion and cytotoxicity in 565P and 505P. a) Western immunoblots of PcrV and ExoS in 565P and 505P. b) Cytotoxicity of AECs induced by 565P or 505P as measured by LDH release (data adapted from Malet et al. [38]). Results are shown as median ± IQR; N = 9 replicates from 3 independent experiments; statistical comparisons were performed using Mann-Whitney test.

4.8 Supplementary table

Supplementary table S4.1 *In vitro* bacterial phenotypic characteristics of each strain from a representative subset of Persistent and Eradicated isolates.

Strains	Eradication status	Biofilm formation (OD_{600nm})	Congo red binding (OD_{495nm})	Psl binding by ELISA (OD_{450nm})	Psl binding by confocal (μm^3/100000 μm^3 biofilm),	Psl binding by flow cytometer (MFI)	PcrV expression
50E	Eradicated	0.07	-0.25	2.14	132	838902	++
263E	Eradicated	0.15	-0.18	1.87	1608	1330782	++++
288E	Eradicated	1.33	-0.07	2.50	143	2021134	++
325E	Eradicated	0.28	-0.25	2.35	1296	1711373	++
404E	Eradicated	0.29	-0.31	2.17	1141	1442342	+++
549E	Eradicated	0.02	-0.31	2.01	373	2272601	+++
513E	Eradicated	0.18	-0.16	0.90	-	-	+
558E	Eradicated	0.21	-0.07	2.08	4432	1598024	+
342P	Persistent	0.21	-0.30	1.70	15443	2046835	++
375P	Persistent	0.21	-0.24	1.81	14151	1139841	++++
505P	Persistent	0.24	-0.32	1.93	13609	1048245	++
551P	Persistent	0.12	-0.29	1.71	5393	1631175	++
565P	Persistent	0.16	-0.38	1.81	14105	1631175	++++
580P	Persistent	0.12	-0.32	2.08	10512	1631175	++++
572P	Persistent	0.49	-0.23	2.34	-	2272601	++++

Data adapted from Table 4.1. Representative Persistent and Eradicated isolates were selected based on their similarity in mucoid status, protease production, swimming and twitching motility, biofilm Crystal Violet assay, and tobramycin minimum inhibitory concentration as previously described [39].

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Chapter 5: Discussion

5.1 Summary of major findings

The failure of PA eradication treatment is likely multifactorial, and it has been a challenge to identify predictors of failed inhaled tobramycin therapy. Few studies have examined the contribution of PA-host interactions [1–5], and none have previously identified a significant risk factor to predict patients' clinical outcomes following PA eradication treatment. Our study presented in chapter 2 is the first to report a significant association between clinical outcomes and PA-neutrophil responses. Using PA isolates collected from CF children with new-onset infections, we observed that PA isolates recovered from children who failed eradication (Persistent isolates) were more resistant to *in vitro* neutrophil phagocytosis and intracellular bacterial killing than PA isolates from children who successfully cleared their infection (Eradicated isolates). Moreover, loss of pilus-mediated twitching motility and overproduction of EPS were important strain-specific bacterial phenotypes linked to resistance to neutrophil phagocytosis, and the latter was a significant independent predictor of failed eradication treatment.

Strain-specific phenotypes such as wrinkly colony morphology and loss of pilus-mediated twitching motility are typical of chronic infection. Specifically, how Persistent isolates contribute to the evasion of neutrophil antibacterial functions is still unclear. In chapter 3, we demonstrated that Persistent isolates displayed impaired complement C3 deposition on the bacterial surface and reduced bacterial adhesion to neutrophils compared to Eradicated isolates. These mechanisms likely contributed to the Persistent isolates' resistance to *in vitro* neutrophil OPK, and ultimately impaired *in vivo* bacterial clearance. Furthermore, total leukocytes, neutrophils, and systemic inflammatory cytokine and chemokine responses measured in mice did

not fully explain why Persistent isolates failed *in vivo* bacterial clearance compared to Eradicated isolates. Finally, we showed that complementation of PilA in a Persistent isolate that lacked twitching motility restored the isolate's susceptibility to neutrophil OPK.

The engagement and interactions between PA and neutrophils are important to bacterial clearance, and our findings support the notion that such interactions are impaired in response to Persistent isolates. To improve *in vivo* PA clearance, we used monoclonal antibodies, which enhance neutrophil-mediated OPK by targeting the bacterial determinants Psl EPS and PA T3SS injectisome protein PcrV. We tested mAb developed by AstraZeneca (previously MedImmune) [6], namely the monospecific anti-Psl mAb Psl0096 and the bispecific anti-Psl-PcrV mAb MEDI3902. We showed that both Psl0096 and MEDI3902 demonstrated equivalent efficacy at potentiating *in vitro* OPK against Persistent and Eradicated isolates. Moreover, the potentiation effect of the intracellular bacterial killing of clinical PA isolates by MEDI3902 was correlated with strain-specific T3SS PcrV expression. Lastly, MEDI3902 significantly reduced the lung bacterial burden in mice infected with Persistent isolates at 24 h p.i. Altogether, these results suggest that PA-neutrophil antibacterial functions are important mechanisms that can be targeted therapeutically. Strategies aiming to potentiate PA-neutrophil interactions could improve the eradication of new-onset PA infections.

5.2 Modeling PA-neutrophil interactions *in vitro* and *in vivo*

5.2.1 In vitro assay using immortalized human HL-60 cells

We chose wild-type (WT) HL-60 cells as our experimental system for a large number of morphologically distinct PA clinical isolates because it avoids neutrophil donor variability, and thus generates robust and reproducible results. Although this model does not address CTFR-

associated host defects, it allows us to examine the role of bacterial determinants in PA-neutrophil interactions.

HL-60 cells are immortalized human cells which can be differentiated into neutrophil-like cells (dHL-60) and were used to test for neutrophil OPK in response to 71 clinical PA isolates. The differentiated HL-60 cells are equipped with certain antibacterial killing mechanisms comparable to primary human neutrophils. For example, both primary human neutrophils and dHL-60 cells are capable of phagocytosing IgG-opsonized bacteria, releasing primary granules (a potent antibacterial function against microbes), and generating ROS depending on Nox2 (one of the superoxide-generating enzymes) activation [7]. However, compared to primary human neutrophils, dHL-60 cells lack specific, gelatinase and secretory granules; and ROS-mediated antibacterial killing mechanisms are only suboptimal in dHL-60 cells [7]. It has been observed that PA may be more resistant to ROS-mediated killing mechanisms than non-ROS-mediated antibacterial functions. This is supported by the fact that neutrophils from chronic granulomatous disease (CGD) patients lack microbicidal oxygen radicals. Yet, patients with CGD do not suffer from PA infections [8], but pathogens (e.g., *Staphylococcus aureus*, *Burkholderia cepacia*, and *Salmonella spp*) that are particularly susceptible to ROS-mediated antibacterial functions [9], suggesting that non-ROS-mediated antibacterial functions are critical for optimal PA eradication [10]. Given the suboptimal ROS-mediated antibacterial killing mechanisms in dHL-60 cells [7], primary murine neutrophils were also used in our study to validate ROS-dependent and independent antibacterial functions in response to representative Persistent and Eradicated PA isolates.

We recognize that using WT HL-60s may not be representative of CF neutrophils. However, the role of CFTR and the CF lung milieu on neutrophil functions is complex and

controversial. Studies attempting to address whether bacterial clearance by CF neutrophils from the early stage of infection is effective compared to non-CF neutrophils have resulted in divergent conclusions [11]. CFTR was previously detected in the phagolysosome [12], and defective chlorination and degranulation were observed in CF blood neutrophils following infection with PA compared to the healthy blood neutrophils [12,13]. It is thus plausible that neutrophil-mediated responses elicited by clinical PA isolates in non-CF neutrophils may differ from CF neutrophils. Interestingly, CF human neutrophils ΔF508-CF HL-60 cells have recently been developed and characterized. Compared to WT dHL-60 cells, the CFTR-deficient ΔF508-dHL-60 cells have diminished antimicrobial activities, ROS production, HOCl-mediated antimicrobial, and OPK [14]. We could therefore compare OPK in response to representative Persistent and Eradicated isolates using CF and WT dHL-60 cells.

Conversely, a study by Leuer and colleagues showed that CF blood neutrophils demonstrated similar phagocytic function as non-CF blood neutrophils [15]. Mckeon et al. also reported that ROS-mediated antibacterial functions did not differ in CF vs. non-CF neutrophils after infection with PA [16]. Interestingly, multicenter clinical studies of early PA eradication have reported that up to 40% (N = 82/204) of young CF patients with new-onset PA infections often result in spontaneous PA eradication without antibiotic treatments following the initial sputum sampling and culture [17,18], implicating that young CF children may clear PA infections in many cases during early infection.

At the current stage, whether our model of *in vitro* phagocytosis assay using dHL-60 cells could serve as a clinical diagnostic/predictive test remains unknown. Although we recognize that cell culture-based assays to phenotype PA isolates would be difficult to implement within clinical care, they are valuable and can be used in research settings. Regardless of the potential of

this assay as a diagnostic/predictive tool, it allowed us to identify impaired neutrophil functions in response to PA strain-specific phenotypes as candidate mechanisms that contribute to failed tobramycin eradication treatment.

To validate our *in vitro* findings in an independent patient cohort from the United States, we tested a collection of clinical isolates collected from CF children (1-12 years old) undergoing inhaled tobramycin treatment from the Early Pseudomonas Infection Control (EPIC) clinical trial [17] using our *in vitro* neutrophil phagocytosis assay. We saw a trend towards a lower *in vitro* phagocytosis elicited by Persistent isolates (n = 7) compared to Eradicated isolates (n =7), but no statistical significance was observed with this small subset of PA isolates (Appendix Figure A1). We note that PA clinical isolates from the EPIC study were recovered from oropharyngeal swabs in contrast to isolates from the SickKids PA eradication study were from patients' sputum cultures and this may reflect the isolates infecting the lower respiratory tract [19].

5.2.2 Intravital imaging to examine the interplay between PA and neutrophils in vivo

One of our key observations from chapter 2 showed that impaired *in vitro* neutrophil phagocytosis was significantly associated with persistent infection. To demonstrate this *in vivo*, we have previously attempted to measure *in vivo* phagocytosis of PA using confocal microscopy by staining extracellular and intracellular PA with an anti-PA antibody followed by differential staining with conjugated secondary antibodies [20]. Unfortunately, we encountered several limitations with this technique. First, we noticed an increased background noise in both uninfected and infected samples, and it was likely caused by non-specific binding of conjugated secondary antibodies and autofluorescence. Second, our murine PA infection model allowed for measurement of bacterial burden at specific endpoints but could not distinguish bacterial

phagocytosis vs. killing. Third, we noted that a high variability in the proportion of infected neutrophils between experiments. It is possible that our results are influenced by the number of newly recruited neutrophils, which may contribute to the variability. Overall, this method requires further optimization and development and does not capture the dynamic interplay between PA and neutrophils *in vivo*.

To study PA-neutrophil interactions *in vivo*, spinning disk intravital microscopy is a powerful imaging tool that can be used to study the dynamic interplay between host and pathogen *in vivo*. This technique has been used in various infection models to demonstrate host-pathogen interactions in specific tissues [21–24]. For this technique, neutrophils would be labeled with conjugated murine neutrophil marker Ly6g 12 h before the experiment. To locate PA in the lung vasculature, mice would be infected with a fluorescently-tagged representative clinical PA isolate. Following the infection, mice would be anesthetized to expose the trachea and part of the lung to visualize the intravascular neutrophils and PA. Volocity software would be used to identify individual captured bacteria by neutrophils. Using this approach, we could visualize and quantify the initial neutrophil recruitment and infiltration to the lung vasculature after infection, especially phagocytic killing specific to neutrophils, and thus examine *in vivo* phagocytosis and intracellular bacterial killing of representative Persistent and Eradicated isolates. Nonetheless, we recognize that there are many challenges in performing intravital microscopy to image lung tissue *in vivo*, including restricted organ accessibility, limited penetration depth, surgical procedures to expose the lung, and imaging difficulties due to the motion from the respiring lung and cardiac contractions [25].

In addition to neutrophil antibacterial functions essential for PA eradication, the host inflammatory responses may also define the eradication outcome [26]. Studies by others have

shown that key proinflammatory cytokines and chemokines, notably IL-1β, IL-6, IL-8, GM-CSF, G-CSF, and TNF-α, are significantly dampened following infection with PA strains that are typical of chronic infection (e.g., non-motile, non-piliated, overproduction of EPS) compared to their respective WT [24,27–31]. In chapter 3, we showed that mice infected with Persistent isolates exhibited higher bacterial burden than Eradicated isolates at each endpoint. However, among the two Persistent isolates, only 505P-infected mice induced significantly lower cytokine responses (IL-1β, IL-6, and MCP-1) than those infected with Eradicated isolates and 565P. Although this discrepancy in cytokine responses among Persistent isolates may, in part due to the strain-specific phenotype (overproduction of Psl and Pel) found in 565P, the host responses to each infection could be varied between animals, and this is likely an important factor that contributes to the variability. To address this, we could perform a mixed infection competition *in vivo*. Using a mixed infection model would increase the sensitivity and accuracy of direct comparison between different PA strains within the same mouse infection, hence reducing the variability of innate immunity in response to different PA strains [32]. To test this, we would select a pair of Persistent and Eradicated isolates, and each isolate could be tagged with a unique marker (e.g., gentamicin or tetracycline-resistant marker) that differentiates each isolate. Mice would be co-infected with an equal amount of gentamicin-resistant Persistent PA and tetracycline-resistant Eradicated PA to achieve a final 50 μL inoculum of $1x10^7$ bacteria intratracheally. Lungs would be harvested at 6, 48, and 96 h p.i. to measure viable lung bacterial burden by CFU plating on LB agar with gentamicin or tetracycline to select for Persistent or Eradicated PA. The competitive index (CI) for each strain would be calculated to determine whether Persistent PA would outcompete Eradicated PA at each endpoint.

5.2.3 In vivo imaging of neutrophil degranulation elicited by clinical PA isolates

In chapter 3, we demonstrated that representative Persistent isolates exhibited impaired *in vitro* neutrophil degranulation activity compared to Eradicated isolates. Thus, neutrophil degranulation may be an important antibacterial mechanism against Persistent isolates. In our study, we did not examine this process *in vivo*. Therefore, measuring degranulation activity induced by representative Persistent and Eradicated isolates *in vivo* would complement our *in vitro* findings. The release of NE is associated with degranulation activity [33]. An approach to monitor neutrophil degranulation activity *in vivo* is to inject mice with a fluorescent activatable sensor technology probe NE680 fast. This probe would generate a fluorescent signal after cleavage by elastase and could be visualized in mice via a fluorescence molecular tomography (FMT) imaging system [34]. This measurement could serve as a surrogate to measure neutrophil degranulation *in vivo* in response to representative Persistent and Eradicated PA isolates.

5.3 Potential anti-EPS therapies that enhance neutrophil-mediated bacterial clearance

5.3.1 Anti-Psl antibody treatments

Individuals with CF with new-onset PA infections who fail eradication therapy will undergo stepwise or repeated antibiotic regimens, but the success of each additional antibiotic regimen subsequently decreases even in the absence of antibiotic resistance [35]. Thus, more effective therapies are needed to improve PA eradication. Developing antibodies that harness the innate immune response against PA and neutralize bacterial virulence factors may be a promising therapeutic approach to prevent or treat PA infections alone or adjunctively with antibiotics [36]. Conserved surface antigens are typically targeted for antibacterial antibody development. MEDI3902, which targets Psl and PcrV, was reported to have superior activity at

potentiating neutrophil-mediated antibacterial functions against PA than other monospecific mAbs such as Psl0096 (only targets Psl) [37], or V2L2 (targets PcrV only) [38], although we found no differences *in vitro* compared to Psl0096. MEDI3902 initially passed phase I safety and tolerability clinical studies [39], and was tested in a randomized phase IIa clinical trial to prevent nosocomial ventilator-associated pneumonia (VAP) in mechanically ventilated patients [40]. Although the results from the phase IIa trial were not favorable since treatment did not reduce PA incidence in VAP patients, MEDI3902 may have therapeutic value in people with CF with new-onset PA infections.

Our study in chapter 4 is the first to test the effect of MEDI3902 using a collection of CF clinical PA isolates. We have shown that mAb MEDI3902 significantly reduces the bacterial burden in mice infected with Persistent isolates at 24 h p.i. However, complete bacterial clearance was only modest at 96 h p.i. especially for one of the Persistent isolates, suggesting that further testing and optimization are needed. Other regimens, such as using MEDI3902 in combination with antibiotics (e.g., tobramycin and meropenem), have shown significant improvement in PA clearance *in vitro* and *in vivo* compared to MEDI3902, IgG control, and antibiotic treatment alone [41–43]. Although the exact mechanism by which MEDI3902-antibiotic combinations potentiate bacterial clearance is still unclear, it is speculated that antibiotics alleviate the bacterial burden and provide better access to mAb and neutrophils, resulting in increased engagement between neutrophils and PA [41]. We could also adapt our *in vivo* prophylactic model to test whether MEDI3902 treatment with anti-PA antibiotics adjunctively would enhance PA clearance *in vivo* compared to MEDI3902, IgG, and anti-PA antibiotic treatment alone. We would first test the dosage of the antibiotic of interest needed in order to see optimal protection in mice alone or in combination with MEDI3902. Lungs would

be harvested to quantify viable bacterial burden at 24 or 96 h p.i. We expected that mice treated with MEDI3902 alongside antibiotics would provide superior activity against PA than those treated with MEDI3902 or antibiotic therapy alone.

There are several limitations of using MEDI3902 as a prophylactic treatment. First, repeated doses may be needed in order to maintain its effect to enhance neutrophil-PA clearance. Second, the impracticality of receiving MEDI3902 in individuals with CF to prevent PA infections regardless of patients' microbiological status (PA+ or PA-) is inconvenient and not cost-efficient. Thus, it would be most beneficial for therapeutic purposes to develop MEDI3902 as a treatment for established PA infections.

5.3.2 Recombinant hydrolases PslG and PelA

Two recombinant glycoside hydrolases, PslG and PelA, which target Psl and Pel have been previously developed by the Howell group to treat established biofilms [44]. These non-cytotoxic enzymes have been shown to prevent and directly degrade Psl and Pel-dependent biofilms and augment biomass reduction from PA biofilms with and without tobramycin [44]. Psl and Pel hydrolases, which show no negative effects on lung fibroblasts could be beneficial to prevent and degrade biofilms directly [44], especially in the context of chronic infection where Psl-dependent microaggregates have been identified in CF sputum. In addition, hydrolase treatment increases bacterial killing mediated by dHL-60 cells against PA laboratory strains *in vitro* [44] and in an *in vivo* wound infection model [45], suggesting hydrolase treatment may enhance neutrophil-mediated bacterial clearance against Psl and Pel expressing PA isolates. We have previously compared the neutrophil potentiation effect by hydrolase treatment (PslG and PelA) to anti-Psl mAbs (Psl0096 and MEDI3902) using clinical Persistent isolates. However, our

preliminary data showed that hydrolase treatment was less efficacious than mAb treatment at ameliorating *in vitro* neutrophil OPK (Appendix Figure A2). Thus, we decided not to proceed with the hydrolase treatment and prioritized our studies with MEDI3902. Furthermore, the CF lung environment contains many proteases or other molecules which could interfere with the activity of hydrolase therapy. Therefore, further validation and testing of the activity of hydrolase treatment in the presence of sputum supernatant would be required to evaluate the efficacy of hydrolase therapy in the context of CF infections.

5.4 Future directions

Several future directions for our studies are to be considered. In order to test and validate *in vivo* clearance in response to representative Persistent and Eradicated isolates, we will consider using intravital microscopy to examine and visualize the interplay of PA and neutrophils. Next, a mixed infection model within the same host will add additional value to further demonstrate and verify whether Persistent isolates would outcompete Eradicated isolates and lead to bacterial persistence *in vivo*. These experiments are discussed in section 5.2.2. Finally, since others have shown that MEDI3902 alone or with antibiotics conjunctly can be used to treat established PA infections in a murine [42] or a rabbit model [43], we will develop MEDI3902 as a treatment to treat established CF PA infections. We will test whether the administration of MEDI3902 alone or combined with anti-PA antibiotics such as tobramycin or meropenem will afford protection in mice after infection with Persistent isolate.

5.5 Conclusion

Our findings extend our current knowledge of PA-neutrophil interactions that can be targeted to improve PA eradication treatment or delay persistent infections. Studies in this thesis provide valuable biological insights into the mechanisms that contribute to the failure of eradication treatment of new-onset PA infections in some CF patients. Our data also shed light on developing novel therapies targeting specific bacterial determinants or neutrophil-dependent PA clearance to improve the outcome of eradication therapy in CF patients with new-onset infections. Lastly, the use of MEDI3902 to be optimized as a therapeutic treatment would benefit people with CF who are not eligible for any CFTR modulator therapies and would significantly contribute to alternative treatments for CF.

Appendices

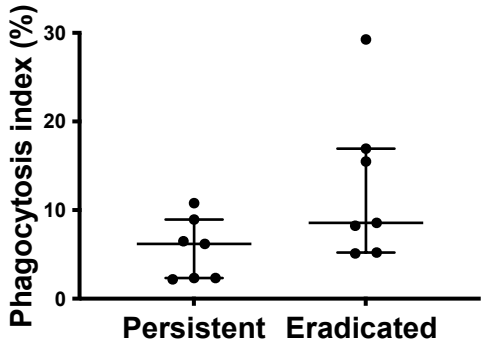

Figure A1. A trend towards a lower *in vitro* neutrophil phagocytosis elicited by Persistent (n = 7) compared to Eradicated (n = 7) PA isolates from the EPIC clinical study. Results are shown as median ± IQR; N ≥ 6 replicates from 2 independent experiments; statistical comparisons were performed using Mann-Whitney test.

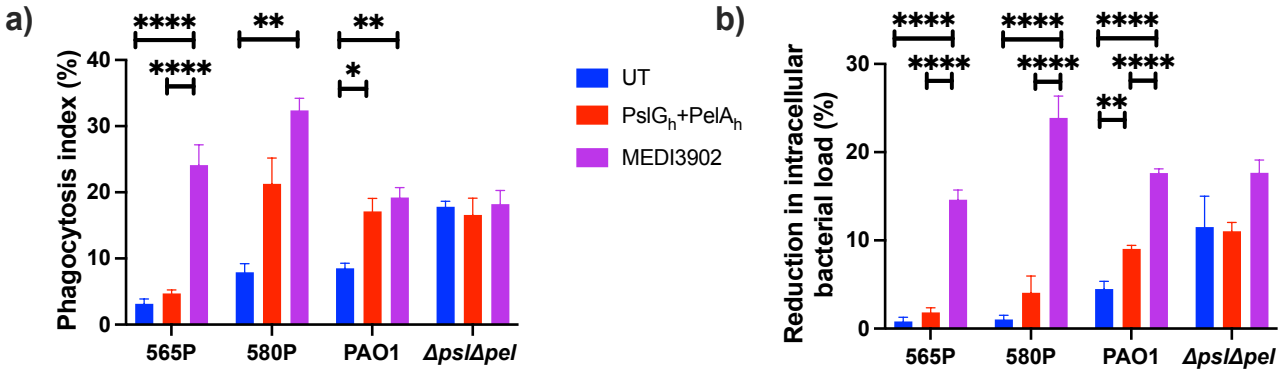

Figure A2. *In vitro* neutrophil OPK by hydrolase or MEDI3902 treatment in response to clinical Persistent PA isolates. a) Phagocytosis and b) Intracellular bacterial killing of Persistent isolates 565P and 580P, PAO1 and EPS-deficient mutant with and without hydrolase (PslGh and

PelAh) or MEDI3902. For *In vitro* OPK by hydrolase treatment, bacteria were pretreated with 50 μg/mL of PslGh and 100 μg/mL of PelAh for 1 h at 37 °C, washed, and opsonized with 10% baby rabbit serum. Opsonization, phagocytosis, and intracellular bacterial killing assays were performed as described in chapter 4. N ≥ 9 biological replicates from 3 independent experiments. Results are shown as mean ± SEM; statistical comparisons were performed using ANOVA (*$P <$ 0.01,**$P < 0.01$, ****$P < 0.0001$).

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