The Impact of *Pseudomonas aeruginosa* Genetic Adaptation to the Cystic Fibrosis Airway on Inflammation and Bacterial Persistence

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Abstract

By adulthood, the lungs of most Cystic Fibrosis (CF) patients become chronically infected by the bacterium *Pseudomonas aeruginosa.* Once chronic, these infections are virtually ineradicable and often fatal, and are characterized by severe neutrophilic inflammation and a persistent bacterial biofilm infection. In the early stages of infection, *P. aeruginosa* strains found in CF airways are indistinguishable from environmental strains, but as *P. aeruginosa* adapts to the CF lung, it undergoes extensive genetic adaptation and microevolution. Phenotypic changes commonly occurring in CF-adapted *P. aeruginosa* strains include mucoidy, the loss of motility, loss of acute virulence factors like secreted proteases, and loss of LasR quorum sensing. Despite the loss of functions used to invade and injure the host, the emergence of CF-adapted *P. aeruginosa* strains in CF airways is paradoxically associated with more severe lung disease and worst patient outcomes. We hypothesized that CF-adapted *P. aeruginosa* isolates alter host pulmonary immune responses in a manner that increases neutrophil-mediated inflammation and impedes bacterial clearance, thereby contributing to lung disease progression.

Using *in vitro* human airway epithelial cell (AEC) culture models and a murine model of chronic *P. aeruginosa* airway infection, we determined that *P. aeruginosa* isolates defective in LasR quorum sensing, or in the production of the LasR-regulated secreted protease LasB, were unable to degrade pro-inflammatory cytokines secreted by AEC, unlike wild-type *P. aeruginosa* strains. Genetically engineered Δ*lasR* and Δ*lasB* mutant strains were associated with hyper-inflammatory cytokine responses and increased neutrophil recruitment *in vitro* and *in vivo*. Additionally, infection with the Δ*lasR* and Δ*lasB* mutants led to greater lung immunopathology and, in the case of the Δ*lasB* strain, greater morbidity. In CF patients, we found that the frequency of *lasR* mutants in the lungs was positively correlated with plasma IL-8 levels, a marker of neutrophil inflammation.

Next, we compared the *in vivo* virulence and immunogenicity of two clonally-related *P. aeruginosa* isolates recovered from a single patient 7.5 years apart: the Late isolate, which had numerous CF-adapted phenotypic changes including a loss of LasR quorum sensing and secreted LasB activity, and a non-adapted Early isolate. We found that the Late isolate caused less early mortality and morbidity in a murine model of chronic airway infection compared to the Early isolate, but displayed enhanced persistence by day 10 post infection (p.i.). We demonstrated that the kinetics of cytokine responses in the lungs of Early and Late-infected mice differed; the Early isolate stimulated a robust early pro-inflammatory and pro-neutrophilic response that was quickly shut down, and the Late isolate stimulated less vigorous pro-inflammatory response but a more prolonged neutrophil chemokine response. The Late isolate elicited greater neutrophil recruitment into the lungs by days 4 and 10 p.i. compared to the Early isolate, demonstrating that this CF-adapted isolate was able to persist despite causing a robust neutrophil response.

Together, our studies suggest that bacterial adaptive changes may worsen pulmonary inflammation and contribute directly to the pathogenesis and progression of chronic lung disease in CF patients. Understanding how phenotypic changes occurring in CF-adapted *P. aeruginosa* isolates modulate host inflammatory responses and promote bacteria persistence *in vivo* can provide us insights into the pathogenesis of chronic infections, a paradigm distinct from acute virulence.

Résumé

La majorité des patients adultes atteints de fibrose kystique (FK) sont chroniquement infectés par la bactérie *Pseudomonas aeruginosa*. Ces infections sont souvent mortelles, et sont caractérisées par une infiltration neutrophilique excessive et délétère, ainsi que la formation de biofilm bactériens persistants et résistants aux traitements. Lorsque surviennent les premières infections, les souches de *P. aeruginosa* retrouvées dans les voies respiratoires des patients FK sont indiscernables des souches environnementales. Mais au fil du temps, lorsque les infections deviennent chroniques, *P. aeruginosa* subit des changements génétiques et phénotypiques importants. Le caractère mucoïde, la perte de la motilité, la perte de facteurs de virulence aiguë comme les protéases sécrétées, et la perte du système de quorum-sensing de LasR sont des caractéristiques phénotypiques typiques des souches de *P. aeruginosa* adaptés aux voies aériennes FK. Malgré la perte de fonctions utilisées pour envahir et endommager l'organisme hôte, l'émergence de souches de *P. aeruginosa* adaptées aux voies respiratoires FK est paradoxalement associée à une diminution de la fonction pulmonaire et des pires résultats pour les patients. Nous avons émis l'hypothèse selon laquelle les souches de *P. aeruginosa* adaptées modifient la réponse immunitaire pulmonaire de l'hôte, induisant une infiltration neutrophilique plus importante, empêchant la clairance bactérienne, et contribuant ainsi à la progression de la maladie pulmonaire FK.

En utilisant des cultures de cellules épithéliales bronchiques (CEB) et un modèle murin d'infection bronchique chronique par *P. aeruginosa*, nous avons mis en évidence que les isolats de *P. aeruginosa* ayant un système de quorum-sensing de LasR inactif, ou une production de la protéase sécrétée LasB diminuée, étaient incapables de dégrader les cytokines pro-inflammatoires sécrétées par les CEB à la différence des souches sauvages. Nous avons également montré que les mutations de *lasR* et de *lasB* chez les souches de *P. aeruginosa* induisent une augmentation de la sécrétion de cytokines pro-inflammatoires et du recrutement des neutrophiles *in vitro* et *in vivo*. En outre, l'infection par les mutants Δ*lasR* et Δ*lasB* a induit une plus grande réaction inflammatoire endommagent dans les poumons des souris et, dans le cas de la souche Δ*lasB*, une plus grande morbidité des souris. Chez les patients FK, nous avons observé

que la fréquence de l'isolation de mutants *lasR* dans les poumons était positivement corrélée avec le niveau d'IL-8 dans le sang, un marqueur de l'inflammation neutrophilique.

Nous avons ensuite comparé la virulence et la réponse immunitaire induite par un isolat de *P. aeruginosa* adapté aux voies respiratoires d'un patient FK à une souche parentale (non-adaptée) isolée chez le même patient 7,5 années auparavant. L'isolat adapté présentait de nombreux changements phénotypiques, y compris une perte du système de quorum-sensing de LasR et une réduction de la sécrétion de LasB. Nous avons constaté que l'isolat adapté induisait moins de létalité et de morbidité dans un modèle murin d'infection bronchique chronique par rapport à l'isolat non-adaptée, mais que cette souche pouvait persister mieux à long terme dans les poumons des souris. Nous avons démontré que la cinétique de la réponse des cytokines dans les poumons des animaux infectés par les deux isolats était différente; l'isolat précoce induit une réponse pro-inflammatoire et neutrophilique importante qui est rapidement arrêtée, alors que l'isolat adapté induit une réponse pro-inflammatoire moins vigoureuse, mais une sécrétion de chémokine neutrophilique plus longue. L'isolat adapté est responsable d'un recrutement de neutrophiles plus important au niveau pulmonaire aux jours 4 et 10 après infection comparé à l'isolat non-adapté, ce qui démontre que l'isolat adapté peut persister malgré le fait qu'il provoque une réponse neutrophilique importante.

L'ensemble de nos résultats suggère que les changements phénotypiques adaptatifs qui se produisent dans les souches de *P.* aeruginosa au cours des infections des voies respiratoires peuvent aggraver l'inflammation pulmonaire et contribuer directement à la pathogénicité et à la progression de la maladie pulmonaire chronique chez les patients atteints FK. Comprendre comment les changements phénotypiques se produisent dans les souches de *P. aeruginosa* adaptées, peuvent moduler les réponses inflammatoires de l'hôte, et induisent une persistance des bactéries *in vivo* peut nous fournir des indications sur la pathogenèse des maladies pulmonaires chroniques causées par *P. aeruginosa*.

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List of abbreviations

ΔF508 mutation	Three base pair deletion leading to the deletion of a phenylalanine residue at position 508 of CFTR amino acid sequence
3D	Three dimensional
3-oxo-C12-HSL	N3-oxo-dodecanoyl-L-homoserine lactone
7-AAD	7-Amino-actinomycin D
AEC	Airway epithelial cells
AHL	Acyl homoserine lactone
ANOVA	Analysis of variance
ASL	Airway surface liquid
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchoninic acid assay
bp	Base pair
BSA	Bovine serum albumin
C5a	Complement component 5a
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CEB	Cellules épithéliales bronchiques
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CHUM	Centre Hospitalier de l'Université de Montréal
COPD	Chronic obstructive pulmonary disease
CpG	Cytidine phosphate guanosine dinucleotides
CR3	Complement receptor 3
CXCR1	Chemokine (C-X-C motif) receptor 1
DAMP	Damage associated molecular pattern
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ECR	Elastin congo red
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
ENA-78	Epithelial-derived neutrophil activating peptide 78 (CXCL5)
ENaC	Amiloride sensitive epithelial sodium channel
ERK	Extracellular regulated kinase
ExoS	Exoenzyme S
Ex /Em	Excitation and emission wavelengths
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FEV1	Forced expiratory volume in the 1st second
FK	Fibrose kystique
FREQ-seq	Illumina-based allele frequency sequencing

FVC	Forced vital capacity
G542X mutation	Glycine-to-STOP nonsense mutation at position 542 of CFTR amino acid sequence
G551D mutation	Glycine-to-aspartate non-synonymous substitution at position 551
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
GI	Gastrointestinal
GM-CSF	Granulocyte monocyte colony stimulating factor
H&E	Hematoxylin and eosin
HI	Heat inactivation
hrs	Hours
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
IQR	Interquartile range
kb	Kilobase
KC	Keratinocyte chemoattractant (CXCL1)
kDa	Kilodalton
LB	Luria Bertani
LBP	Lipopolysaccharide-binding protein
LDH	Lactate dehydrogenase
LIX	Lipopolysaccharide-induced CXC chemokine (CXCL5)
LOD	Limit of detection
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
Ly6G	Lymphocyte antigen 6 complex, locus G
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MD2	Myeloid differentiation protein 2
MDR-PA	Multi-drug resistant *Pseudomonas aeruginosa*
MIP	Macrophage inflammatory protein
MOI	Multiplicity of infection
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MRSA	Methicillin resistant Staphylococcus aureus
MYD88	Myeloid differentitation primary response gene 88
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor of kappa B
NLRC4/Ipaf	Nod-like receptor caspase recruitment domain containing protein 4
NOD	Nucleotide-binding oligomerization domain
NS	Not significant
NT	No treatment
OD	Optical density
ORCC	Outwardly rectifying chloride channel
ORF	Open reading frame

PA	Phosphoramidon
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PAR	Protease-activated receptor
PBS	Phosphate buffered saline
PE	Phycoerythrin
p.i.	Post infection
PMN	Polymorphonuclear leukocytes
PRR	Pathogen recognition receptor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
RBC	Red blood cell
rh	Recombinant human
RI-MUHC	Research Institute of Mcgill University Health Centre
ROS	Reactive oxygen species
r.p.m	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCFM	Synthetic Cystic Fibrosis sputum media
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SP	Surfactant protein
Spp.	Species
T3SS	Type 3 secretion system
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
USA	United States of America
WT	Wild-type

Original contribution to knowledge

Chapter 2: Cystic fibrosis–adapted *Pseudomonas aeruginosa* quorum sensing *lasR* mutants cause hyperinflammatory responses.
Originally published in *Science Advances*. 2015, (*1*).

We examined the effect of LasR quorum sensing inactivation, which occurs in 30-65% of *P. aeruginosa* isolates recovered from chronically-infected CF airways, on airway epithelial cell (AEC) IL-8 and IL-6 mediated inflammatory responses. Using *in vitro* and *in vivo* models of *P. aeruginosa* chronic airway infection, we demonstrated that *lasR* mutants drive increased IL-6 and IL-8 responses, leading to increased neutrophil recruitment. We found a positive correlation between the frequency of *lasR* mutants in the lungs of CF patients chronically infected with *P. aeruginosa* and plasma levels of IL-8, a marker of neutrophil inflammation. Finally, we dissected the mechanism by which *lasR* mutants caused hyper-inflammatory IL-6 and IL-8 responses, determining that it was due to the loss of the LasR-regulated secreted protease LasB, which can degrade IL-8 and IL-6, thereby dampening host inflammatory responses to *P. aeruginosa*.

Chapter 3: Loss of elastase activity in *Pseudomonas aeruginosa* and its impact on host pulmonary inflammation. (In preparation for submission).

We demonstrated that the secreted protease LasB, an important *P. aeruginosa* acute virulence factor, was capable of altering AEC pro-inflammatory cytokine transcriptional responses, and was capable of decreasing pro-inflammatory cytokines protein levels in AEC cultures, likely through proteolytic degradation. We found that the loss of secreted LasB, which occurs in protease-negative CF-adapted *P. aeruginosa* isolates, led to hyper-inflammatory cytokine responses in a murine model of chronic airway infection, greater neutrophil recruitment, increased lung damage and caused greater morbidity. This suggests that the loss of LasB, which attenuates acute virulence, may actually exacerbate the damaging immunopathology occurring during CF chronic airway infections.

Chapter 4: Altered bacterial persistence and host responses to a CF-adapted *Pseudomonas aeruginosa* clinical isolate in vivo. (In preparation for submission).

We performed an *in vivo* comparison of a pair of fully-sequenced clonally-related longitudinal *P. aeruginosa* clinical isolates recovered from a single CF patient 7.5 years apart using a murine model of chronic airway infection. We found that the CF-adapted "Late" isolate, which has 68 mutations and has undergone several CF-adapted phenotypic changes, caused less lethality and morbidity in mice compared to the non-adapted "Early" isolate. The Late isolate, however, was better able to persist in the lungs of mice over time compared to the Early isolate, despite eliciting a greater neutrophil response in the lungs. We characterized the cytokine responses to chronic pulmonary infection with the Early and Late isolates overtime and found that the magnitude and kinetic of pro-inflammatory cytokine responses were different and this may be responsible for the differences in neutrophil recruitment and bacterial clearance during chronic infection with the two strains.

Contribution of authors

Chapter 2: Cystic fibrosis–adapted *Pseudomonas aeruginosa* quorum sensing lasR mutants cause hyperinflammatory responses
Shantelle L. LaFayette, Daniel Houle, Trevor Beaudoin, Gabriella Wojewodka, Danuta Radzioch, Lucas R. Hoffman, Jane L. Burns, Ajai A. Dandekar, Nicole E. Smalley, Josephine R. Chandler, James E. Zlosnik, David P. Speert, Joannie Bernier, Elias Matouk, Emmanuelle Brochiero, Simon Rousseau, Dao Nguyen.

SLL prepared bacterial filtrates, performed AEC stimulations, prepared agar beads for mouse infections, processed mouse samples, did FACS and analysis, performed all western blots and analysis, performed all cell culture sample and animal sample ELISAs and analysis. DH performed murine intratracheal infections. DH and SLL performed murine necropsies. EB contributed nasal polyp samples and TB and SLL performed nasal polyp explant stimulation experiments. GW performed IL-8 ELISA on CF patient plasma samples. DR contributed reagents for mouse experiments. LRH contributed *P. aeruginosa* strains and edited manuscript. JL contributed *P. aeruginosa* strains. AAD, NES and JTC performed the FREQ-seq of *lasR* alleles in patient samples. DS, LRH, JEZ and JLB, contributed *P.* aeruginosa clinical isolates. JB and EM oversaw CF patient clinical studies and collected patient sputum and blood samples SR contributed cells and reagents for airway epithelial cell culture. DN and SLL designed all the studies, oversaw all analyses, wrote and edited the manuscript.

Chapter 3: Loss of elastase activity in *Pseudomonas aeruginosa* and its impact on host pulmonary inflammation (*manuscript in preparation)*
Shantelle L. LaFayette, James Schafhauser, Daniel Houle, Simone Périnet, Danuta Radzioch and Dao Nguyen.

SLL prepared bacterial filtrates, performed all mouse infections (with the assistance of DH), *in vivo* sample analyses (including FACS analyses, cytopsin, luminex,), qRT-PCR and protease assays. SLL and SP processed mouse lungs for flow cytometry. SLL and JS performed AEC stimulations and cell line ELISAs. DR contributed reagents for animal experiments. SLL and DN designed all the studies, wrote and edited manuscript.

Chapter 4: Altered bacterial persistence and host responses to a CF-adapted *Pseudomonas aeruginosa* clinical isolate in vivo.
Shantelle L. LaFayette, Daniel Houle, Danuta Radzioch and Dao Nguyen

SLL performed all mouse infections (with the assistance of DH), *in vivo* sample analyses (cytopsin, luminex). DR contributed reagents for animal experiments.

Chapter 1: Introduction

1.1. Cystic fibrosis

History

While cystic fibrosis (CF) likely has existed in human populations for centuries, it has only been recognized as a distinct clinical syndrome since the late 1930s (*2*). In 1938, Dr. Dorothy Anderson, an American pathologist, provided the first comprehensive description of CF as a unique medical disorder that encompassed pancreatic insufficiency, excessively salty sweat and recurring bronchiectasis, which were previously thought to be unrelated pathologies (*3*). Infants dying of malnutrition had mucous plugs in the glandular ducts of the pancreas and the condition was therefore named "cystic fibrosis of the pancreas"(*4*). Dr. Anderson was also the first to develop a comprehensive treatment plan for CF patients that included a special diet and pancreatic enzyme supplements (*4*).

In 1982 and 1983, Quinton *et al* published seminal studies demonstrating that abnormally salty sweat in CF patients was caused by impaired cAMP-mediated chloride transport in sweat glands (*5, 6*). Although the CF defect was assigned to chromosome 7 by genetic linkage analysis in 1986 (*7*), it was only in 1989 that the CF disease causing gene was identified by Dr Tsui and colleagues at Toronto's Hospital for Sick Children by positional cloning (*8, 9*). The gene was named "Cystic Fibrosis Transmembrane Conductance Regulator" (CFTR) to reflect its predicted protein structure as a transmembrane ion transporter (*8*).

Epidemiology

CF is the most common lethal recessive disease in Caucasians of Northern European ancestry, occurring in approximately 1 in 3000 newborns and with a carrier rate of approximately 1 in 26 individuals (*10-13*). In some parts of

the world, such as Quebec in Canada, the incidence of CF can be much higher (1 in 900) (*13*). In addition to affecting individuals of Northern European ancestry, CF also frequently affects other Caucasian ethnic groups. For instance, individuals of Eastern European and Ashkenazi Jewish ancestry have relatively high incidences of CF (*14, 15*). CF disease in other racial groups, however, remains quite rare (*16*). Currently, it is estimated that 70 000 individuals worldwide are living with CF, and half are found in the United States of America (USA) and Canada.

In the past six decades, the life expectance of CF patients has increased dramatically. In the 1950s, the median age of survival for a child with CF was eight years (*4*). Today, the estimated median survival age of a child born and diagnosed with CF is 39 years in the USA and 50.9 years in Canada (*17*). A study comparing the median age of death in CF patients in ten countries between 1974 and 1994 also observed significant increases in CF life expectancy as a function of time in every case (*18*). The dramatic increase in patient life expectancy over the past thirty years have been largely attributed to significant improvements in the early diagnosis and management of CF, particularly the management of lung, pancreatic and gastrointestinal diseases (*19*)

Structure and Function of CFTR

Normal CFTR structure and function

Located on the long arm of chromosome 7 at position 7q31.2 (Fig. 1.1A), *CFTR* is a large gene that spans 250 kb of genomic DNA and contains 27 exons (Fig. 1.1B) (*20*). Transcription yields a 6128bp full-length mRNA that is translated and processed into a 1480 amino acid glycoprotein with a molecular mass of 170 Kd. A unique member of the ABC transporter family, CFTR functions predominantly as a chloride channel (*21*). The CFTR protein is made up of five domains (Fig. 1.1C): two transmembrane domains (TM1 and TM2) that form the channel pore, two nucleotide-binding domains (NBD1 and NBD2) that control opening and closing of the channel via ATP hydrolysis, and a regulatory domain

(RD). In normal cells, CFTR is found in the apical membrane of cells where it regulates transepithelial salt transport, fluid flow and ion concentrations (*8, 22*). Besides its ability to directly transport anions, CFTR can influence transepithelial ion homeostasis through the regulation of other ion channels. For instance, CFTR inhibits the amiloride sensitive epithelial sodium channel (ENaC), a channel that mediates the first step of active sodium reabsorption(*23*). The activation of the outwardly rectifying chloride channel (ORCC) is also reliant on CFTR (*23*).

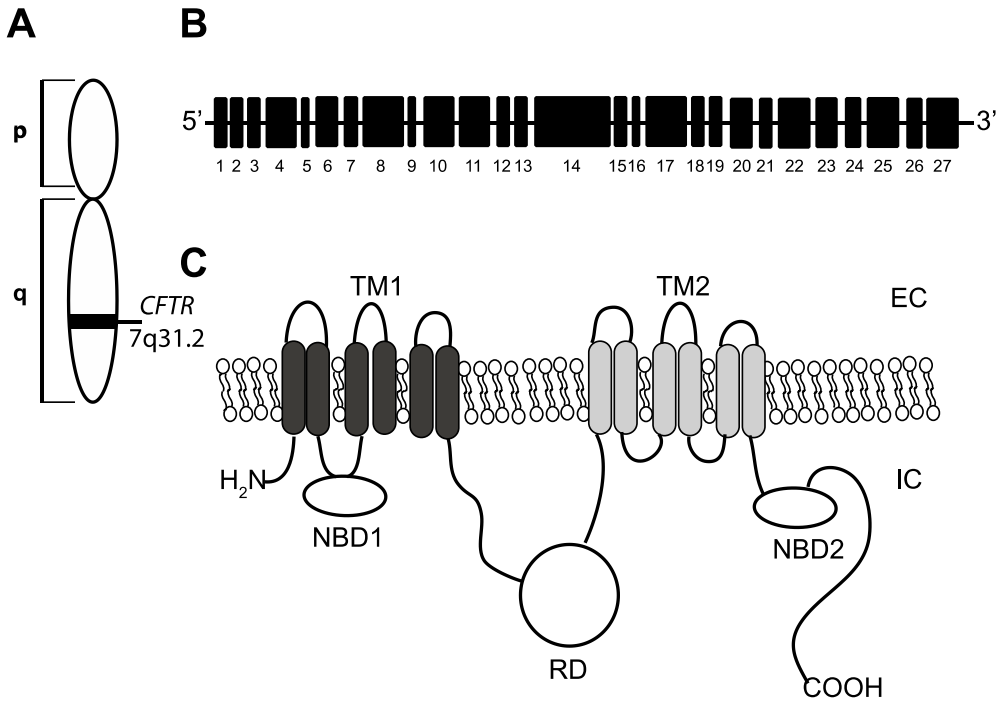

Fig. 1.1: CFTR gene and protein

(A) Depiction of *CFTR* location on human chromosome 7 (position q31.2)
(B) Depiction of the *CFTR* open-reading frame, which contains 27 exons. Adapted from (*24*)
(C) Depiction of CFTR protein structure in apical membrane of a cell. TM: transmembrane domain, NBD: nucleotide binding domain, RD: regulatory domain, EC: extracellular space, IC: intracellular space. Adapted from (*24*)

CF-causing CFTR mutations

CFTR is a gene with astounding allelic heterogeneity, with over 2000 mutations described to date on the CFTR1 Cystic Fibrosis Mutation Database (*25*). However, it remains unclear exactly how many of these mutations cause CF (*26*). *CFTR* mutations can decrease CFTR function through several different mechanisms and are thus organized into six functional classes to reflect these differences (Fig. 1.2) (*27*). Class I and class V mutations produce premature termination of *CFTR* biosynthesis through frameshift and nonsense mutations (class I), or by introducing mRNA splice site abnormalities (class V) (*27*). The G542X mutation, a glycine-to-STOP nonsense mutation at position 542, is an example of a class I mutation. Class II mutations prevent proper folding and post-translational processing of the protein, causing it to be degraded (*28*). These include the highly prevalent ΔF508 mutation, a three base pair deletion in the *CFTR* gene leading to the loss of a phenylalanine residue at the amino acid position 508. Class III mutations prevent the appropriate opening and closing of the ion channel, such as a glycine-to-aspartate non-synonymous substitution at position 551 (G551D). Class IV mutations lead to defective ion conduction by CFTR (*27*). Finally, the most recently described class of mutations, class VI, include mutations that affect CFTR protein stability, reducing the protein's half-life (*24*).

Class I and II mutations tend to result in the most severe CF phenotypes because they either completely prevent CFTR from reaching the apical membrane, or they reduce the amount significantly (*27*). Class III mutations can vary in severity with some, like G551D, resulting in very little CFTR function and thus being quite severe (*27*). Class IV and V mutations tend to be less severe as they allow for the proper biosynthesis, processing and transport of functional CFTR to the apical membrane, even if the activity or amount of CFTR is reduced (*27*). Class VI mutations can also vary in severity, depending on how much the mutation influences CFTR's stability (*24*).

The ΔF508 mutation is by far the most common CF-causing mutation, accounting for 66% of CFTR mutations worldwide (*25*). In contrast, no other single CFTR mutation accounts for more than 5% of CF-causing mutations (*13*). The next most common CFTR mutations are the G542X and G551D mutations that account for approximately 2.4% and 1.6% of all *CFTR* mutations respectively (*25*).

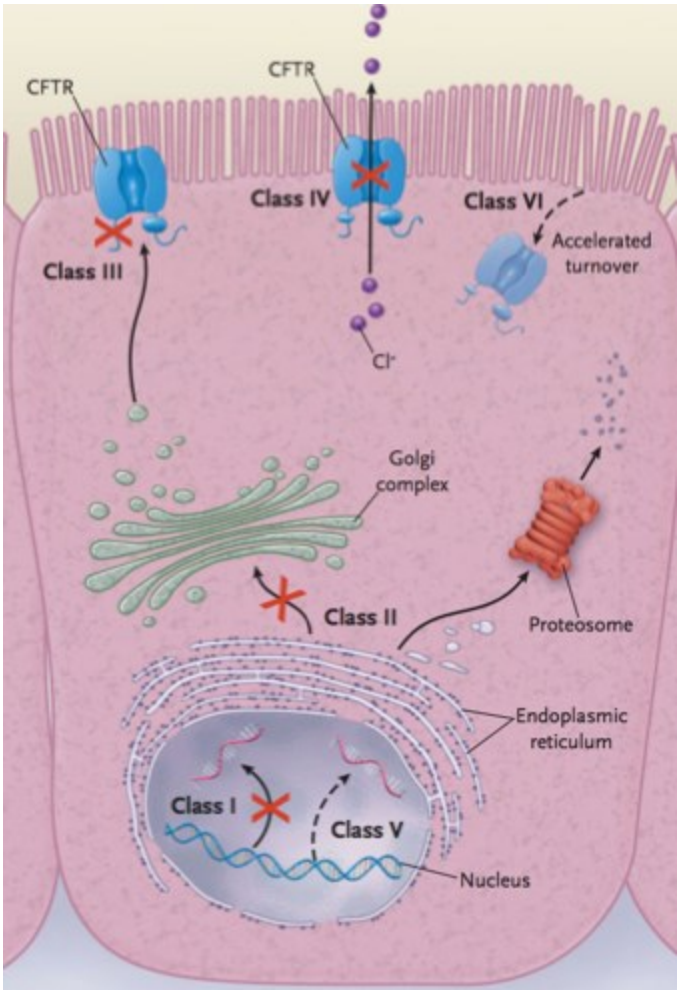

Fig. 1.2: Classes of CFTR mutations

I: Lack of CFTR synthesis.
II: Defective CFTR processing.
III: Defective CFTR channel regulation or gating.
IV: Defective chloride conductance.
V: Reduced amount of CFTR protein
VI: Increased turnover of CFTR channel at cell surface.
Image is reproduced from (*29*)

Extrapulmonary clinical manifestations of CF disease

In CF sweat glands, as primary secretions travel from the glandular coil to the surface of the skin, chloride absorption is hindered by defective CFTR, causing unusually high levels of sodium chloride to accumulate in the skin sweat. In the mucosal epithelia, CFTR dysfunction leads to the overproduction of thick sticky mucus that obstructs exocrine glands, resulting in deleterious effects on multiple organs in the body. Gastrointestinal symptoms are often the first to arise. Around 15% of CF infants are born with meconium ileus, an obstruction of the GI tract caused by inspissated material in the small and large bowels (*13*). Distal intestinal obstructions increase in incidence with age and first present in adulthood in ~80% of cases (*30*). Approximately 85-90% of CF patients develop exocrine pancreatic insufficiency that causes fat malabsorption leading to steatorrhea (feces with increased fat content), fat-soluble-vitamin deficiency, and malnutrition (*13, 31*). In ~20% of CF adults, pancreatic disease is also associated with the destruction of islet cells resulting in CF-related diabetes mellitus (*32*). Finally, CF patients are also at risk for focal biliary cirrhosis caused by obstruction of the intrahepatic bile ducts, and male infertility due to obstruction of the vas deferens (*13*).

1.2. CF lung disease

Overview

While CF affects many different organs, it is CF lung disease that poses the greatest challenge to the management of CF, and that has the greatest impact on the life span and quality of life of affected individuals (*32*). CF lung disease is characterized by worsening bronchiectasis and airway obstruction, associated with chronic bacterial infections, neutrophil inflammation and lung tissue destruction Although the GI manifestations of CFTR defects are often apparent shortly after birth, the development of CF lung pathology can be more variable (*13*). This is due to the fact that CF lung disease is influenced by a combination of genetic, stochastic and environmental factors, whereas GI disease is influenced mainly by genetics. Thus CFTR mutation severity correlates

with GI disease severity, whereas no such correlation occurs with CF lung disease (*30*).

Progressive lung disease is a major cause of morbidity and mortality in CF, with respiratory failure causing 80 to 95% of all deaths (*13, 33*). As lung disease progresses, CF patients experience gradual declines in lung function, measured as the forced expiratory volume in the 1st second (FEV1) and forced vital capacity (FVC) by spirometry (*34, 35*). CF patients with lung disease suffer from chronic symptoms of cough, sputum production and breathlessness, but also develop intermittent and recurrent episodes of increasing pulmonary symptoms termed exacerbations (*36*). These pulmonary exacerbations are typically associated with changes in cough, sputum production, labored breathing, decreased energy level and appetite, weight loss and short- and long-term decreases in lung function, and thus have important effects on the immediate and long-term morbidity of patients (*36*). Pulmonary complications such as pneumothorax and hemoptysis also may also require invasive interventions and can be life-threatening (*37*).

The pathogenesis of CF lung disease

Although the initial event in CF is clearly CFTR dysfunction, how this predisposes individuals to chronic bacterial infections and acute bacterial exacerbations remains incompletely understood. How bacteria persist in CF lungs, resisting clearance by the host immune system and eliciting a massive and damaging neutrophil pro-inflammatory response, remains an ongoing question. The prevailing hypothesis is that the basic CFTR defect compromises host defenses in the airways and leads to the build up of a thick viscous mucus, which provides an environment that promotes bacterial growth (*38*). The persistence of bacteria in the CF airways directly damages host tissues and fuels a vigorous neutrophil response that also causes collateral damage to the host. The neutrophils, however, fail to clear the bacterial infection, leading to a vicious cycle of inflammation and tissue damage (*38*).

Impact of CFTR dysfunction on pulmonary host defenses

In the lung, CFTR is mainly expressed on the apical membrane of surface airway epithelial cells (AEC) and the cells of the submucosal glands (*39*). However, alveolar epithelial cells and some migratory cells, like phagocytes, can also express CFTR at the cell surface (*39*). AEC, cells of the submucosal glands and phagocytes play important roles in innate defense mechanisms in the airways (*39*). The airway epithelial layer not only forms a physical barrier, but expresses motile cilia, which are critical to mucociliary clearance (*39*). Additionally, AEC produce antimicrobial proteins and peptides, sense pathogens and initiate innate immune responses (*40*). Cells of the submucosal glands produce mucins that can trap inhaled particles and pathogens but must also be removed by mucociliary clearance to avoid obstructing the airways (*39*). Phagocytic defense mechanisms in the lungs, carried out by resident macrophages and circulating neutrophils, are critical for eliminating pathogens in the airways. Evidence suggests that many of these defenses are hampered by the loss of CFTR function in the lungs (*39*)

Airway surface liquid, mucus and mucociliary clearance

Mucociliary clearance is an important primary innate defense mechanism that protects the lungs from inhaled particles and pathogens. The mucociliary clearance system is composed of ciliated epithelial cells, a protective mucus layer and the airway surface liquid (ASL) layer that traps and clears particles and pathogens from lower respiratory tract. A body of literature suggests that CFTR dysfunction increases isotonic fluid reabsorption, thus depleting the ASL volume and dehydrating the overlying mucus layer (*39*). This would lead to the collapse of epithelial cilia, thus disrupting coordinated and directional mucociliary clearance. Supporting this model, the overexpression of the ENaC in transgenic mice, leading to increase Na+ epithelial reabsorption, caused ASL volume depletion associated with mucus obstruction, impaired bacterial clearance and neutrophilic inflammation (*41*). The loss of CFTR function further impairs mucociliary clearance due to increased production of MUC5AC and MUC5B, the

predominant respiratory mucins (*42*). A recently developed CF piglet model (ΔF508 homozygous) also revealed abnormal mucus production from CF submucosal glands, which remains tethered to gland ducts and inhibit mucociliary clearance (*43*).

Antimicrobial proteins and peptides

CFTR dysfunction also impairs the function of secreted innate defense molecules, thus contributing to the increased susceptibility to infections seen in CF patients. In addition to chloride, CFTR functions as a transporter of bicarbonate ($HCO3^-$), an important pH buffer *in vivo*. Pezzulo *et al*, demonstrated in the CF pig model that the ASL was more acidic than in non-CF pigs and impaired bacterial killing by lactoferrin and lysozyme, two major antimicrobial proteins secreted in the airways. (*44*). It remains unclear whether this occurs in CF patients, as no significant differences in nasal or lower airway pH have yet been observed between CF and control individuals (*45*). AEC also produce antimicrobial peptides belonging to the β-defensin and cathelicin families (*46*), whose antimicrobial activity is markedly reduced in hypertonic conditions (*46*). Decreased CFTR chloride transport is thought to increased the salt concentration in CF ASL, which would further inactivate antimicrobial peptides in the lungs (*47*).

Airway epithelial cells and innate immune responses

Normal AEC express a broad range of toll-like receptors (TLRs), pathogen recognition receptors (PRRs) that recognize conserved structural motifs expressed by microbial pathogens, known as pathogen-associated molecular patterns (PAMPs), or dying cells, known as danger-associated molecular patterns (DAMPs) (*48, 49*). TLR-2 is the predominant TLR expressed on the surface of AEC (*48*). By heterodimerizing with either TLR-1 or TLR-6, TLR-2 recognizes a wide array of bacterial PAMPs including lipoteichoic acid and peptidoglycan, two major constituents of Gram-positive bacterial cells walls, and a diverse array of Gram-negative and Gram-positive lipoproteins (*48*). AEC also

express TLR-4, TLR-5, and TLR-9 on the cell surface; which recognize bacterial lipopolysaccharide (LPS), flagellin, and unmethylated cytidine- phosphate-guanosine (CpG) dinucleotides in bacterial DNA (CpG DNA) respectively (*48*).

Although several studies suggested that CF AEC express the same TLRs as non-CF AEC, the localization of the TLRs may differ, impacting host-pathogen signaling (*50-53*). Increased apical surface expression of TLR2 and TLR5 on human CF AEC correlates with increased inflammatory responses to bacterial products recognized by these PRRs (*52*). On the other hand, poor apical surface expression of TLR-4 on CF AEC results in poor stimulation by LPS (*54*). In one study, CF AEC were shown to rely almost exclusively on TLR-5 to sense *P. aeruginosa* and *Burkholderia spp. (53).* TLR-5-flagellin interactions in CF AEC resulted in an over-exuberant production of the inflammatory cytokine IL-6, and the administration of neutralizing antibodies against TLR-5 could completely ablate CF AEC inflammatory responses driven by bacterial stimulation (*53*). Finally, some researchers have proposed that CFTR is itself a PRR that binds *P. aeruginosa* LPS directly, and that its down-regulation on the surface of CF AEC prevents the internalization and destruction of this microbe (*55*) (*56*).

CFTR and phagocytic function

Immunohistological and physiological data suggest that *CFTR* is transcribed and functional in many cell types, including neutrophils (*57-59*), Several functional abnormalities have been described in CF neutrophils including cleavage of CXCR1 leading to disabled bacterial killing (*60*), reduced phagolysomal bacterial killing (*60, 61*), defects in degranulation (*59*) and delayed apoptosis (*62*). Other studies, on the other hand, have failed to demonstrate differences in innate immune functions of CF neutrophils compared to normal neutrophils (*52*). Interestingly, myeloid *CFTR*-inactivated mice have higher mortality after *P. aeruginosa* lung infection compared to controls, suggesting that CFTR function in myeloid cells does contribute to normal lung defenses against infection (*61*). CF neutrophils have a slower rate of apoptosis, compared to

normal neutrophils, which may prevent the timely removal of neutrophils and the resolution of inflammation (*52, 63*). Interestingly, TLR-5 expression is significantly increased on airway neutrophils in CF patients compared to circulating neutrophils in the same patients, or neutrophils from bronchiectasis patients or healthy controls (*64*). This suggests a critical role for TLR-5 in neutrophil-pathogen interactions, in addition to AEC-pathogen interactions, in CF.

Bacterial infections in CF lung disease

Bacteria that are harmless to healthy individuals, or that even form a part of their normal microflora, can be a serious threat to individuals with CF (*38, 39*). The airways of most CF patients are colonized with bacteria starting in infancy or early childhood, and many remain chronically infected for the remainder of their lives (*38*). While mucus plugging of the airways and reduced host defenses clearly create a favorable environment for bacterial growth, it remains unclear why CF patients are highly susceptible to some bacterial infections but not others. Numerous studies have reported on "classical" CF bacterial pathogens, namely *Staphylococcus aureus, Haemophilus influenzae, P. aeruginosa* and *Burkholderia spp.* (*38*). The acquisition of these CF pathogens is partly age-dependent (Fig. 1.3), with *H. influenzae* and *S. aureus* being the most prevalent pathogens found early in CF infants and children. Once patients reach adulthood, *P. aeruginosa* is the most prevalent pathogen and chronically infects up to 80% of CF adults (*10*). In older CF patients, other organisms such as *Burkholderia spp., Stenotrophomonas maltophilia, Achromobacter xylosoxidan* and non-tuberculosis mycobacteria species are increasingly prevalent (*38*).

Substantial clinical data have linked the recognition of bacterial infection in the lung, with the onset of symptomatic lung disease, which is marked by excessive airway inflammation and the eventual loss of pulmonary function (*38*). With the exception of *H. influenzae*, whose role in CF lung disease remains unclear, all the above-mentioned "classical" CF pathogens have been associated with increased pulmonary risk in CF patients (*38, 65*). Several clinical studies

have demonstrated that chronic colonization of the airways by *P. aeruginosa* has a negative impact on the prognosis and outcome of CF patients. For example, CF patients infected with *P. aeruginosa* have increased morbidity, greater lung function decline, increased hospital stay and frequency of exacerbations compared to CF patients without *P. aeruginosa* infections (*66-71*). The *P. aeruginosa* pulmonary bacterial burden is also negatively correlated with measures of pulmonary function (FEV1, FVC) (*72, 73*). *P. aeruginosa* infections are associated with lower survival and a significant predictor of mortality in CF patients (*69, 74*). Finally, antibiotics that have anti-pseudomonas activity (such as tobramycin, colistin, ciprofloxacin, meropenem) improve clinical symptoms and lung function in CF patients (*75-78*).

Chronic infection of CF airways with *Burkholderia spp.* is associated with worse pulmonary status and higher mortality than any other single pathogen, including *P. aeruginosa* (*38, 79-81*). Certain *Burkholderia spp.* cause a rapidly fatal acute pneumonia in CF patients, known as “fulminant cepacia syndrome”, that can be transmitted patient-to-patient (*82*). Early lung infections with *S. aureus,* and methicillin-resistant *S. aureus* (MRSA) in CF children are associated with lung function deterioration, poorer nutrition parameters and increased inflammation (*83-86*). On the other hand, the clinical significance of the opportunistic pathogens *S. maltophilia* and *A. xylosoxidans* is still only emerging.

The vast literature on CF microbiology has been mainly focused on the primary CF pathogens described above, largely owing to the ability of conventional laboratory culture-based methods to detect them. In the last ten years, the advent of microbiome studies using 16S rRNA sequencing have allowed scientists to probe the CF respiratory bacterial communities using molecular rather than culture-based methods. These studies have highlighted that the CF respiratory tract contain a complex microbial community comprising multiple distinct bacterial phylotypes, in addition to fungi and viruses (*87-89*). They have confirmed the presence of well-established pathogens like *P.*

aeruginosa but have also identified previously unrecognized bacterial species, including members of the *Streptococcus milleri* group and anaerobes such as *Prevotella oris, Fusobacterium gonidoformans*, and *Bacteroides fragilis* (*39, 65, 90*). Whether bacterial species such as *Streptococcus milleri* group or respiratory anaerobes are bona fide pathogens or indirectly contribute to CF lung disease and exacerbations remains an unresolved and active area of research (*65*) While these exciting studies reveal new perspectives on the CF airway polymicrobial communities, we are still years away from understanding how these dynamic and complex polymicrobial communities contribute to the pathogenesis and progression of CF lung disease.

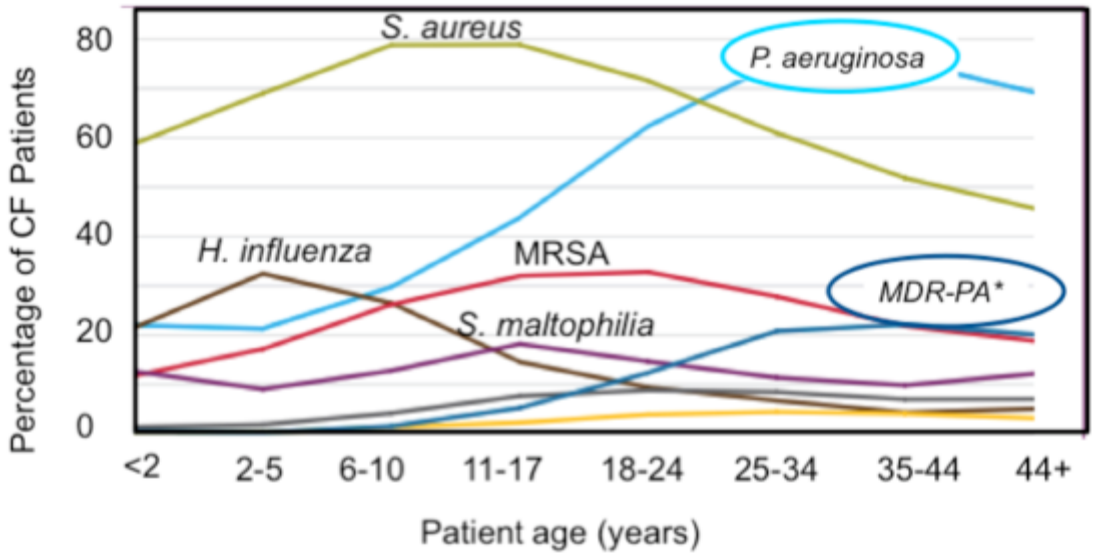

Fig. 1.3: Age-specific frequency of bacterial infections in CF patients

Adapted from (*91*).*Multi-drug resistant *P. aeruginosa (MDR-PA)*

Inflammation in CF lung disease

Although neutrophils are innate immune cells essential for host defenses against bacterial infections, the CF lung is characterized by a vigorous neutrophilic inflammatory response that results in tissue destruction and contributes to the pathophysiology of CF lung disease. Pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor (TNF)-α, are increased in the sputum and bronchoalveolar lavage fluid from patients with

CF, while the anti-inflammatory cytokine IL-10 is reduced (*92-95*). Circulating levels of TNF-α, IL-1 and IL-8 can also be increased in CF patients compared to healthy controls (*96, 97*). Increased sputum levels of IL-1β, IL-6, IL-8, TNF-α are significantly correlated with reduced lung function, further supporting the notion that excessive inflammation is an important contributor to the progression of CF lung disease (*35, 98*).

Whether the inflammation begins strictly after bacterial colonization, or is pre-existing is still debated. There are two scenarios that may explain the excessive inflammation occurring in the CF airway (*99*). The first theory is that the inflammation is disproportionate to the infection, with CF AEC and phagocytes responding too vigorously to bacteria present in the airways. The second theory maintains that inflammation in CF airways is inherent and independent of infection, but may be worsened by infection. The first theory is supported by many *in vitro* studies showing that immortalized and primary CF AEC produce increased amounts of IL-6 and IL-8 in response to bacterial antigens compared to non-CF AEC, but baseline levels of cytokines do not differ significantly between the two groups (*100-102*). Evidence suggests that CF macrophages and neutrophils contribute directly to the exaggerated cytokine response to bacterial ligands by producing increased levels of IL-1α, IL-1β, IL-6, IL-10, granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF) and monocyte-chemoattractant protein (MCP-1) (*103-106*). The second theory is also supported by several *in vitro* studies that demonstrate that different CF cells models have constitutively high nuclear factor of kappa B (NF-κB) and extracellular regulated kinase mitogen activated protein kinase (ERK MAPK) activities (*52, 107, 108*). Both NF-κB and ERK MAPK are important signaling molecules that control the production of pro-inflammatory cytokines (*109*). Studies have also noted reduced levels of the anti-inflammatory cytokine IL-10 produced by CF AEC, which would prevent the dampening of inflammation (*110*). Most compelling are clinical studies of CF-newborns and CF-fetuses that demonstrate that pro-inflammatory priming in the

CF airways precedes bacterial infection (*39*).

In addition to cytokines, eicosanoid lipid mediators such as prostaglandins, leukotrienes and lipoxins derived from arachidonic acid, also play important roles in determining the balance between pro- and anti- inflammatory responses (*111*). Several studies suggest that eicosanoids are dysregulated in the CF airways, with CF sputum showing increased levels of leukotriene B4 (LTB4), a potent pro-inflammatory neutrophil chemoattractant and activator, and reduced levels of the anti-inflammatory lipid mediator lipoxin A4 compared to controls (*112-114*). Interestingly, a clinical trial was carried out to test the efficacy of a LTB4 receptor agonist, BILL 284 BS, as an anti-inflammatory drug for use in CF patients with chronic lung infections (*115*). However, the trial was terminated early due to severe adverse pulmonary events caused by acute bacterial exacerbations (*115*). This demonstrates that pro-inflammatory eicosanoids like LTB4 are critical for controlling chronic infections in CF patients. Finally, the prostaglandin-endoperoxide genes *COX1* and *COX2* involved in prostaglandins biosynthesis may be modifier genes of CF disease severity (*116*).

Increased pro-inflammatory cytokine, chemokine and eicosanoid levels in CF airways undoubtedly contribute to the excessive recruitment and activation of neutrophils associated with CF airway infections. In turn, activated neutrophils produce many antimicrobial substances, such as secreted proteases, myeloperoxidase (MPO), extracellular DNA and reactive oxygen species (ROS) that can also cause collateral host tissue damage (*117*). Sputum neutrophil elastase (NE) and MPO concentrations negatively correlate with lung function and are closely associated with CF lung disease (*118, 119*). *In vitro* and *in vivo* studies have shown that NE can degrade host tissues, impair mucociliary clearance, increase airway secretions and antagonize opsonin-mediated phagocytosis (*120, 121*). MPO, an enzyme that generates hypohalous acids, can cause oxidant-mediated damage to host tissues when released extracellularly (*122*). Such neutrophil-derived products may directly contribute to lung damage CF patients.

Current treatments for CF lung disease

The management of CF lung disease includes chest physiotherapy and inhaled medications (such as mucolytics or hypertonic saline) that improve mucus clearance. Antibiotics play a major role in preventing, eradicating or controlling bacterial infections, and are used in inhaled or systemic forms. Chronic antibiotic therapies with inhaled tobramycin or azithromycin are commonly used to improve symptoms and lung function. Antibiotics, such as beta-lactams, aminoglycosides and quinolones, are also used to treat acute pulmonary exacerbations (*123*). Unfortunately, the effectiveness of antibiotic therapy is increasingly undermined by the emergence of antibiotic-resistant organisms, such as MRSA or multidrug resistant *P. aeruginosa*. There has been long standing and increasing interest in using anti- inflammatory drugs to directly target the inflammatory process in CF lung disease. Anti-inflammatory therapies currently used in CF patients include corticosteroids and non-steroidal anti-inflammatory drugs (e.g. ibuprofen) but both classes cause significant side-effects (*123, 124*). CF lung disease can be associated with airway hyper-responsiveness that respond to bronchodilators (*125*). Most recently, the development of CFTR modulators that restore CFTR protein activity to correct the initial CFTR defect, rather than mitigating the effects downstream of CFTR dysfunction, has raised great hopes. To date, ivacaftor (a CFTR potentiator) and ivacaftor/lumacaftor (CFTR corrector/potentiator combination) are the only FDA-approved CFTR modulators. Finally, lung transplantation is often the only remaining option for CF patients with end stage lung disease.

1.3. Pseudomonas aeruginosa in chronic CF lung disease

Pseudomonas aeruginosa: a ubiquitous bacteria and successful opportunistic pathogen

P. aeruginosa is a Gram-negative rod of the Gammaproteobacteria class which includes several other medically-important bacteria such as *Escherichia coli, Salmonella* spp., *Vibrio cholera* and *Yersinia pestis* (*126*). This ubiquitous bacterium is found in soil, aquatic and man-made environments (*127*). *P. aeruginosa* is an opportunistic pathogen in diverse hosts including mammals, insects, plants, amoebas, and nematodes (*128-134*). Its environmental adaptability is attributed to its unusually large genome (6.3Mb), which encodes a versatile set of metabolic genes, in addition to a plethora of genes involved in transcriptional regulation, virulence and environmental stress responses (*134, 135*). *P. aeruginosa* rarely causes disease in healthy individuals, but can cause severe life-threatening acute infections including bacteremia, pneumonia, and bone and joint infections in compromised hosts (*136*). It is the most common cause of fatal hospital-acquired infections, such as acute pneumonia, in critically ill patients and those on mechanical ventilation (*127, 137, 138*).

P. aeruginosa in CF lung disease

In contrast to the acute and rapidly fatal nosocomial pneumonia, *P. aeruginosa* lung infections occurring in CF patients are chronic, slowly progressive and not invasive. The natural history of *P. aeruginosa* infections in CF patients is typically as follows (Fig. 1.4). CF infants or young children are first infected at a very young age, with *P. aeruginosa* detected in the lower respiratory tract as early as 6 months of age. Infections remain intermittent for a variable period of time, during which re-infection may be caused by one or more *P. aeruginosa* strain (*139*). Over time, intermittent *P. aeruginosa* infections transition into chronic infections defined by the repeated recovery of *P. aeruginosa* in airways secretions and the development of *P. aeruginosa*-specific antibodies

(*140*). By adulthood, the majority of CF patients (up to 80%) are chronically infected by *P. aeruginosa.* Chronic *P. aeruginosa* infections are associated with greater pulmonary inflammation and neutrophilia and account for the majority of morbidity and mortality in CF patients (*141-143*).

Once chronic, *P. aeruginosa* airway infections are virtually impossible to eradicate. Within the CF airway mucus, *P. aeruginosa* grows as biofilms, which are multicellular bacterial aggregates encased in a self-produced extracellular polymeric substance (*144, 145*). The switch to biofilm growth in the CF airways renders the organism significantly more resistant to antibiotics and immune killing and is thus a growth strategy that promotes the persistence of chronic infections despite antibiotic therapy (*146*). Chronic *P. aeruginosa* infections in the CF lung contribute to CF lung disease directly, by damaging of host tissues, and indirectly, by eliciting a vigorous and equally damaging inflammatory response (*38*).

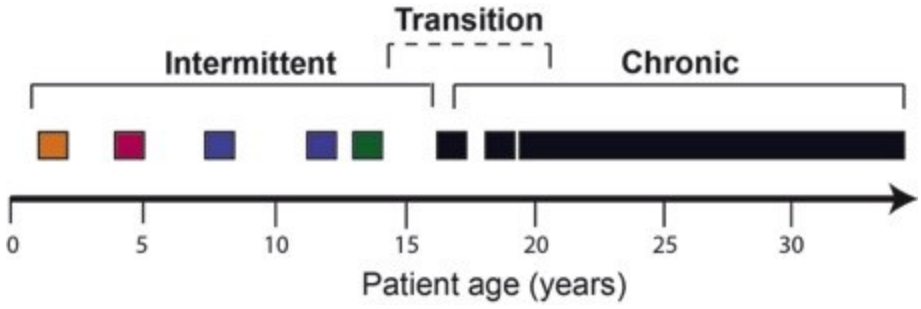

Fig. 1.4: Typical progression of *P. aeruginosa* airway infections in a CF host

Colored boxes represent phylogenetically independent clones. Intermittent infections can be eradicated, and patients may remain negative for *P. aeruginosa* for years, until the establishment of a chronic infection (black box). Adapted from (*143*).

Innate immune responses to *P. aeruginosa* in the airways

Given the long-term and intimate contact of *P. aeruginosa* with CF lungs during chronic infections, there are many opportunities for this pathogen to be sensed by the host and to trigger innate immune responses. *P. aeruginosa*

produces many PAMPs that are recognized by TLRs, nucleotide-binding oligomerization domain family (NOD)-like receptors and other PRRs found on the cell surface, in endosomal compartments, and within the cytoplasm of AEC and immune cells (*147*). Some well characterized host PRR- *P. aeruginosa* PAMP interactions are summarized in Table 1.1. Interactions between *P. aeruginosa* PAMPs and host PRRs are critical for mediating appropriate immune responses leading to elimination of this pathogen.

Table 1.1: Pathogen recognition receptor (PRR) sensing of *P. aeruginosa* pathogen associated-molecular patterns (PAMPs)

PRR	PAMP	References
TLR-2	Mannuronic acid (component of alginate) C-terminal domain of ExoS LPS Flagellin Slime-GLP Lipoproteins	(*148*) (*149*) (*150*) (*151*) (*152*) (*153*)
LBP-CD14-MD2-TLR-4	LPS	
TLR-4	N-terminal domain of ExoS Mannuronic acid (component of alginate)	(*149*) (*148*)
TLR-5	Flagellin	(*154*)
TLR-9	Unmethylated CpG DNA	(*155*)
Nod1	Peptidoglycan	(*156*)
NLRC4/Ipaf	Flagellin T3SS apparatus	(*157*) (*157*)

Recognition of *P. aeruginosa* by TLRs is especially important for initiating innate immune responses against this pathogen. The involvement of TLR-9 in responses to *P. aeruginosa* during lung infection has not been well characterized, but TLR-2, TLR-4 and TLR-5 have been shown to be very important. TLR2, TLR4 and TLR5 are somewhat redundant in response to *P. aeruginosa* lung infections, since the loss of any one of these TLRs alone does not compromise effective bacterial control. Loss of signaling through all three TLRs, however, significantly blunts TNF-α, IL-6, G-CSF and KC responses to *P. aeruginosa* and neutrophil

recruitment in murine models of acute pneumonia (*158, 159*). Both LPS-TLR4 and flagellin-TLR5 interactions likely contribute the most to host defenses against acute *P. aeruginosa* pneumonia since absence of both TLR-4 and -5 signaling also results in hypersusceptibility to this pathogen (*160*).

The protective role of TLR signaling is further supported by studies showing that *MYD88*$^{-}$*/MYD88*$^{-}$ mice, that lack the adaptor molecule common to most TLRs, do not mount early IL-6, MIP-1β, KC, MIP-2, GM-CSF and MCP-1 cytokine responses to *P. aeruginosa* lung infection (*158*). As a result, these mice fail to control bacterial replication resulting in necrotizing pneumonia and death (*158*). Restoration of MYD88 signaling in AEC alone is sufficient to restore protective innate immune responses to *P. aeruginosa*, demonstrating that AEC-mediated TLR signaling is critical for innate immune responses during acute *P. aeruginosa* lung infections. *In vitro,* stimulation of murine epithelial cells with *P. aeruginosa* flagellin or LPS lead to the production of KC and IL-6, and loss of MYD88 function in these cells significantly reduced levels of both cytokines (*161*). Human AEC have been shown to respond to *P. aeruginosa* flagellin by producing IL-8 and IL-6 (*162*).

The first immune cells likely to encounter *P. aeruginosa* in the lungs are resident alveolar macrophages. Macrophages can internalize and kill bacterial pathogens; however, their role in pathogen sensing is also of primary importance during *P. aeruginosa* infections as they express most TLRs and NOD-like receptors (*52*). *In vitro*, *P. aeruginosa* LPS and flagellin activate TLR-4 and TLR-5 signaling in murine alveolar macrophages leading to the secretion of KC, TNF-α and IL-6 (*161*). Both flagellin and the type 3 secretion system (T3SS) apparatus of *P. aeruginosa* are sensed by the NOD-like receptor NLRC4/Ipaf, leading to the activation of caspase-1 and production of IL-1β in macrophages (*163*). Despite these observations, the importance of macrophages for effective immune responses to *P. aeruginosa* remains unclear. Depletion of macrophages using clodronate liposome in rat and mouse models of acute *P. aeruginosa* pneumonia

significantly attenuated KC and MIP-2 cytokine responses, immune cell recruitment and bacterial clearance in some studies (*164, 165*), but not in others (*166*).

Neutrophils clearly play a critical role in the defense against acute *P. aeruginosa* infections. For example, neutropenia is a major risk factor for severe *P. aeruginosa* infections in clinical settings (*167*). *P. aeruginosa* infections elicit a significant neutrophilic response, and complete resolution of acute infections relies critically on this response. In order to recruit neutrophils to the lungs during infections, AEC and alveolar macrophages must generate a neutrophil chemokine response. Among host-produced neutrophil chemoattractants, IL-8 (CXCL8) is the most important in humans (*168, 169*). In mice, KC (CXCL1), MIP-2 (CXCL2), LIX (CXCL5) and lungkine (CXCL15) can all contribute to neutrophil recruitment (*166, 168, 170, 171*). Additionally, LTB4 and complement-derived mediators such as C5a can also recruit neutrophils (*168*). Once recruited and activated, neutrophils generate and release antimicrobial compounds including ROS, NE, MPO, defensins, lactoferrin and lysozyme (*172*). Many of these microbicidal components are released in a complex extracellular structure, along with neutrophil chromatin, known as a neutrophil extracellular trap (NET). Neutrophils can also perform both opsonic (CR3/CD11b) and non-opsonic phagocytic uptake of *P. aeruginosa*, leading to bacterial killing within the phago-lysosome (*173*).

Genetic diversification and adaptation of *P. aeruginosa* in the CF airway

The interactions between *P. aeruginosa* and the CF host during chronic airway infections are dynamic and evolve over time. While *P. aeruginosa* clearly elicits host responses, the host also shapes the bacteria. During decades-long persistence in the CF lung, *P. aeruginosa* adapts to its habitat, the heterogeneous and fluctuating CF lung environment. For example, lung microenvironments likely contain fluctuating levels of oxygen and nutrients, varying concentrations of antibiotics, host inflammatory responses, and

heterogeneous microbial communities (*143*). All of these conditions represent environmental stressors with strong selective pressures that can shape the subsequent evolution of *P. aeruginosa* within the host. Many studies have documented the extensive genetic diversification, adaptation and microevolution of *P. aeruginosa* during the course of chronic infection in the CF lung (*143, 174-177*).

Mutations are the key source of genetic variability fueling the evolution of *P. aeruginosa* in the CF lung. The genetic mutation rate is a major factor that determines the adaptive capacity of *P. aeruginosa* in this environment, and the CF lung environment may increase the mutation rate and accelerate the generation of *de novo* spontaneous mutations through several mechanisms. Elevated levels of ROS and sub-lethal concentrations of antibiotics in the CF airway are mutagenic to *P. aeruginosa* (*178, 179*). *P. aeruginosa* variants with a hypermutable phenotype (mutator strains), that have spontaneous mutation rates up to 1000-fold higher than wild-type strains, may also be selected for (*180, 181*). Finally, biofilm growth also enhances oxidative damage mediated mutagenesis and the emergence of genetic variants (*181*).

Chronic infection of the CF airways by *P. aeruginosa* likely starts with one "founding clone" that is acquired from environmental reservoirs (*139, 182*). The accumulation of mutations over successive generations leads to new clonal lineages with phenotypic characteristics that differ from the founding strain. Several models have been proposed to explain the genetic adaptation and microevolution of *P. aeruginosa* within the CF lung (*183*). The 'dominant-lineage' model proposes that variants with beneficial mutations are selected for and become dominant in *P. aeruginosa* populations (Fig. 1.5A). The 'diverse-community' model on the other hand proposes that mutations generate multiple adaptive lineages that rise to intermediate frequency and coexist (Fig. 1.5A). Supporting this idea, a growing number of studies report that CF lungs are colonized by coexisting *P. aeruginosa* variants derived from a common ancestral strain or even clonally distinct strains (*139, 184-189*). The "insurance hypothesis"

suggests that genetically diverse populations may be more resilient and better at surviving unpredictable stresses (*190*). The simultaneous presence of multiple genetic variants may thus increase the fitness of *P. aeruginosa* populations in the CF lung environment (*183, 191*).

These two models, however, are not mutually exclusive. Short-term genetic diversification of *P. aeruginosa* populations in the CF lung may generate the variants on which longer-term evolution operates, with the selection of certain adapted lineages. Several lines of evidence support the idea that both genetic diversification and selection operate in *P. aeruginosa* populations within the CF lung. In the first study using whole genome sequencing to examine the genetic adaptation of *P. aeruginosa* during a longitudinal infection, Smith *et al.* reported up to five separate lineages of *P. aeruginosa,* arising from a common ancestor, co-existing in the airways of a young CF patient (*177*). Over time, a single clone became the dominant lineage, although the study's sampling strategy could not exclude the co-existence of other lineages. The increased ratio of non-synonymous to synonymous single nucleotide polymorphisms (SNPs) in this dominant clonal lineage suggested that it was under positive selection in the host (*177, 184*). Since the Smith *et* al study, other genomic studies have been carried out and have confirmed the coexistence of different clonal lineages within single patients, extensive genetic diversity within clonal lineages due to the accumulation of mutations, and positive selection of certain lineages within patients (*184, 192*).

Numerous longitudinal studies of *P. aeruginosa* CF clinical isolates have found evidence of parallel genetic and phenotypic adaptations occurring in different CF patients, suggesting that common selective pressures operate during chronic infections in the CF lung (*177, 184, 192, 193*). Many CF-adapted mutations are loss-of-function mutations, mostly non-synonymous SNPs or insertion/deletions, and many of these occur in global regulators, resulting in large-scale changes in gene expression profile and phenotypes of the mutated strains (*143, 177, 184*). Common CF-adapted phenotypes observed at later

stages of infections include mucoidy (overproduction of alginate), antibiotic resistance, loss of motility, alterations in LPS, reduced production of acute virulence factors (such as proteases, pyocyanin) and adaptation of metabolic pathways (such as changes in the uptake and utilization of different amino acids and carbon sources) and the loss of LasR quorum sensing (*38, 143, 177, 194*). These CF-adapted phenotypes differ significantly from those observed in *P. aeruginosa* isolates from early stage CF infections and the environment (Fig. 1.5B). The appearance of parallel "chronic CF-adapted phenotypes" might indicate that there is a limited number of adaptive peaks in the fitness landscape of adaptation to the CF lung environment (*143*).

Common CF adapted *P. aeruginosa* phenotypes and their association with clinical outcomes in CF patients

CF airways exert strong selective pressures on *P. aeruginosa* and lead to a number of convergent adaptations in this bacterium. Whether the emergence of these phenotypic traits enhances the fitness of *P. aeruginosa* in the CF lung or causes deterioration in the clinical status of the patient is less clear but is an area of active study. Here, we review a few of these *P. aeruginosa* adaptations and discuss their association with clinical outcomes in patients.

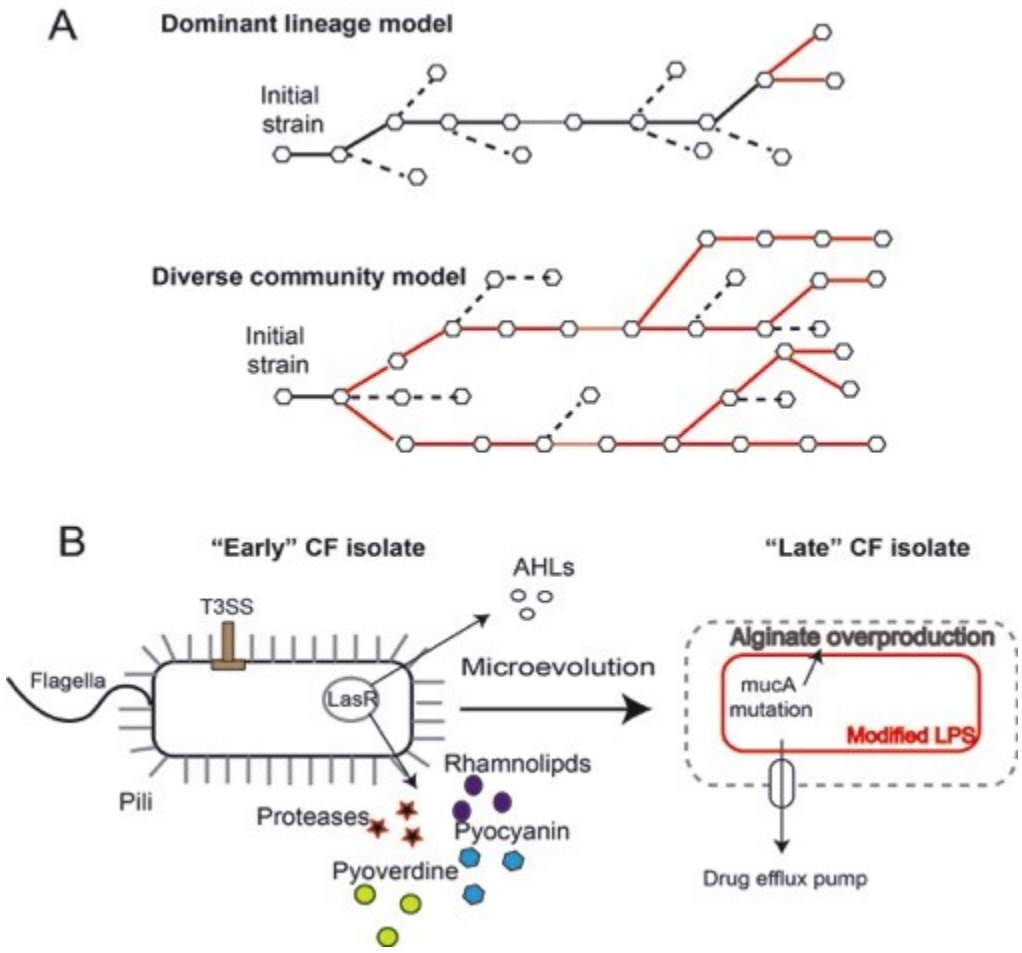

Fig. 1.5: Microevolution and adaptation of *P. aeruginosa* during chronic CF lung infections.

(A) Alternative models of *P. aeruginosa* evolution within the airways of CF patients. Black lines correspond to lineages with beneficial mutations that become fixed in the population. Dashed lines correspond to less fit lineages or lineages that emerge only temporarily. Red lines correspond to clonal lineages with polymorphic mutations. Adapted from (*195*).
(B) Representation of *P. aeruginosa* microevolution within the CF host. At early stages of infection, *P. aeruginosa* is fully equipped with cell-associated virulence factors including a flagellum, type IV pili, type three secretion system (T3SS). Cells are also equipped with LasR-quorum sensing (mediated by acyl homoserine lactone (AHL) signaling) that leads to the secretion of acute virulence factors like proteases, pyoverdine, rhamnolipids and pyocyanin. At late stages of infection, *P. aeruginosa* exhibits host-adapted phenotypes including alginate overproduction (leading to mucoidy), loss of virulence factors, increased expression of efflux pumps (leading to antibiotic resistance), loss of motility and modifications in LPS. Adapted from (*196*).

Mucoidy

The mucoid phenotype is the best-studied adaptation of *P. aeruginosa*. Over half of CF patients chronically infected with *P. aeruginosa* harbor mucoid strains (*38, 197*). Mucoidy is caused by the overproduction of the exopolysaccharide alginate, which gives the bacterial colonies a characteristic slimy appearance (*198*). The switch to mucoidy is most often caused mutations in *mucA*, which encodes an anti-sigma factor that represses alginate biosynthesis (*198*). Mucoidy protects *P. aeruginosa* against phagocytosis, toxic oxygen radicals and antibiotics, and may enhance biofilm formation (*198, 199*). In CF patients, the presence of mucoid *P. aeruginosa* in the lungs is strongly associated with chronic stages of infection, accelerated rates of lung function decline and increased risk of pulmonary exacerbations (*200-202*).

Loss of bacterial motility

CF adapted *P. aeruginosa* strains are frequently immotile with impaired swimming and/or twitching motility. Swimming motility relies on the rotation of a single polar, monotrichous flagellum for motility in a liquid or semi-liquid environment (*203*), while twitching motility is a form of surface motility mediated by type IV pili (*204*). The loss of motility occurs through mutations in genes encoding structural components of the motility appendages (ex. *fliC, pilA*) or their regulators (ex. *rpoN, vfr*) (*38*). The expression of genes involved in swimming motility is also down-regulated during biofilm growth, in mucoid strains and in the presence of NE (*205, 206*). Interestingly, in a large longitudinal study of 649 CF children, reduced twitching and swimming motility were significantly associated with pulmonary exacerbations (*202*). Additionally, the loss of flagellin production, which occurs in some immotile CF-adapted strains, may have important implications on TLR-5 mediated signalling and inflammatory responses in the CF lungs (*53*).

Inactivation of LasR *quorum sensing*

Quorum sensing is a cell-cell communication system employed by bacteria to regulate their gene expression in a cell density dependent manner. *P. aeruginosa* has three main quorum sensing systems: the Las and Rhl systems, which use acyl-homoserine lactone molecules as their signal, and the PQS system which uses 4-hydroxy-2 alkyl-quinolones as the signal (*207*). The Las quorum sensing system is composed of LasI which synthesizes the signal N3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL) and LasR, its cognate transcriptional regulator that binds 3-oxo-C12-HSL and regulates gene transcription. LasR-mediated quorum sensing directly and indirectly regulates the expression of hundreds genes in *P. aeruginosa*, including several secreted acute virulence factors such as proteases, cyanide, haemolysin, pyoverdine, rhamnolipids and pyocyanin (*208-211*).

Spontaneous *lasR* mutants emerge readily, both *in vitro* and in the lungs of CF patients (*177, 192, 212, 213*). Why *lasR* mutants emerge at such high frequencies *in vitro* and *in vivo* remains unclear, but it may be the result of fitness advantages incurred by the loss of LasR quorum sensing under certain conditions. Loss of *lasR* function confers a growth advantage in the presence of the amino acids phenylalanine, isoleucine and tyrosine, and confers enhanced ability to use diverse carbon, nitrogen, phosphorus and sulphur compounds (*214*). *lasR* mutants may also have a survival advantage during alkaline stress (*215*). In mixed *P. aeruginosa* populations, *lasR* mutants can act as social cheaters and benefit from LasR-regulated secreted products without having to produce them (*216*).

Some studies have examined the prevalence of *lasR* mutants in upper and lower respiratory samples by looking for the characteristic iridescent sheen often associated with *lasR* mutant colonies on agar (*217, 218*). Through these indirect observations, *lasR* mutants likely represent up to 30 to 65% of *P. aeruginosa* isolates in chronic infections, a prevalence comparable to mucoidy. *lasR* mutants

have also been commonly observed in genomic studies of *P. aeruginosa* diversity in CF lungs using sequencing methods (*177, 184, 192*). Interestingly, *P. aeruginosa lasR* mutants can also emerge in non-CF patients, such as mechanically-ventilated patients (*219*). The clinical significance of *lasR* mutants in chronic CF lung disease remains largely unknown. *lasR* mutants are attenuated in invertebrate and murine models of acute infection, which suggests their emergence in the CF lung would be beneficial to the host (*220-224*). However, a single cross-sectional study by Hoffman *et al* reported that CF patients with *lasR* mutant *P. aeruginosa* isolates have lower age-specific lung function (FEV1) compared to those without *lasR* mutants isolated from their sputum, similar to the effect of mucoidy (*218*).

Loss of protease activity

P. aeruginosa produces several secreted enzymes that have diverse proteolytic activities. Of the proteases characterized, alkaline protease (AprA), elastase A (LasA), elastase B (LasB) and protease IV (PrpL) have been studied extensively (*225*). AprA is a 50 kDa zinc metallo-protease that is most active under alkaline conditions *(225).* LasA is a 27 kDa zinc metallopeptidase also known as staphylolysin due to its ability to lyse staphylococci (*225*). LasB (also named pseudolysin), a 33kDa zinc metalloprotease, is the most abundant secreted protease produced by *P. aeruginosa* and is distinguished by its ability to degrade elastin (*225*). LasB also possesses potent proteolytic activity against other substrates and largely accounts for most of the secreted proteolytic activity by *P. aeruginosa*; for example, LasB's activity on casein or azocasein is five- to ten- fold higher than that of other *Pseudomonas* endopeptidases (*225*). Finally, Protease IV is a serine protease with a molecular mass of approximately 26 kDa that has strict specificity for cleaving lysine residues (*225*). During infection, these secreted proteases degrade a wide array of host proteins, both independently and synergistically, leading to the impairment of host defenses and the destruction of host tissues (*225*).

Protease-deficient isolates can be isolated from ~30-40% of newly or intermittently infected CF airways, and strikingly, from up to 65% of chronic *P. aeruginosa* infections in CF patients (*226*). While many of the CF-adapted protease-deficient strains are *lasR* mutants (*177, 217, 218*), loss of secreted protease activity in clinical isolates of *P. aeruginosa* can occur through other mutations (*227*). Total secreted protease activity was shown to be a good marker for distinguishing infection stages, with significantly more chronically-infected CF patients having protease-deficient isolate compared to intermittently-infected patients (*226*).

CF adapted *P. aeruginosa* and the pathogenesis of chronic infections

Several CF-adapted phenotypes are highly prevalent in chronic *P. aeruginosa* infections. Why certain *P. aeruginosa* phenotypic variants emerge and are selected for in CF lungs, and how they impact host-pathogen interactions and pathogenesis during CF lung infections, remains poorly understood. The loss of motility and expression of acute virulence factors may be a strategy for the bacteria to subvert host immune responses (*203, 228*). However, this raises a striking paradox as chronic CF lung disease is associated with a vigorous immune response while CF-adapted *P. aeruginosa* phenotypic variants appear attenuated. Furthermore, these variants are often associated with worst, not better, lung disease in CF patients. While their association with the later stages of CF lung disease is well established, it is not clear whether these phenotypic variants can contribute to disease progression and, if so, through what mechanisms.

Murine models of *P. aeruginosa* chronic airway infection

A major challenge in the study of CF lung disease has been the lack of good animal models. Shortly after the discovery of the CFTR gene, mouse models of CF were created including CFTR null mice and ΔF508 homozygous mice (*229*). CF mouse models exhibit key phenotypes present in clinical CF GI disease, but not those of clinical CF pulmonary disease (*229*). CF mice do not develop obstructive lung disease nor spontaneous bacterial lung infections,

making them poor surrogates for the study of CF lung disease (*229*).

Some progress was made with the development of a transgenic mouse with airway specific overexpression of ENaC leading to increased airway epithelial Na^+ reabsorption (*41*). This mouse model exhibits several key features of CF lung disease including ASL volume depletion, increased mucus production and defective mucociliary transport leading to mucus obstruction, goblet cell metaplasia, neutrophilic inflammation and poor bacterial clearance. However, the significant anatomical differences in the lower airway between humans and mice make it unlikely that any mouse model will ever accurately reflect the pathology occurring in human CF lung disease (*230*). For instance, the lower airways of humans are composed primarily of ciliated cells, whereas the murine lower airways contain mostly Clara cells. Murine airways also lack submucosal glands, which are present in human airways and have been implicated in CF lung disease progression.

To overcome the limitations of mouse models of CF, other CF animal models have been developed in mammals that share more anatomical and physiological features with humans, notably pigs and ferrets. Both CFTR null pigs and CFTR ΔF508 pigs have been developed (*231, 232*). While CFTR piglets initially demonstrate no signs of lung disease, infection, or inflammation after birth, they develop classic CF lung disease phenotypes including airway inflammation, airway remodeling, mucus accumulation, and infection with multiple bacterial species within the first few months of life (*231*). Much like the CF pigs, CFTR null ferrets also show evidence of lung infections early in life (*230, 232*). In fact, the spontaneous occurrence of severe lung infections in newborn CF ferrets necessitates antibiotic treatment prior to weaning. The disadvantage of these models, other than the cost and the impracticality of large animal husbandry, is the fact that reliable models of *P. aeruginosa* chronic airway infections have not yet been developed in these hosts, and immunological tools are lacking for pigs and ferrets

The inflammatory and immunological events leading to the establishment of chronic CF lung infections with *P. aeruginosa* remain largely undefined, and many research groups have developed animal models that attempt to recreate key features of these processes. The development of chronic *P. aeruginosa* CF lung infection animal models has been complicated by the fact that many laboratory animals effectively clear large burdens of *P. aeruginosa* from their lungs (*233*). To create models where bacterial clearance is impaired leading to chronic infection, *P. aeruginosa* is embedded in an immobilizing agent (e.g. agar or seaweed alginate) (*233*). Cash *et al.* first developed a rat model with *P. aeruginosa* persisting up to 1 month using bacteria embedded in agar beads (*234*). This model was later adapted to different hosts such as mice (including CF mice), guinea pigs, cats and monkeys (*233, 234*). Although the artificial embedding of the bacteria in agar and anatomical differences between human lungs and small laboratory animal lungs are limitations of the agar bead model, the advantage is that it displays several key features of human CF lung infections, namely a sub-acute localized and persistent bacterial infection, bronchial and peribronchial inflammation, and mechanically obstruction of the airways. Additionally, the murine agar bead chronic infection model benefits from widely available experimental tools and reagents to probe the inflammatory and immunologic responses to infection, and different host genetic backgrounds, including knock out and transgenic mice (*233*). Therefore, the *P. aeruginosa* chronic murine agar bead pulmonary infection model is an important research tool for investigating the complex interplay between *P. aeruginosa* and the host during chronic airway infections.

1.4. Thesis hypothesis and objectives

The genetic and phenotypic adaptations of *P. aeruginosa* to the CF airways are well recognized and often associated with later stages of CF lung disease. However, it remains unclear how specific CF-adapted phenotypes alter host responses to affect inflammation or bacterial clearance in the CF lung. In this thesis, we hypothesized that CF-adapted *P. aeruginosa* phenotypes modulate host inflammatory and innate immune responses, leading to excessive neutrophilic inflammation, lung damage and impaired bacterial clearance.

The first objective, presented in chapter 2, was to investigate the effect of CF-adapted *P. aeruginosa lasR* mutants on inflammatory responses both *in vitro,* using various airway epithelial cell culture models, and *in vivo,* using the chronic murine *P. aeruginosa* agar bead pulmonary infection model. We hypothesized that the loss of LasR quorum sensing may increase pro-inflammatory cytokine responses in AEC leading to increased neutrophil recruitment and increase lung tissue damage.

The second objective, presented in chapter 3, was to investigate the role of the bacterial elastase LasB, an important virulence factor regulated by LasR quorum sensing, on inflammation and morbidity during chronic pulmonary infections. We hypothesized that the loss of LasB during chronic infections may worsen inflammation, through the loss of LasB-proteolytic subversion of immune mediators, leading to greater lung injury and morbidity.

Our third and final objective, presented in in chapter 4, was to compare the bacterial persistence and host immune responses to a CF-adapted *P. aeruginosa* isolate and the non-adapted clonally related ancestral strain in the chronic murine *P. aeruginosa* agar bead pulmonary infection model. We hypothesized that the phenotypic changes occurring in the CF-adapted isolate alter host inflammation and bacterial persistence in murine lungs.

Chapter 2: Cystic fibrosis-adapted *Pseudomonas aeruginosa* quorum sensing *lasR* mutants cause hyper-inflammatory responses.

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2.1. Abstract

Cystic fibrosis lung disease is characterized by chronic airway infections with the opportunistic pathogen *Pseudomonas aeruginosa* and severe neutrophilic pulmonary inflammation. *P. aeruginosa* undergoes extensive genetic adaptation to the CF lung environment, and adaptive mutations in the quorum sensing regulator gene *lasR* arise very commonly. We sought to define how mutations in *lasR* alter host-pathogen relationships. Here we demonstrate that *lasR* mutants induce exaggerated host inflammatory responses in respiratory epithelial cells, with increased accumulation of pro-inflammatory cytokines and neutrophil recruitment due to the loss of bacterial protease-dependent cytokine degradation. In subacute pulmonary infections, *lasR* mutant infected mice show greater neutrophilic inflammation and immunopathology compared with wild-type infections. Finally, we observed that CF patients infected with *lasR* mutants have increased plasma IL-8, a marker of inflammation. These findings suggest that bacterial adaptive changes may worsen pulmonary inflammation and contribute directly to the pathogenesis and progression of chronic lung disease in CF patients.

2.2. Introduction

Progressive lung disease is the primary cause of symptoms and early death in patients with the genetic disease cystic fibrosis (CF). In the CF airways, chronic bacterial infections are associated with an exuberant neutrophil-dominant inflammatory response that causes lung damage. The majority of CF patients are chronically infected with the opportunistic pathogen *Pseudomonas aeruginosa* for decades. Since host defenses fail to clear bacteria, the ongoing interplay between pathogen and host drive inflammation and the immunopathology associated with CF chronic *P. aeruginosa* infections (*52, 235*).

During its residence within the host, *P. aeruginosa* evolves and genetically adapts to the CF lung environment (*143, 174, 177, 183, 236, 237*). *P. aeruginosa* isolates from chronic infections differ genotypically and phenotypically from those isolated at early stages of infection or from the environment, and commonly display adaptive changes such as conversion to mucoidy or loss of motility. Strikingly, CF-adapted *P. aeruginosa* isolates have reduced expression of acute virulence factors such as pilus, extracellular toxins and enzymes that cause invasive disease (*143, 174, 177, 236, 237*), suggesting that bacterial factors required for acute virulence are not necessary for chronic infections.

Quorum sensing is a bacterial communication system that allows organisms to coordinate the expression of genes implicated in infection pathogenesis and social microbial behavior in a cell-density dependent manner (*238*). The *P. aeruginosa* transcriptional factor LasR is one of the major quorum-sensing regulators and controls the expression of several exoproducts and acute virulence factors (*239*). Interestingly, *lasR* mutants arise from *P. aeruginosa* populations in both in-vitro laboratory conditions (*222, 240*) and in vivo during human infections (*177, 192, 212, 213*). Remarkably, at least a third of chronically-infected CF patients harbor loss-of-function *lasR* mutants (*177, 218, 241*) and these patients are associated with worse lung function (*218*). Given that *lasR*

mutants are highly attenuated in models of acute infections (*221, 236, 242*), this striking paradox raises the possibility that CF-adapted *P. aeruginosa* variants contribute to the progression of CF lung disease through mechanisms distinct from those involved during acute infections. How the adaptive micro-evolution of *P. aeruginosa* modulates host-pathogen relationships and inflammatory responses remains incompletely understood.

In this study, we defined the impact of *P. aeruginosa lasR* mutants on inflammatory responses in vitro, in vivo and in CF patients. We observed that *lasR* mutants induced an exaggerated neutrophil-dominant hyper-inflammatory response, and dissected the mechanism for this pathogen-host interplay. Our findings suggest a mechanism by which CF-adapted *P. aeruginosa lasR* variants amplify the inflammation of CF lung disease, thus potentially accelerating disease progression.

2.3. Results

Loss of function *lasR* mutation attenuates acute virulence in a CF-adapted *P. aeruginosa* "Late" isolate.

Using a pair of *P. aeruginosa* clonally-related longitudinal isolates (*139*), Smith et al previously examined the earliest (Early) isolate recovered from a CF patient at six months of age, and the Late isolate from the same patient at age eight (*177*). Using whole genome sequencing, they demonstrated that the CF-adapted "Late" isolate underwent adaptive genetic evolution during chronic infection. Similar to many CF-adapted *P. aeruginosa* isolates, the Late isolate has several loss-of-function mutations resulting in the loss of pilus-mediated twitching motility (*pilA*), pyocyanin (*phzS*), and pyoverdine (*pvdS*) production compared to the Early isolate (Supp. Fig. S2.1). Notably, the Late isolate also carries a loss-of-function nonsense mutation in the *lasR* gene (1 bp deletion at position 147), in contrast to a wild-type *lasR* gene sequence in the Early isolate (*177*).

***lasR* mutants induce an inflammatory cytokine response in airway epithelial cells (AEC).**

To begin understanding the consequences of lasR mutations on host responses, we first examined the airway inflammatory responses to the Early and Late pair of isolates. In the CF lung, *P. aeruginosa* grows as biofilm-like aggregates embedded within the mucus layer overlying the airway epithelial surface (*145*), and the airway epithelium is critical in producing pro-inflammatory cytokines in response to bacterial stimuli to recruit neutrophils to sites of inflammation (*243-246*). We therefore used a biofilm-AEC co-culture model where *P. aeruginosa* biofilm aggregates grew in a gel matrix (*247*) within synthetic CF sputum medium, a defined culture medium that approximates the nutrient composition of CF sputum (*248*). Human immortalized AEC were co-cultured with live biofilms across a permeable membrane (depicted in Fig. 2.1A), and we measured the major pro-inflammatory cytokines Interleukin-8 (IL-8) and Interleukin-6 (IL-6) secreted in the AEC culture supernatant (*100, 249*). Surprisingly, Late isolate biofilms were more pro-inflammatory than were Early isolate biofilms: after 24 hrs in co-culture with *P. aeruginosa* biofilms, secreted IL-8 and IL-6 levels were respectively 1.7 fold ($p \leq 0.01$) and 4.7 fold ($p \leq 0.001$) higher in AEC stimulated with Late compared to Early isolate biofilms (Fig. 2.1B). To examine the specific contribution of the *lasR* mutation to this effect, we also tested the EΔ*lasR* mutant, an Early isolate harboring a genetically engineered lasR knock-out mutation. AEC co-cultured with EΔ*lasR* biofilms also produced IL-8 levels equivalent to those co-cultured with Late isolate biofilms, and 1.8 fold higher ($p \leq 0.05$) than Early isolate biofilms (Fig. 2.1B). Similarly, levels of IL-6 in AEC co-cultured with EΔ*lasR* biofilms were 2.5 fold ($p \leq 0.05$) higher compared to AEC stimulated with Early isolate biofilms.

Since both Late and EΔ*lasR* isolates induced higher secreted cytokine levels than the Early isolate in the biofilm-AEC co-culture system, we hypothesized that the cytokine responses were dampened by LasR-dependent extracellular bacterial products because LasR controls the production of many

secreted factors. To test this hypothesis, we stimulated AEC with *P. aeruginosa* cell-free filtrates that contained diffusible bacterial products, and again noted that lasR mutants (both Late and EΔ*lasR* mutants) elicited higher secreted IL-8 and IL-6 levels than did the parental Early isolate. As shown in Fig. 2.1C, the cytokine response to Late filtrates was dose-dependent, with IL-8 levels ~3 to 10-fold, and IL-6 levels ~6 to 120-fold higher than equivalent stimulation with Early filtrates. Results with the EΔ*lasR* mutant filtrates were similar to those of the Late isolate (Fig. 2.1D), indicating that the increased cytokine response was predominantly attributable to *lasR* mutations. Control experiments showed that all *P. aeruginosa* isolates grew to similar bacterial densities in planktonic and biofilm cultures (Supp. Fig. S2.2). *P. aeruginosa* cell-free filtrates and live biofilms also caused minimal cytotoxicity (Supp. Fig. S2.3A/B) and loss of viability (Supp. Fig. S2.3C) to AEC.

To validate our initial observations with AEC, we tested two additional airway epithelial culture systems. We used CFBE41o- cells which are a CF AEC cell line homozygous for the ΔF508 mutation in the Cystic Fibrosis Transmembrane conductance Regulator gene (*250*) (Fig. 2.1E), and ex vivo primary human nasal tissues (Fig. 2.1F) that contain morphologically intact primary upper respiratory tract epithelial cells, as well as stromal structures and immune cells. Stimulation with filtrates from *lasR* mutants induced the highest IL-8 and IL-6 levels in both systems.

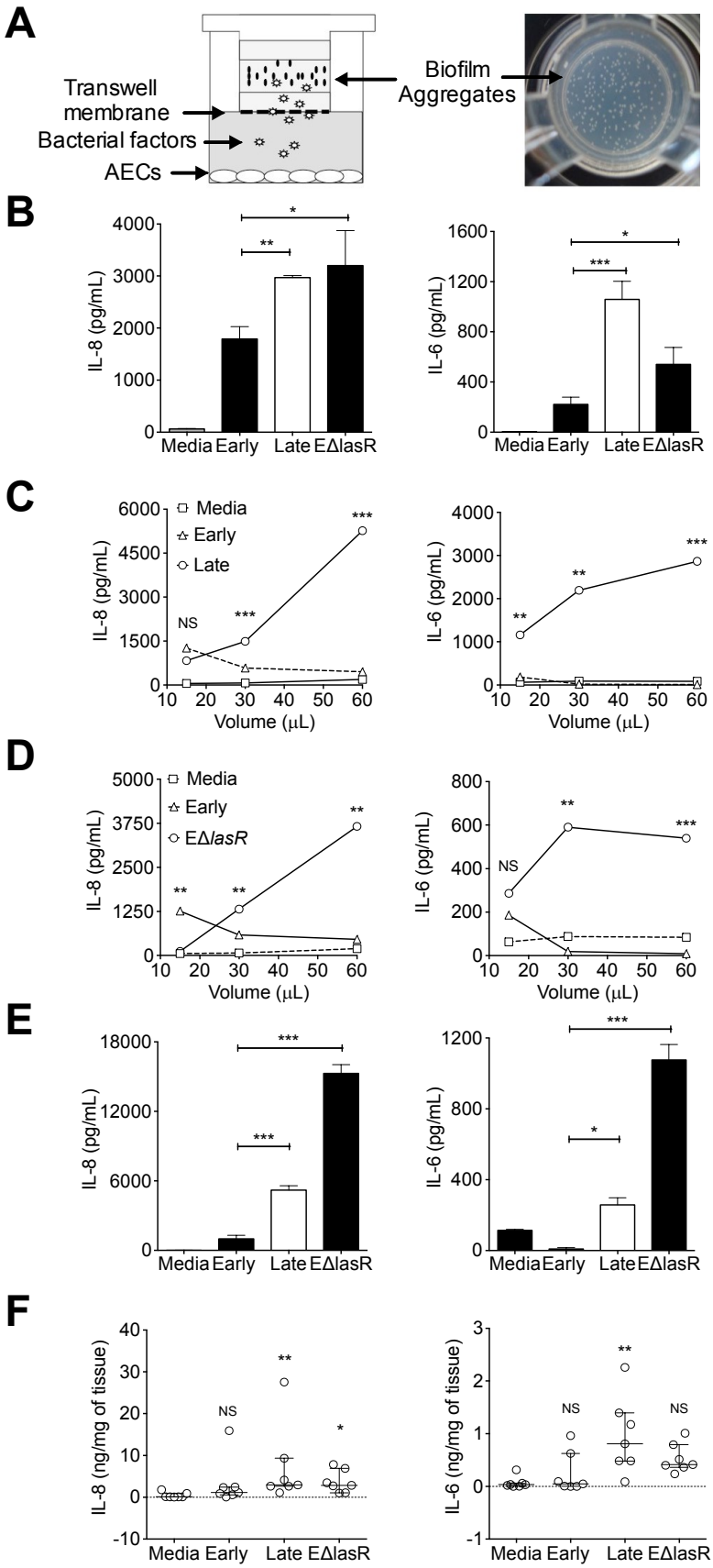

A
Transwell membrane
Bacterial factors
AECs
Biofilm Aggregates
B
IL-8 (pg/mL)
IL-6 (pg/mL)
Media Early Late EΔlasR
C
Media
Early
Late
NS
Volume (μL)
D
EΔlasR
E
F
IL-8 (ng/mg of tissue)
IL-6 (ng/mg of tissue)

Fig. 2.1: *lasR* mutants induce a pro-inflammatory cytokine response in several airway epithelial culture systems

(A) Schematic and photograph (top view) of a biofilm-AEC co-culture system with *P. aeruginosa* biofilm aggregates grown for 48 hrs in SCFM with 0.8% agar in a Transwell permeable support.
(**B)** BEAS-2B cells co-cultured with the Early, Late and EΔ*lasR* biofilm aggregates (or media control) for 18 hrs.
(C-D) BEAS-2B cells stimulated with filtrates from the Early, Late or EΔ*lasR* for 8 hrs at indicated volumes.
(E) CFBE41o- (ΔF508/ΔF508) cells stimulated with 30 µL of Early, Late and EΔ*lasR* filtrates for 18 hrs.
(F) Ex vivo nasal explants stimulated with 60 µL filtrates or media control for 24 hrs.
IL-6 and IL-8 levels were measured in the AEC culture conditioned supernatants after co-culture with biofilm aggregates or stimulation with bacterial filtrates by sandwich ELISA. Results in B to E are shown as mean +/- SEM (n ≥ 3 independent biological replicates, representative of ≥ 2 independent experiments). Statistical comparisons for B to E were done using a two-tailed t-test (vs. Early group). Results in F are shown as median (+/- IQR) of independent biopsies (n=7 patients), and statistical comparison was done using the Kruskal-Wallis test (vs. control group). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Loss of LasR function causes an exaggerated neutrophil-dominant hyper-inflammatory response in vivo.

The results from our in vitro experiments demonstrated that *lasR* mutants caused a hyper-inflammatory IL-8 and IL-6 cytokine response in AEC. We therefore hypothesized that infection with *lasR* mutants will increase pulmonary recruitment of neutrophils and an exaggerated inflammatory response in vivo compared with the Early isolate, which carries a wild-type *lasR* gene. To test this hypothesis, we infected C57BL/6 mice with *P. aeruginosa* embedded in agar beads to create a chronic airway infection model (*251, 252*). *P. aeruginosa*-embedded agar beads approximate endoluminal bacterial aggregates observed in chronic CF airway infections by impairing bacterial clearance and create a subacute and non-lethal pulmonary infection.

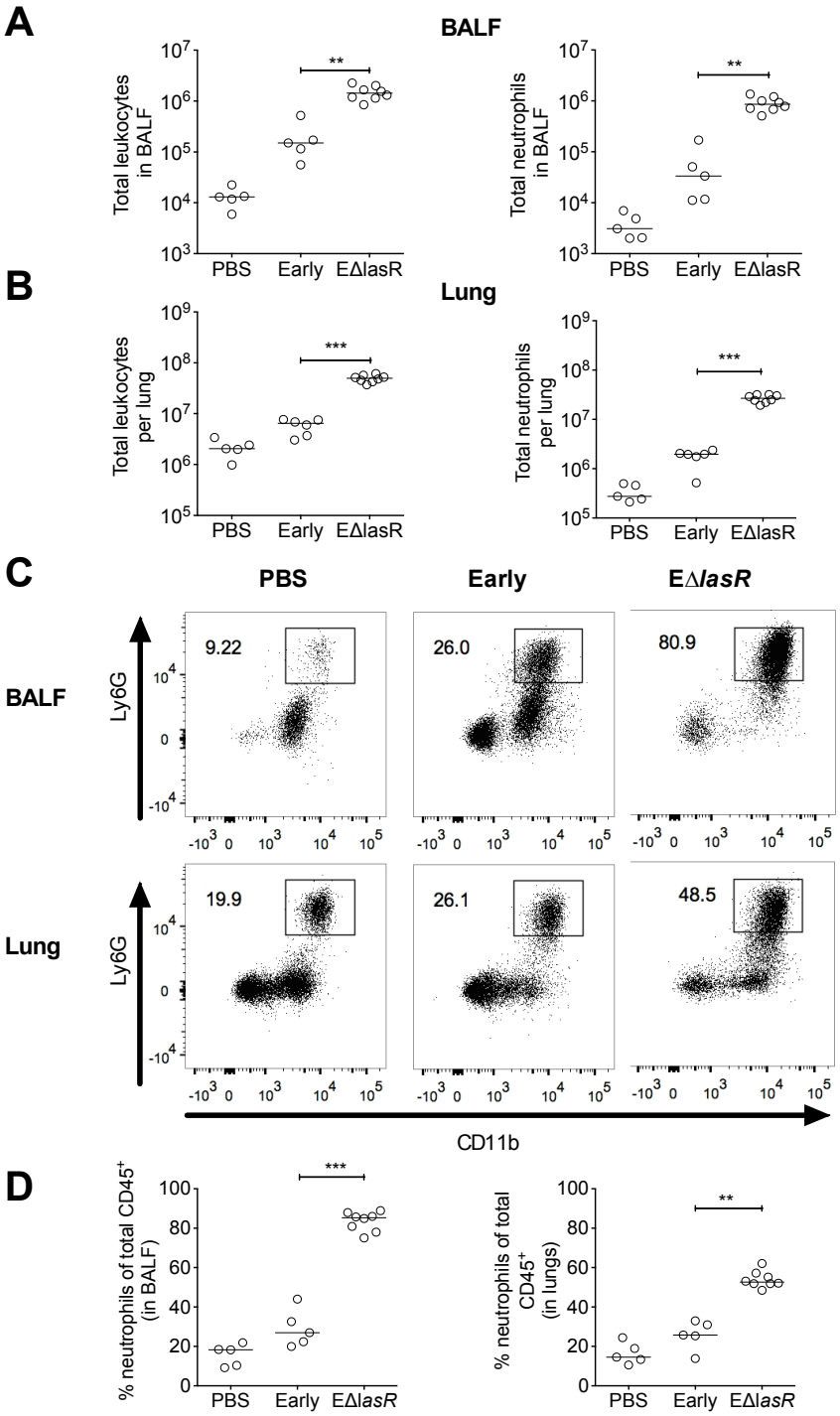


Fig. 2.2: Loss of LasR function causes a neutrophil-dominant hyperinflammatory response in murine *P. aeruginosa* pulmonary infections

(A) Number of leukocytes (CD45+) and neutrophils (CD45+ CD11b+ Ly6Ghi) in BALF.
(B) Number of leukocytes (CD45+) and neutrophils (CD45+ CD11b+ Ly6Ghi) in whole lung homogenates.

(C) Representative FACS scatter plots of neutrophils (CD45+ CD11b+ Ly6Ghi) in the BALF and whole lung homogenates. Insets show the proportion of CD11b+ Ly6Ghi cells of total live CD45+ cells.
(D) Proportion of neutrophils (CD45+ CD11b+ Ly6Ghi) of all leukocytes (CD45+) in BALF and whole lungs.
Mice in the PBS (phosphate buffered saline) control groups are inoculated with sterile PBS agar beads. All results are from samples collected at day 4 p.i. Results in A, B, and D are shown as median (n≥5 mice) of each group. Statistical comparison was done using the Mann Whitney test as indicated. **=$p \leq 0.01$; ***=$p \leq 0.001$

Despite identical bacterial burden at both day 1 and day 4 p.i (Supp. Fig. S4A), mice infected with the EΔ*lasR* mutant showed significantly greater neutrophil-dominated pulmonary inflammation than did those infected with the Early isolate. At day 4 p.i., the bronchoalveolar lavage fluid (BALF) of EΔ*lasR*-infected mice contained 9.5-fold ($p \leq 0.01$) more leukocytes (CD45+), and 25-fold ($p \leq 0.01$) more neutrophils (CD45+ CD11b+ Ly6Ghi) compared to BALF of Early isolate-infected mice (Fig. 2A) ($p \leq 0.01$). Similarly, we observed 8-fold ($p \leq 0.001$) more leukocytes and 14-fold ($p \leq 0.001$) more neutrophils in the whole lung homogenates of EΔ*lasR*-infected mice compared to Early-isolate infected mice (Fig. 2B). Overall, neutrophils accounted for a greater proportion of leukocytes in EΔ*lasR* compared to Early-infected mice (93% vs. 27% in BALF, $p \leq 0.01$; 53% vs. 26% in the lung, $p \leq 0.01$) (Fig. 2C/D).

To further characterize the inflammatory cytokine response in vivo, we measured BALF and plasma cytokine levels at day 4 p.i. Both BALF levels of KC (Keratinocyte-derived Cytokine, a murine functional homolog of the human neutrophil chemokine IL-8) and IL-6 were significantly higher in the EΔ*lasR* infected group, with 8-fold increase for KC ($p \leq 0.001$) and 10.5 fold increase for IL-6 ($p \leq 0.01$) compared to the Early group (Fig. 2.3A), consistent with the vigorous neutrophil recruitment to the lung. Remarkably, blood KC and IL-6 levels also showed similar differences between the Early and EΔ*lasR*-infected groups (Fig. 2.3B).

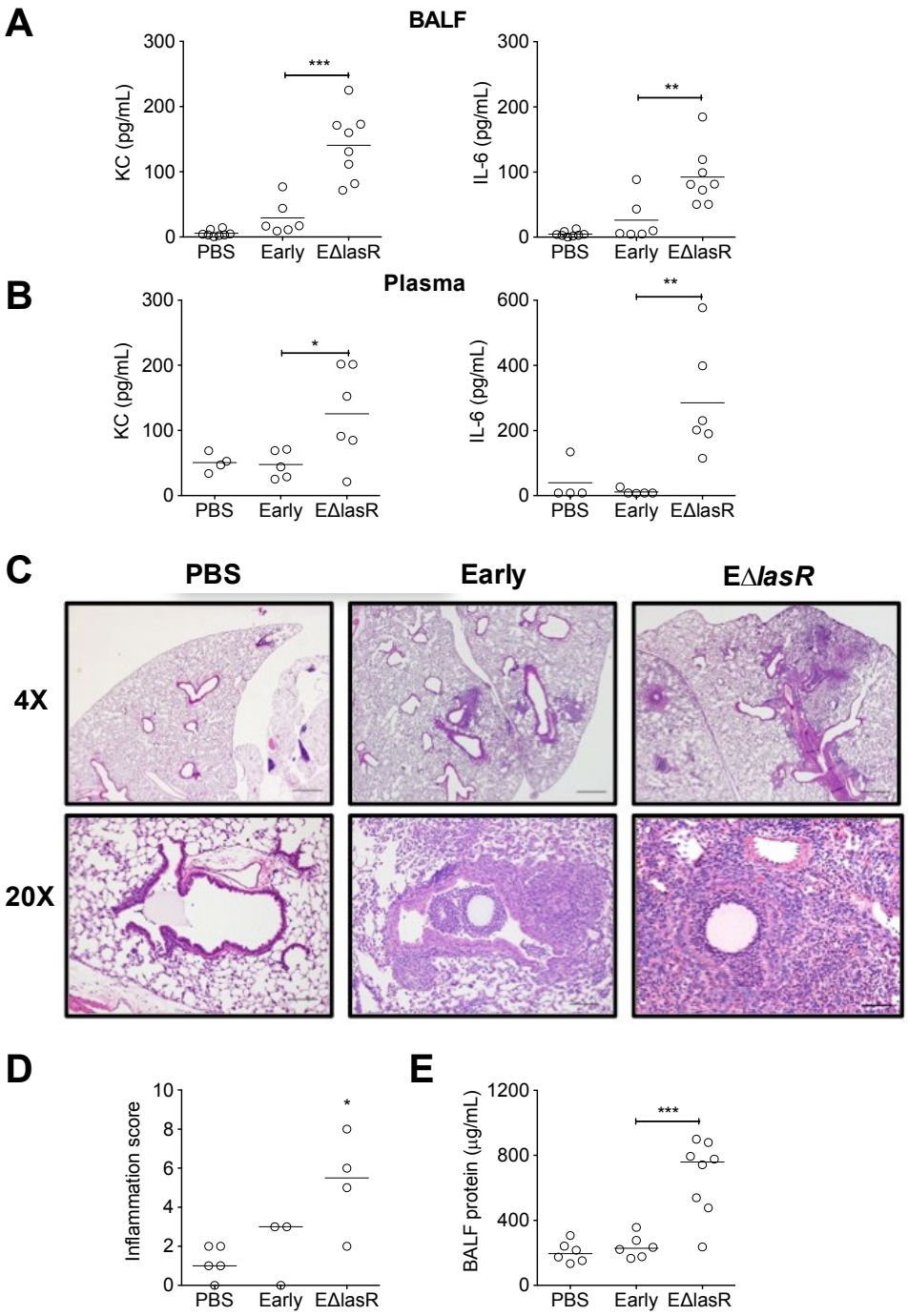


Fig. 2.3: Greater pulmonary inflammation, systemic inflammation and lung injury in EΔ*lasR* infected mice

(A) Cytokine measurement in BALF
(B) Cytokine measurements in plasma obtained from whole blood.
(C) Representative H&E stained lung sections visualized with a 4X (scale bar=500 μm) or 20X (scale bar=100 μm) objective.
(D) Total inflammation score.
(E) Total protein in BALF.

All results are from samples collected at day 4 p.i. Results in A, B, and E are shown as mean (n≥4 mice) of each group and results in D is shown as the median (n≥3 mice). Statistical comparison was done by two-tailed unpaired student's t-test for A, B and E, and by Mann Whitney test for D. *=$p \leq 0.05$;**=$p \leq 0.01$; ***=$p \leq 0.001$.

Histology revealed peribronchial PMN-dominant inflammatory foci associated with endoluminal *P. aeruginosa* embedded beads. We also observed scattered areas of polymorphonuclear leukocyte-dominant parenchymal inflammation (Fig. 2.3C). Lungs infected with the EΔ*lasR* mutant, but not the Early isolate, displayed rare areas of airway necrosis and alveolar fibrin accumulation, suggesting more pronounced localized tissue damage. We scored the airway and parenchymal inflammation in histological sections of lung tissue, and only EΔ*lasR*–infected lungs showed inflammation significantly above the control group (Fig. 2.3D). We also found higher total protein levels in the BALF, a marker of lung tissue damage, in EΔ*lasR*-infected mice compared to Early isolate-infected ones (Fig. 2.3E). Taken together, these results show that the loss of LasR function in *P. aeruginosa* increases neutrophil recruitment and worsen lung immunopathology in vivo.

LasR-regulated proteases directly degrade secreted cytokines.

Cytokine levels in the conditioned supernatants of AEC stimulated with the Early isolate were at times below those in control conditions or nearly undetectable (Fig. 2.1D and 2.1E), leading us to hypothesize that secreted cytokines were degraded by LasR-controlled factors absent in the Late isolate and EΔ*lasR* mutant. To test this hypothesis, we first measured cytokine mRNA expression and secreted cytokine levels simultaneously in AEC stimulated with Early and Late filtrates in a time-course experiment and observed that the differences in secreted cytokines could not be attributed to the IL-8 and IL-6 mRNA response (Supp. Fig. S2.5). Furthermore, heat treatment of Early filtrates restored AEC cytokines to levels greater than the Late filtrate treatment group (Fig. 2.4A). These findings suggested that heat-labile bacterial factors significantly dampened secreted cytokine levels, likely through a post-secretion

degradation process, and heat inactivation of the Early filtrates uncovered the cytokine response to other bacterial factors present in those filtrates.

Since LasR regulates the expression of many extracellular factors, including heat-labile proteases, we next asked whether LasR-controlled proteases directly degraded IL-8 and IL-6. Consistent with our hypothesis, the Late isolate and EΔ*lasR* mutant both had nearly undetectable extracellular total protease and elastase activities compared to the Early isolate, and both activities in the Early isolate were heat labile (Fig. 2.4B and 2.4C). To confirm that LasR-regulated factors directly degraded IL-6 and IL-8, we incubated recombinant human IL-6 (rhIL-6) and IL-8 (rhIL-8) proteins with *P. aeruginosa* filtrates that contained secreted bacterial products. The Early isolate filtrate degraded both cytokines, and this proteolytic degradation activity was abrogated by heat treatment (Fig. 2.4D). In contrast, both rhIL-6 or rhIL-8 protein levels remained intact when incubated with Late or EΔ*lasR* filtrates (Fig. 2.4D).

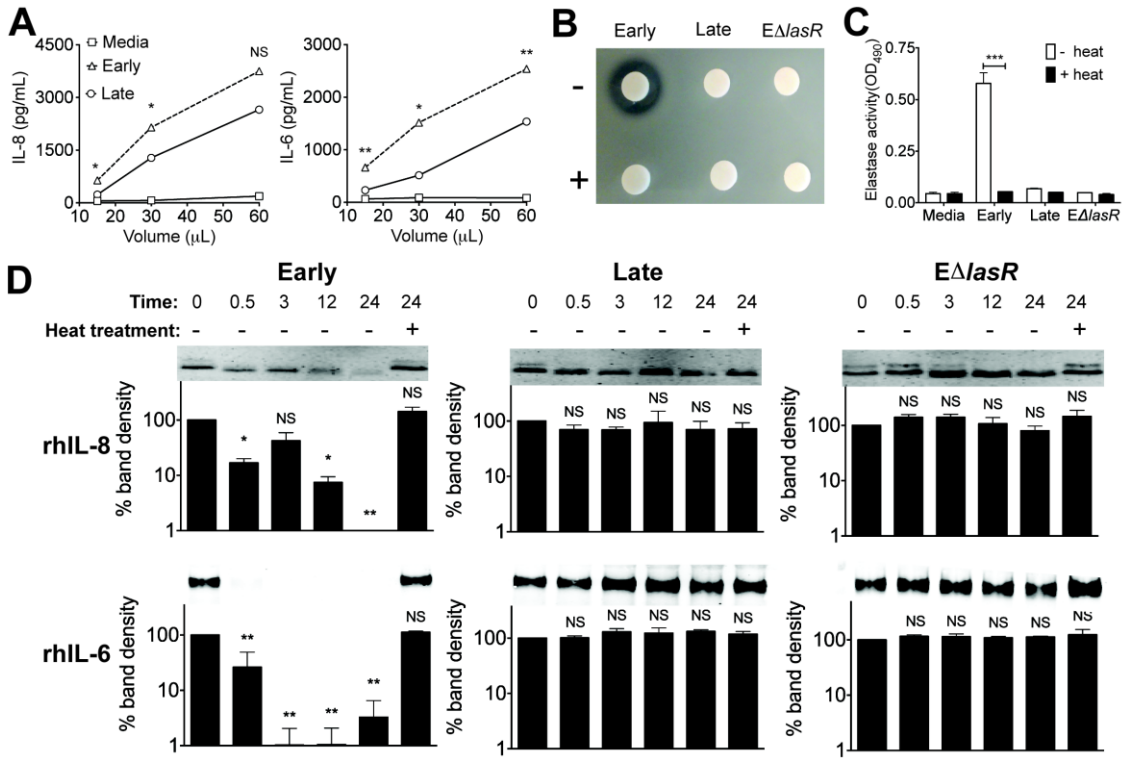


Fig. 2.4: LasR-regulated proteases directly degrade cytokines

(A) BEAS-2B cells stimulated with Early or Late heat-treated filtrates for 8 hrs at the indicated volumes. IL-6 and IL-8 levels were measured in the AEC conditioned supernatant after stimulation by sandwich ELISA.
(B) Total secreted protease activity in filtrates (+/- heat treatment) on skim milk agar plates.
(C) Specific elastolytic activity in filtrates (+/- heat treatment) using the elastin congo red assay.
(D) Recombinant human IL-6 (rhIL-6) or IL8 (rhIL-8) (10 µg/mL) incubated with filtrates (+/- heat treatment) starting at Time = 0. The rhIL-6 or rhIL-8 levels were measured by SDS-PAGE followed by Western blot and bar graphs indicate the relative amounts of protein measured by band densitometry analysis relative to T=0.
Results in A, C and D are shown as mean (+/- SEM) and are representative of at least three independent experiments. Statistical comparisons were done using a t-test compared with the Early group for A and C, and with T=0 in the same condition for D. *=$p \leq 0.05$;**=$p \leq 0.01$; ***=$p \leq 0.001$.

Since LasR-dependent regulation of proteases varies among different strains of *P. aeruginosa* (*253*), we then measured protease production by the laboratory strain PAO1-V (*254*) and four different CF clinical isolates carrying functional wild-type *lasR* alleles (*217*), and compared them to their respective genetically engineered *lasR* knock-out mutants. As observed with the Early isolate, all of the *P. aeruginosa* isolates displayed extracellular total protease (Fig. 2.5A) and elastase activities (Fig. 2.5C) that were abolished by lasR mutations. All wild-type parental strains also induced significantly less IL-6 and IL-8 compared with their isogenic *lasR* mutants (Fig. 2.5D). Since loss of protease activity is commonly seen in CF-adapted *P. aeruginosa* isolates (*226, 255*), we also examined pairs of clonally related longitudinal *P. aeruginosa* isolates collected from three other CF patients (described in Supp. Table S2.1). The CF-adapted isolates L1, L2 and L3 produced less elastase (Fig. 2.5E) and induced more IL-6 and IL-8 production in AEC compared to their paired early isolates E1, E2 and E3 respectively (Fig. 2.5F). Taken together, these results indicate that LasR-dependent proteolytic degradation of cytokines occurs in multiple *P. aeruginosa* isolates, and this is a major mechanism in the hyper-inflammatory cytokine response associated with protease-deficient isolates.

Bacterial proteases degrade cytokines more efficiently than neutrophil elastase.

Previous studies have reported that neutrophil elastase (NE) can degrade IL-6 and IL-8 (*256, 257*). Since this enzyme is abundant in the airways of CF patients (*98*), we compared the cytokine-degrading activities of NE with those of *P. aeruginosa* proteases. At quantities titrated to a specific elastase activity equivalent to 2µg/mL purified human NE (Supp. Fig. S2.6), filtrates from *P. aeruginosa* clinical isolates potently degraded both rhIL-6 and rhIL-8, but NE did not (Fig. 2.5B). Extracellular bacterial proteases thus likely contribute significantly to cytokine degradation.

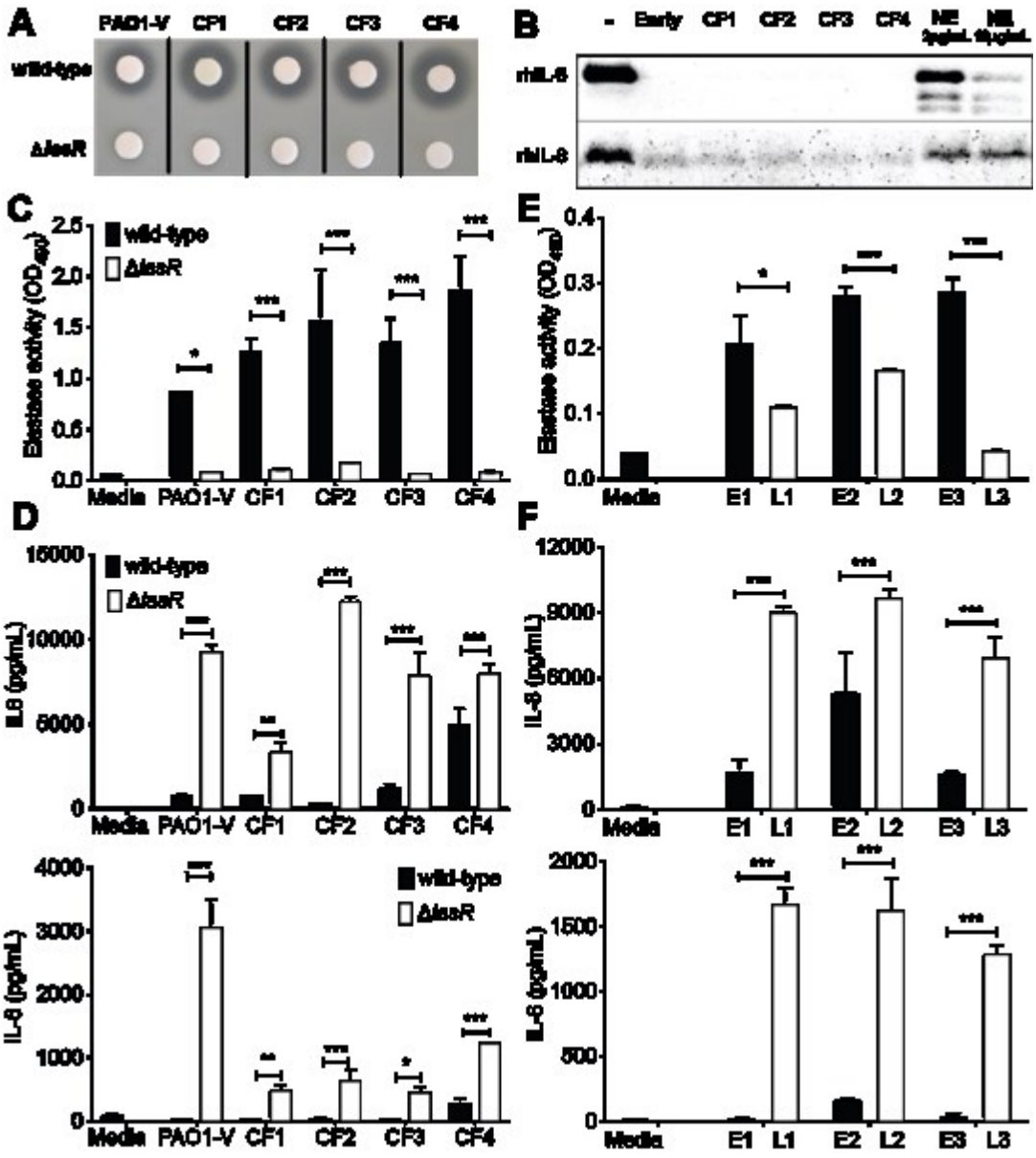


Fig. 2.5: *P. aeruginosa* CF clinical isolates degrade cytokines and protease-deficient isolates induce greater IL-8 and IL-6 responses in AEC

(A) Total secreted protease activity in filtrates from the laboratory strain PAO1-V, clinical isolates (CF1-4) and their isogenic Δ*lasR* mutants assessed on skim milk agar plates.
(B) Recombinant human (rh) IL-6 or IL-8 (10 µg/mL) incubated with filtrates or purified human neutrophil elastase (NE, 2 or 10 µg/mL) for 3 hrs (rhIL-6) or 24 hrs (rhIL-8). CF1-4 filtrates were dosed at equivalent elastase activity as NE 2 µg/mL (as demonstrated in Supp. Fig S6).
(C) Specific elastase activity in filtrates using the elasting congo red assay.
(D) BEAS-2B cells stimulated with 60 µL filtrates or media control for 24 hrs.

(E) Specific elastase activity in filtrates using the elasting congo red assay. E1-L1, E2-L2 and E3-L3 are clonally related longitudinal *P. aeruginosa* isolates from three CF patients (as outlined in Supp. Table S2.4).
(F) BEAS-2B cells stimulated with 60 µL filtrates or media control for 24 hrs.
For panels D and F, IL-6 and IL-8 levels were measured in the AEC conditioned supernatant after stimulation by sandwich ELISA. Results in C to F are shown as mean (+/- SEM) of n=3 independent biological replicates and are representative of n≥2 independent experiments. Statistical comparisons were done using a two-tailed t-test as indicated. *=$p \leq 0.05$;**=$p \leq 0.01$; ***=$p \leq 0.001$.

LasB, a LasR-regulated elastase, is required for IL-8 and IL-6 degradation.

LasR positively regulates the expression of several *P. aeruginosa* extracellular proteases, including LasA, LasB and AprA. To determine which LasR-regulated proteases mediate cytokine degradation, we stimulated AEC with culture filtrates from the PAO1-V wild-type strain and its isogenic *aprA*, *lasA* and *lasB* mutants. The *lasB* mutation had the same effect on cytokine degradation as did *lasR* mutations, while deletion of aprA and lasA had no significant effect (Fig. 2.6A). To confirm the role of LasB, we deleted *lasB* in the Early isolate, and complemented the Late isolate with an inducible lasB gene, thus creating the EΔ*lasB* and L+*lasB* strains respectively. As shown in Fig. 2.6B, the EΔ*lasB* isolate induced a pro-inflammatory cytokine response, while overexpression of lasB in the Late isolate restored cytokine degradation. Taken together, our results show that the LasB is necessary and sufficient to degrade IL-6 and IL-8 in AEC cultures.

Loss of *P. aeruginosa* LasB activity stimulates neutrophil recruitment.

If the LasB-mediated cytokine degradation contributes to the hyper-inflammatory responses of *lasR* mutants, we reasoned that the loss of LasR and LasB function should be sufficient to increase neutrophil recruitment. To test this hypothesis, we used a neutrophil transmigration assay to determine the functional consequences of AEC cytokine response on primary human neutrophil recruitment and chemotaxis in vitro.

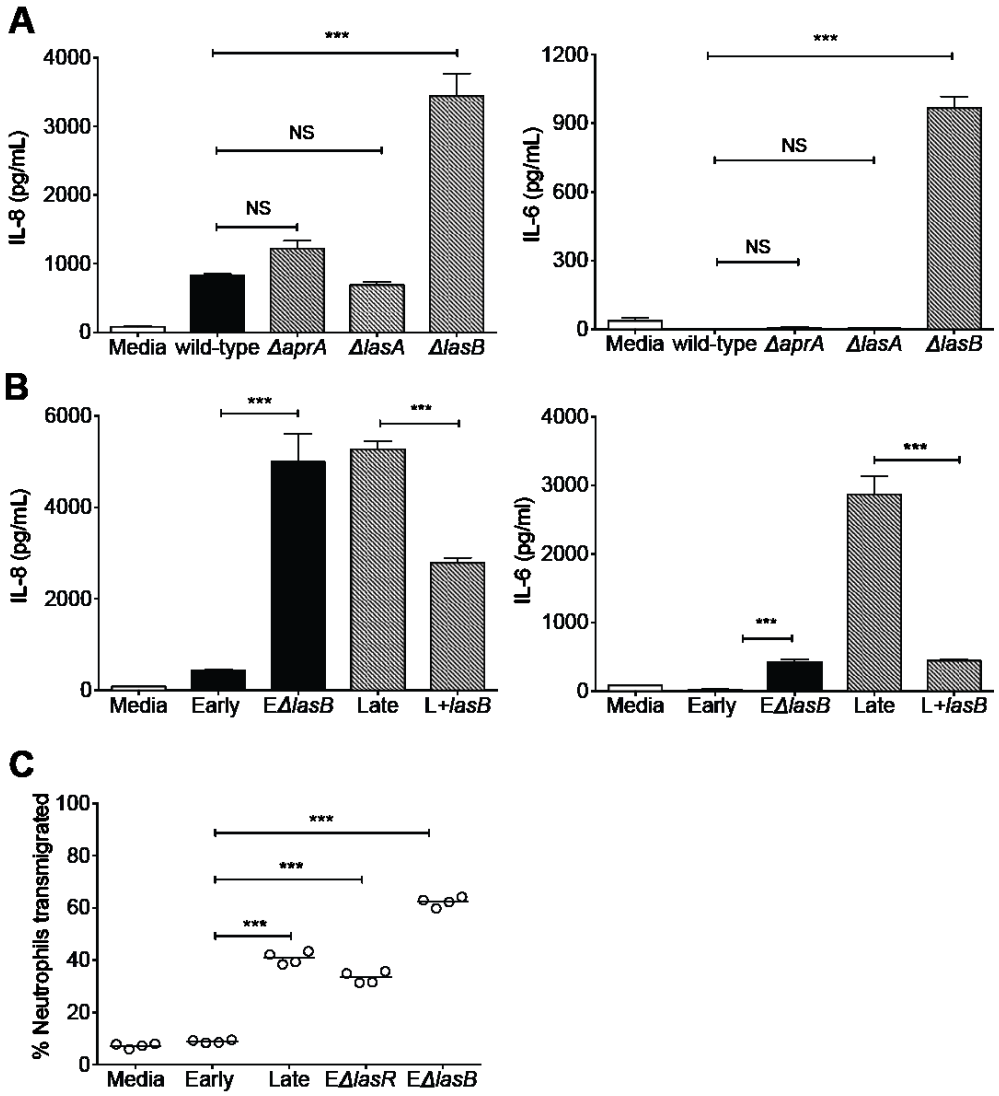


Fig. 2.6: Loss of LasR-regulated LasB abrogates IL-6 and IL-8 degradation and induces more neutrophil recruitment *in vitro*

(A) BEAS-2B cells stimulated with 60 µL filtrates or media control for 8 hrs from PA01-V (wild-type), and its derived Δ*aprA*, Δ*lasA* and Δ*lasB* mutants.
(B) BEAS-2B cells stimulated with 60 µL filtrates or media control for 8 hrs from Early, EΔlasB, Late and L+lasB (complemented with lasB) isolates.
(C) Human neutrophil transmigration assay with conditioned medium from BEAS-2B cells stimulated with 60µL filtrates or media control for 24 hrs.
For panels A and B, IL-6 and IL-8 levels were measured in the AEC conditioned supernatants after stimulation by sandwich ELISA. Results are shown as mean (+/- SEM) of n=3 independent biological replicates and are representative of at least n≥3 independent experiments. Statistical comparisons were done using a two-tailed t-test as indicated. *=$p \leq 0.05$;**=$p \leq 0.01$; ***=$p \leq 0.001$.

We collected the conditioned supernatant from AEC stimulated with Early, Late, EΔ*lasR* and EΔ*lasB* filtrates, and measured the effects of these supernatants on neutrophil chemotaxis and transmigration. As shown in Fig. 2.6C, conditioned supernatant from AEC stimulated with Late, EΔ*lasR* and EΔ*lasB* filtrates induced 4.5, 3.5 and 6.8-fold greater neutrophil transmigration respectively compared to AEC stimulated with Early filtrates (all $p<0.001$). These results therefore demonstrate that *P. aeruginosa* mutants lacking LasB function, either through mutation of *lasB* or inactivation of its transcriptional regulator *lasR*, induce greater neutrophil recruitment than do wild-type *P. aeruginosa*.

Chronic airway infection with *P. aeruginosa lasR* mutants is associated with higher levels of plasma IL-8 levels in CF patients.

Based on our *in vitro* and *in vivo* results, we predicted that chronic *lasR* mutant infections would also be associated with a hyper-inflammatory response in CF patients. In a prospective cohort of 17 adult CF patients chronically infected with *P. aeruginosa*, we analyzed patients' whole sputum samples by FREQ-seq analysis (*258*) to determine the relative frequency of *lasR* mutant alleles, defined by the identification of non-synonymous mutations within the *lasR* coding sequence. As shown in Fig. 2.7A and Supp. Fig. S2.7A, some patients were colonized with mixed *P. aeruginosa* populations with both wild-type and mutant *lasR* alleles. We defined patients' *lasR* status as *lasR* mut if their sputum *P. aeruginosa* populations contained >50% lasR mutant alleles, and as *lasR* wt if they contained <50% *lasR* mutant alleles. Based on the frequency of *lasR* mutant alleles in their sputum samples, 8 out of 17 patients were considered *lasR* mut (Fig. 2.7A), and both *lasR* mut and *lasR* wt groups were similar in age (Supp. Fig. S2.7B) and sex (female sex 50% vs 55% in *lasR* mut and *lasR* wt group respectively). We concurrently measured the subjects' plasma levels of IL-8, a systemic marker of inflammation in CF lung disease (*97, 259*). Remarkably, the *lasR* mut group had higher IL-8 levels compared to *lasR* wt group during a stable clinical state (median (IQR) 6.2 (4.7-18.7) vs 3.4 (2.7-5.2), $p\leq0.03$) (Fig. 2.7B), and results remained similar even when the patients' *lasR* mut status was defined as >20% or >90% *lasR* mutant alleles instead of >50%. Notably, the *lasR*

mut group was still associated with higher IL-8 levels when patients were assessed longitudinally (median (IQR) 6.4 (5.0-16.0) vs 3.9 (3.2-7.1), p≤0.005) (Supp. Fig. S2.7C), with a positive correlation between *lasR* mutant allele frequency and IL-8 levels (Spearman r=0.33; 95% CI [0.03-0.58]; p≤0.05) (Fig. 7C7). These results thus show a significant association between airway infections with *lasR* mutants and a marker of inflammation in CF patients.

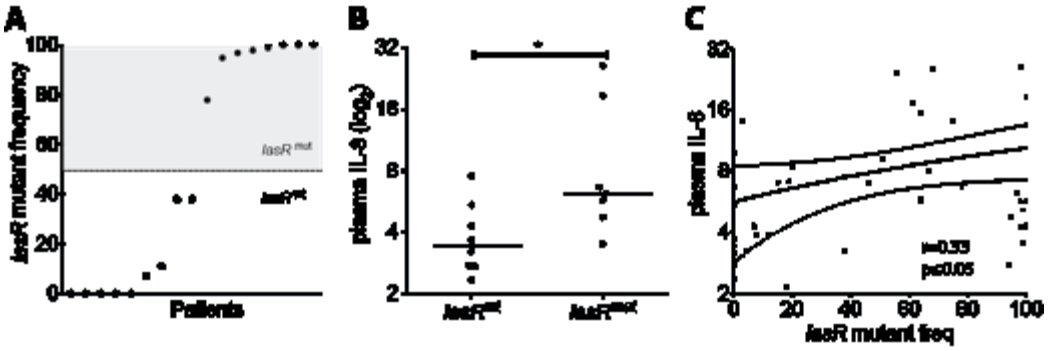


Fig. 2.7: Colonization with *lasR* mutant *P. aeruginosa* is associated with higher plasma IL-8 in CF patients

(A) Frequency of *lasR* mutant alleles measured by FREQ-seq in the 17 CF patients, with each dot representing one patient's sputum sample collected during a clinically stable state. The patients' *lasR* status is defined as the *lasR* wt or *lasR* mut groups as shown.
(B) Plasma IL-8 levels (pg/mL) measured in clinically-stable CF patients according to their *lasR* wt or *lasR* mut status. Results are shown as median (*lasR* wt group n=9 patients; *lasR* mut group n=8 patients). Statistical comparison was done using the Mann Whitney test compared with the *lasR* wt group. **p*≤0.05.
(C) Linear correlation between *lasR* mutant allele frequency and plasma IL-8 levels in longitudinal sampling of CF patients (n=17). Spearman correlation r=0.33; 95% CI [0.03-0.58]. *p*≤0.05.

2.4. Discussion

During the transition from initial to chronic infection, *P. aeruginosa* adapts to the CF lung environment and incurs genotypic and phenotypic changes resulting in reduced expression of invasion and toxicity factors (*143, 174, 175, 177, 183, 184, 192, 236, 237, 260*). Although such CF-adapted *P. aeruginosa* mutants should be attenuated in virulence and induce less pathology, in CF

patients they are in fact associated with later stages of lung disease that exhibit progressive lung inflammation and dysfunction. To explore this paradox, we focused on *lasR* mutants. Our study showed that *lasR* mutants induce greater inflammation in vitro and in vivo, despite reduced expression of acute virulence factors. We demonstrated that LasB, a LasR-controlled protease, degraded cytokines, and loss of LasB-dependent proteolysis was sufficient to cause an exaggerated inflammatory response in vitro under biologically relevant conditions. Notably, the loss of LasR function also induced an exaggerated cytokine response associated with increased pulmonary neutrophilic inflammation and airway tissue damage in murine airway infections. These findings thus support a model wherein adaptive *lasR* mutants directly contribute to exaggerated cycles of infection and inflammation, leading to progression of CF lung disease. Consistent with this model, CF patients predominantly infected with *lasR* mutants had higher plasma IL-8 levels than those predominantly infected with wild-type *P. aeruginosa*, whereby circulating IL-8 levels are markers of inflammation elevated in CF lung disease (*97, 259*). Whether *lasR* mutants cause worsening of lung function over time in CF patients still needs to be addressed in a longitudinal study.

Airway inflammation in CF lung disease is dominated by neutrophils and directly contributes to pulmonary dysfunction and tissue damage (*52, 139, 261, 262*). *P. aeruginosa* typically grows as biofilm aggregates within the viscous mucus layer overlying the airway epithelial surface, and is not invasive in CF chronic infections (*145, 263*). AEC thus interact with extracellular bacterial products to produce pro-inflammatory mediators critical for recruitment of neutrophils to the sites of inflammation. These interactions are modeled in our experimental systems, and we observed the hyper-inflammatory effects associated with *lasR* mutants across several cell culture systems, including CF and non CF AEC, immortalized as well as primary human cells, using both genetically engineered and naturally occurring *lasR* mutants. We recognize that our murine infection model overlooks the contribution of host CFTR mutations

(which cause CF disease) on host defenses and immunity, and is not a model of CF lung disease per se. However, it nonetheless provides a well-established in vivo system to dissect the host-pathogen interactions during chronic infection and their effects on pulmonary immunopathology.

At first glance, our findings contrast with previous studies reporting on the pro-inflammatory effects of LasR-regulated factors. For example, the LasR-dependent quorum sensing signal 3-oxo-C12HSL can interact directly with host cells via multiple different mechanism (*264*), such as activating NF-κB (Nuclear factor kappa-light-chain enhancer of activated B cells), MAPK (Mitogen-activated protein kinase) and Ca2+-dependent signaling pathways, leading to induction of pro-inflammatory cytokine responses (*265-268*). Azghani et al also reported that LasB can activate MAPK and NF-κB inflammatory signaling pathways in host cells (*269, 270*). In contrast to ours, these experimental systems, these studies focus on host intracellular signaling responses, mRNA and intracellular cytokine levels, and do not capture the contribution of post-secretion cytokine degradation, the major mechanism investigated in our studies. Our results with heat-inactivated filtrates or protease deficient mutants are in fact consistent with the notion that additional LasR-regulated factors can induce IL-8 and IL-6 production. However, but the resulting secreted cytokines levels are only elevated in the absence of bacterial protease activity. Since protease-mediated cytokine degradation occurs after cytokines are secreted, this process can prevail over more “upstream” bacterial-host interactions that modulate AEC cytokine expression.

LasR controls the expression of extracellular proteases in many *P. aeruginosa* strains (*271, 272*), including the clinical and laboratory strains tested in this study, and LasB can be detected at levels exceeding 100 µg/mg in the sputum of CF patients (*273*). In protease-deficient *P. aeruginosa* clinical strains, the loss of protease activity is primarily caused by loss of the LasB elastase production (*274, 275*). Although the *P. aeruginosa* elastase LasB is established

as an acute virulence factor that degrades host matrix proteins and causes tissue damage (*276-278*), its dual immune modulatory role is likely under-recognized, as our results show that it degrades cytokines more potently than human neutrophil elastase. In settings where immune responses fail to clear bacteria, such as in the CF airways, the loss of bacterial elastase activity paradoxically amplify pulmonary neutrophilia due to reduced cytokine degradation.

LasR-regulated proteases could modulate host inflammatory responses through multiple pathways. For example, *P. aeruginosa* proteases are capable of degrading in vitro cytokines such as RANTES (Regulated on activation, normal T cell expressed and secreted), MCP-1 (Monocyte chemotactic protein 1) and ENA-78 (Epithelial-derived neutrophil activating peptide 78, or CXCL5) (*279-281*). We focused on IL-8 and IL-6 as AEC secrete them abundantly in response to *P. aeruginosa* in order to recruit and activate neutrophils (*101, 282-284*). Sputum IL-8 levels are higher in CF patients infected with *P. aeruginosa* (*279*) and correlate with decreased lung function (*98, 118, 119*). Interestingly, LasB can degrade surfactant proteins A and D leading to resistance to phagocytosis (*285-287*), and AprA can degrade flagellin monomers, thus dampening TLR5 (Toll-like receptor 5)-mediated responses (*288*). These observations add to the proposed notion that *P. aeruginosa* proteases disrupt host inflammatory responses, and their loss in CF-adapted *P. aeruginosa* isolates could promote further inflammation.

In many *P. aeruginosa* strains, the quorum-sensing regulator LasR controls the expression of a number of acute virulence determinants, including proteases, pyocyanin and other exoproducts (*220, 289*). Accordingly, *lasR* mutants are attenuated in several models of acute virulence such as in invertebrate host models (*222, 223*) and rodent acute pneumonia (*220, 221, 224*). That the loss of LasR-regulated acute virulence factors does not prevent *P. aeruginosa* chronic infections supports the notion that CF-adapted mutants

contribute to the pathogenesis of chronic infection through pathways and host-pathogen relationships different from those involved in acute infections (*183*).

A convergent adaptive evolution is consistently observed across different *P. aeruginosa* populations in spite of the vast genetic heterogeneity within and between CF patients (*177, 184, 192*). Significantly, loss-of function *lasR* mutants commonly arise during chronic *P. aeruginosa* infections to become a dominant variant or occur as part of a mixed *P. aeruginosa* population in the CF lung (*177, 212, 214, 218*). In a retrospective study of 30 CF patients, *lasR* mutant isolates were identified in 63% of patients (*177*). More recently, *lasR* mutant *P. aeruginosa* isolates were isolated in 31% of CF patients, and those patients infected with *lasR* mutants were associated with worse age-specific lung function (*218*), underscoring the clinical relevance of our study. Interestingly, *lasR* mutants can also emerge during the course of non-CF infections, such as in populations of *P. aeruginosa* colonizing intubated patients on mechanical ventilation (*219*). Furthermore, in a large prospective study of children with CF, the presence of protease-deficient *P. aeruginosa* isolates were among the best bacterial phenotypic predictors of chronic infection (*226*). Since many of the protease-deficient CF isolates are *lasR* mutants (*217*), it follows that the loss of LasR-controlled quorum sensing may also be a predictor of more severe chronic infections. Whether this bacterial adaptation is a marker of more severe CF lung disease, or contributes to its progression, was not addressed by these observational clinical studies.

Loss-of-function *lasR* mutations emerge under certain laboratory growth conditions and often co-exist in mixed *P. aeruginosa* populations, as noted in our study and others (*192, 212, 213, 218, 240*). *lasR* mutants may have a fitness advantage, such as a growth advantage on nitrate and aromatic amino acid sources (*214, 290*), they are more resistant to alkaline stress (*215*) and they can act as social cheaters in mixed *P. aeruginosa* populations (*213, 240, 291, 292*). Increased β-lactamase activity may also contribute to their selection under β-

lactam antibiotic treatment (*214*). Despite these observations, it remains unknown why *lasR* mutants emerge so readily in the CF lung.

Our results also raise some concerns regarding the proposed development of inhibitors targeting LasR quorum sensing (*293*) or bacterial proteases (*294*) as novel antimicrobial agents against *P. aeruginosa*. In chronic CF infections, such agents could induce greater host inflammation and thus cause more harm. Understanding how bacterial adaptation during specific chronic infections modulates host responses and contribute to lung inflammation is critical in developing antimicrobial therapies tailored to the different types and stages of infection.

2.5. Materials and methods

Bacterial strains, plasmids and construction of mutants

The bacterial isolates and plasmids used in this study are listed in Supp. Figs. S2.2 and S2.3 respectively. The Early (AMT0023-30) and Late (AMT0023-34) isolates, recovered from the same CF patient at 6 months and 8 years of age respectively, are clonally related and previously described (*177*). The isolates CF1, CF2, CF3 and CF4 were isolated from four different CF patients (*214*), and their isogenic *lasR* mutants were genetically engineered (*214*). The longitudinal isolates E1, E2 and E3 and their respective L1, L2 and L3 CF-adapted isolates are clonally related and were recovered from three different CF patients. The construction of the Δ*lasB* and Δ*lasR* mutants, and the inducible lasB+ expression construct are described in detail in the Supplemental methods.

Bacterial planktonic and biofilm growth conditions

Bacteria were grown at 37°C in synthetic cystic fibrosis sputum media (SCFM), a defined culture medium that approximates the nutrient composition of

CF sputum (*248*), unless otherwise specified. Planktonic cultures were grown in liquid medium with shaking at 250 r.p.m. Biofilms were grown as aggregates embedded in agar as described previously (*247*) with modifications. Briefly, biofilm cultures were inoculated with overnight planktonic cells diluted in 50% SCFM, 20 mM NaNO3, and 0.8% melted agar to a final concentration of 5000 cell/mL. Biofilm cultures were grown in 12 mm Transwell permeable supports (Corning, 0.4 µm polyester membrane) statically at 37°C for 48 hrs. Antibiotics used for selection were gentamicin 50 µg/mL and tetracycline 80 µg/mL for *P. aeruginosa*; gentamicin 10 µg/mL and tetracycline 10 µg/mL for *E. coli*.

P. aeruginosa filtrate preparation

Planktonic bacterial cultures were grown in SCFM for 24 hrs, then centrifuged at 7200 g for 10 min at room temperature. The supernatants were filtered with low-protein binding 0.22 µm cellulose acetate filters (Corning), and aliquots of sterile filtrates were stored at -20°C, with no more than two freeze-thaws before use. Where indicated, the filtrates were heat treated for 10 min at 95°C to inactivate proteases.

Airway epithelial cell cultures and stimulation with _P. aeruginosa_ filtrates

Immortalized wild-type human bronchial epithelial BEAS-2B cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM, Wisent) containing 4.5 g/L D-glucose and supplemented with 10% heat-inactivated fetal bovine serum (FBS, Wisent), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37oC with 5% CO2. For stimulation, 2x105 cells were seeded into 12-well polystyrene tissue-culture treated plates (Corning Costar) and grown to confluence before cells were incubated in starvation medium (DMEM supplemented with 0.5% heat-inactivated FBS and antibiotics) for 16 hrs, then stimulated with 60 µL (or otherwise indicated) of sterile *P. aeruginosa* filtrates or SCFM control medium in 1mL fresh starvation medium, and incubated at 37°C with 5% CO_2 for the indicated time.

Immortalized CFBE41o- cells (*250*) were routinely cultured in Eagle's Minimum Essential Medium (EMEM) containing 1g/L D-glucose and supplemented with 10% heat-inactivated FBS (Wisent), penicillin (100 U/ml) and streptomycin (100 µg/mL) at 37°C with 5% CO_2. For stimulation, $2x10^5$ cells were seeded into 12-well polystyrene tissue-culture treated plates (Corning Costar) coated with human fibronectin 10 µg/mL (VWR), bovine collagen I 29 µg/mL (BD Biosciences), bovine serum albumin 100 µg/mL in LHC basal medium (Invitrogen) and grown to confluence. Prior to stimulation, cells were incubated in starvation medium (EMEM supplemented with 0.5% heat-inactivated FBS and antibiotics) for 16 hrs, then stimulated with 30 µL of sterile *P. aeruginosa* filtrates or SCFM control medium in 1 mL fresh starvation medium, and incubated at 37oC with 5% CO2 for 18 hrs. For all experiments, the conditioned AEC supernatants was collected after stimulation and stored at -20°C until further analyses.

Biofilm - airway epithelial cell co-culture system

Biofilms aggregates were grown for 48 hrs as described above. Biofilm-containing Transwells were then inserted into tissue culture wells containing a confluent monolayer of BEAS-2B cells immersed in 1 mL of starvation medium for co-incubation at 37°C with 5% CO_2 for the indicated time. For all experiments, the conditioned AEC culture supernatants was collected after stimulation and stored at -20°C until further analyses.

Ex Vivo human nasal explant stimulation with *P. aeruginosa* filtrates

Nasal explant specimens were obtained from human subjects 18 years or older diagnosed with chronic rhinosinusitis as previously described (*295*). All subjects participated voluntarily with signed written informed consent, as approved by the institutional review board of the Centre Hospitalier de l'Université de Montréal (CHUM). Surgical specimens were washed in PBS and

cut into pieces (mean weight 6.9 ± SEM 0.64 mg). Each tissue piece was cultured in 3:1 mixture of DMEM (Invitrogen) and Hank's buffered salt solution (Invitrogen) supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C with 5% CO_2 for 72 hrs, with replacement every 24 hrs. After 72 hrs, the samples were stimulated for 24 hrs with 60 µL of *P. aeruginosa* filtrates or media control. The conditioned tissue supernatant was collected after stimulation and stored at -20°C until further analyses.

Cytokine measurements in conditioned AEC supernatants

Conditioned supernatants of AEC cultures or nasal explants were centrifuged at 13,000 xg to pellet cell debris, diluted (1/10 to 1/100 as required) and used for cytokine quantification by ELISA. Levels of interleukin 6 (IL-6) and interleukin 8 (IL-8) were quantified by ELISA (BD OptEIA Human IL-6 and IL-8 ELISA kits, BD biosciences) according to the manufacturer's instructions. All measurements were done with at least three independent biological replicates.

Chronic murine *P. aeruginosa* pulmonary infection

The pulmonary murine infection with agar-embedded *P. aeruginosa* was performed as previously done (*251*). Briefly, planktonic bacterial cultures were grown to OD600=0.5 in LB broth at 37°C shaking at 250 r.p.m. Bacterial cells were pelleted by centrifugation, resuspended in sterile PBS and mixed 1:1 v/v with a solution 2% LB and 3% molten agar. Agar-embedded *P. aeruginosa* beads were generated with continuously stirring heavy mineral oil (Fisher scientific) as previously described (*251*), and the bacterial density was determined by standard dilution methods after homogenization of beads. Sterile PBS LB agar beads were used in control experiments.

Six to nine week old, specific pathogen free, male C57BL/6 mice were purchased from Charles River Laboratories (Kingston, ON, Canada or Raleigh, NC, USA). Anesthetized mice were infected with 50 µL (~5 $x10^5$ CFU/mouse) of

the agar bead suspension using a non-invasive intra-tracheal inoculation method as previously done (*251*). Mice were humanely sacrificed at designated time points. Whole blood was collected by cardiac puncture and lungs were perfused with PBS to reduce blood leukocytes. Bronchoalveolar lavage (BAL) was performed by injecting and aspirating 4 x 500 µL sterile ice cold PBS through an intra-tracheal catheter. After the BAL, the lungs were removed and placed in sterile RPMI 1640 medium (Wisent) containing protease inhibitors. For bacterial counts, whole lungs were homogenized and serially diluted for viable bacteria plate 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 MUHC.

Bronchoalveolar fluid (BALF), lung homogenates and plasma sample preparation and analysis

The BALF was centrifuged to pellet cells and aliquots of the supernatant was frozen at -80°C until analysis. BALF total protein was measured using BCA Protein Assay Kit (Pierce). For lung homogenates, perfused lungs were minced, digested with 150U/mL collagenase (Sigma-Aldrich), filtered through a 100 µm pore size cell strainer (BD). RBCs were removed by hypotonic lysis. Remaining cells were pelleted, resuspended in 1 mL sterile RPMI 1640 and enumerated using an automated cell counter (Z1 cell counter, Beckmann-Coulter). Murine plasma was collected from whole blood mixed with 0.5 M EDTA, and aliquots were frozen at -80°C until analysis. Cytokines were quantified in all samples by ELISA (Mouse IL-6 and KC/CXCL1 DuoSet ELISA kits, R&D).

Flow cytometry of BALF and lung homogenates

We stained $2x10^6$ cells from lung single cell suspensions, or cells from 400 µL BALF with Fixable Viability Dye eFluor780 (Affymetrix eBiocience), then blocked with anti-murine CD16/CD32 (Affymetrix eBioscience). Cells were then surface stained with eFluor610-conjugated anti murine-CD45 (30-F11, Affymetrix

eBioscience), eFluor710-conjugated anti-murine Ly6G (1A8, Affymetrix eBioscience) and V500-conjugated anti-murine CD11b (M1/70, BD. Finally, cells were fixed (Cytofix, BD) and analyzed using a LSRII flow cytometer (BD Biosciences) and FlowJo 10.0.7 software (Tree Star, Stanford, CA).

Lung histopathology

Murine lungs were inflated and fixed overnight with 10% buffered formalin phosphate (Fisher Scientific). Paraffin embedded tissues were sectioned into three 5 µM slices that were 50-500 µm apart. H&E stained sections were evaluated by a veterinary pathologist for inflammation and pathology in a blinded manner. Images were acquired using an Olympus BX51 microscope fitted with an Olympus DP70 CCD camera. To assess the lung inflammation, a semi-quantitative score was used (see Supplemental methods) and the average score of two sections was reported.

Cytokine degradation assay by immunoblotting

Lyophilized recombinant human IL-6 or IL-8 (Peprotech) at 10 µg/mL final concentration was incubated with *P. aeruginosa* filtrates 3% (v/v) final concentration at 37°C. At specified times, 10 µL aliquots were mixed 1:1 (v/v) in Tricine buffer (200 mM Tris-HCl, 40% glycerol, 2% SDS, 0.04% Coomasie Blue G-250, 2% β-mercaptoethanol pH 6.8) and heated to 95°C for 5 min. Samples were then separated by SDS-PAGE using 16.5% Tris-Tricine gels. After transfer of gels onto nitrocellulose paper, the blots were blocked with 2% BSA, then immunoblotted with polyclonal human IL-6 or IL-8 antibodies (R&D) diluted in PBS (with 0.1% BSA, 0.05% Tween-20). The signal was detected using donkey α-goat secondary antibody conjugated to IRDye800 infrared fluorophore (Li-Cor Biosciences) in PBS with 0.05% Tween-20. Images of the fluorescently labeled protein bands were captured with the Odyssey Imaging System (Li-Cor Biosciences) and analyzed using ImageJ software (1.47v, National Institutes of

Health, MD). The protein band density was quantified and normalized to the T=0 control on the same blot.

Skim milk agar protease activity assay

Total protease activity of the *P. aeruginosa* filtrates was assessed using 1.5% agar plates containing 1.5% skim milk as previously described (*296*). Briefly, *P. aeruginosa* filtrates was spotted onto sterile 6mm paper discs placed on the agar surface, and plates were incubated for 24 hrs at 37oC.

Elastase activity assay

Elastase activity in the *P. aeruginosa* filtrates was measured using the elastin-congo red elastase assay as previously described with minor modifications (*297*). Filtrates were incubated with ECR solution (5 mg/mL elastin congo red (Sigma), 100 mM Tris Cl, 1mM CaCl2, pH 7.5) at 37°C for 24 hrs shaking at 250 r.p.m. Samples were then centrifuged at 2200 g for 10 min, and absorbance at 490nm was measured in the supernatant using a microplate reader (Bio-Rad Model 680). All measurements were done with at least three independent biological replicates.

In-vitro PMN transmigration assay

PMNs were isolated from human blood (*298*) and samples contained >99% neutrophils. Neutrophil transmigration assays were performed as previously described using a modified Boyden chamber (*299*). Briefly, $5x10^5$ PMNs were added to the upper compartment of Transwell inserts with 5 μm pore size permeable polycarbonate membrane (Corning). Conditioned supernatants of stimulated AEC were added to the Transwell lower compartment and incubated for 3 hrs at 37°C with 5% CO_2. Cells from both upper and lower compartments were collected and counted using a cell counter (Z1 Coulter, Beckman). The % neutrophil transmigration was defined as the % of neutrophils in the basolateral compartment compared to the total number of neutrophils in both compartments.

Clinical data and cytokine measurements in CF patients.

CF patients from the Adult Cystic Fibrosis Clinic at the Montreal Chest Institute (Montreal, Canada) were enrolled prospectively. Clinical data, plasma sample collection and cytokine measurements were previously described (*299*). Spontaneously expectorated sputum samples were collected and stored at -80°C until *lasR* FREQ-seq analysis. All clinical data and samples were obtained either 1) at baseline during a period of stable disease, defined by the absence of any pulmonary exacerbations requiring antibiotic therapy in the preceding month, or 2) longitudinally over a period of 18 months during both stable and exacerbation clinical states. Informed written consent was obtained from all subjects and the study was approved by the Institutional Review Board of the McGill University Health Centre.

FREQ-seq analysis of *lasR* alleles in CF sputum

Genomic bacterial DNA was extracted from whole sputum using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA). The *lasR* gene was amplified by PCR using *lasR*-specific primers lasR-F and lasR-R (see Supp. Table S3 for primer sequence). PCR products were purified using the QIAquick PCR purification kit (Qiagen). Standard Illumina Nextera libraries were constructed using individual PCR products according to manufacturer's guidelines (Illumina Inc., San Diego, CA). Paired-end libraries for each sample were sequenced with an Illumina MiSeq to generate 150 bp reads for an average of at least 20,000 reads per base per sample. Sample sequences were aligned against the *lasR* PAO1 reference sequence (www. pseudomonas.com) using the BWA-MEM algorithm (*300*). All *lasR* polymorphisms identified were present at a frequency of >5% in at least 10,000 reads of that given sample. Non-synonymous mutations within the *lasR* coding sequence were considered mutant *lasR* alleles and patients were considered to be in the *lasR* mut group if the *lasR* mutant allele frequency was >50% in that sample.

Statistical analyses

All results are expressed as mean ± SEM, or median ± IQR as indicated. Statistical analyses were done using Prism 5 software (GraphPad). Comparisons were performed using an unpaired two-tailed student's t-test, or the Mann Whitney non-parametric test as indicated. Comparisons between three or more groups were performed using one-way ANOVA with Bonferroni's test or Kruskal Wallis non parametric test as indicated. A P value of ≤0.05 was considered to be statistically significant. Not significant (NS) $p>0.05$, *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$.

Accession numbers for genes and proteins mentioned in text

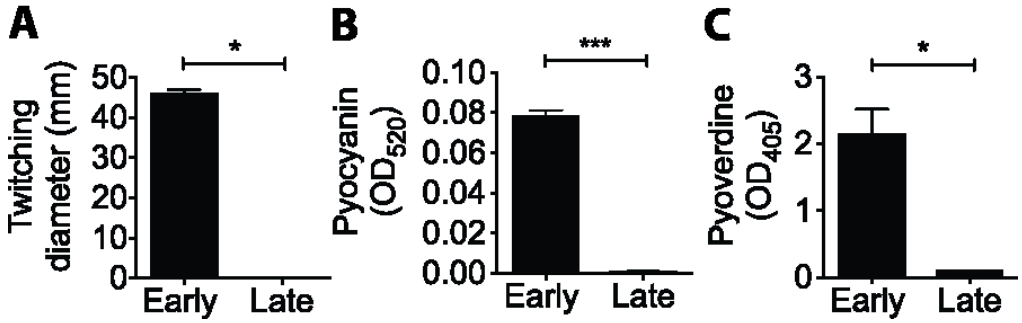
(NCBI Entrez gene ID number) *P. aeruginosa*: pilA (878423); phzS (881836); pvdS (882839); lasR (881789); aprA (881248); lasA (878260); lasB (880368);

Acknowledgements

The PAO1-V, PAO1-V ΔlasA and PAO1-V ΔaprA were kindly provided by Jeffery A. Hobden and Suzanne Fleiszig. We would like to thank Joe J. Harrison for kindly providing us with the Gateway vectors. CFBE41o- cells were a kind gift from Dr. John P. Clancy, and the BEAS-2B cells from Salman Qureshi (McGill University). Human PMNs were obtained from William Powell (McGill University). SLL is supported by scholarships from CIHR and Cystic Fibrosis Canada. DN is supported by a CIHR salary award and funded by grants from Cystic Fibrosis Canada and the Burroughs Wellcome Fund (1006827.01). NES, AD and JRC are supported by grant R565 CR11 from the Cystic Fibrosis Foundation. JLB is supported by grant NIH P30 DK089507. We acknowledge the CRCHUM/IRCM Respiratory Tissue Biobank and the FRQS RNH for access to human explant tissues (EB).

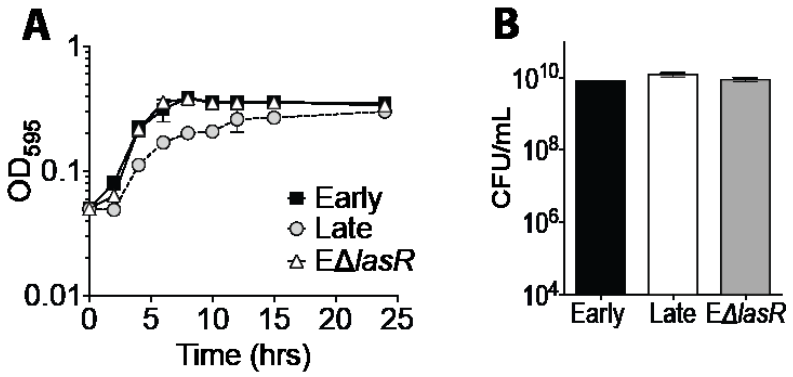
2.6. Supplemental material

Supplemental Figures



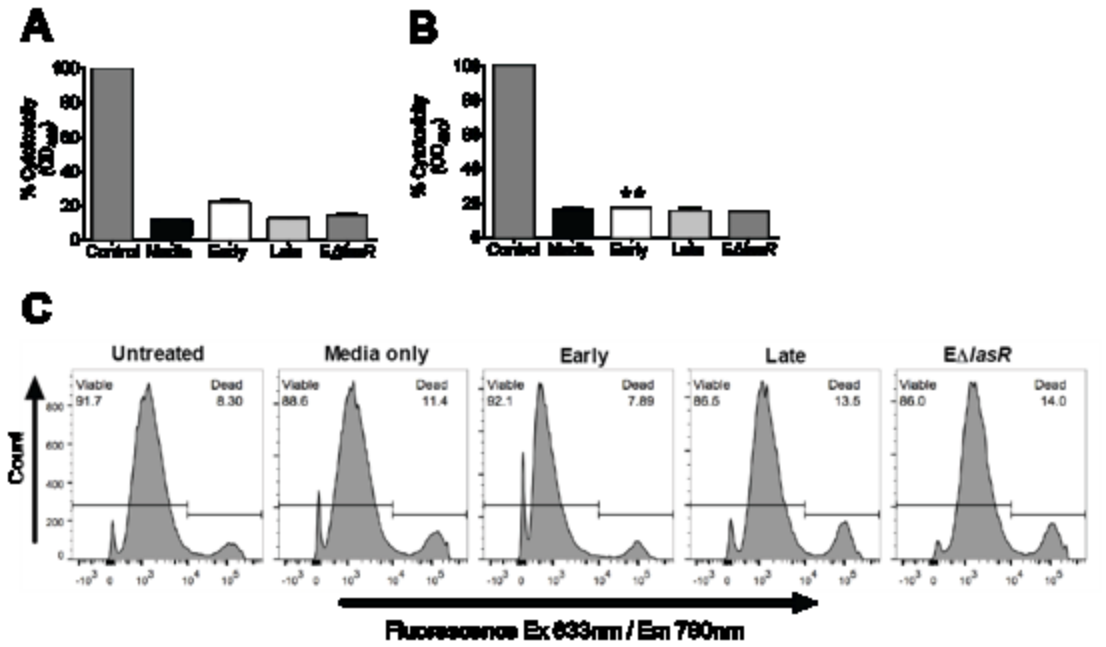
Supp. Fig. S2.1: The Late isolate is impaired in the production of acute virulence factors compared to the Early isolate

(A) Type IV pilus-mediated twitching motility.
(B) Pyocyanin production in planktonic cultures.
(C) Pyoverdine production in planktonic cultures.
Results are shown as mean +/- SEM (n ≥ 3 independent biological replicates). *=$p \leq 0.05$; ***=$p \leq 0.001$. Statistical comparisons were done by Mann-Whitney test for panel A, and by two-tailed unpaired t-test for panels B and C.



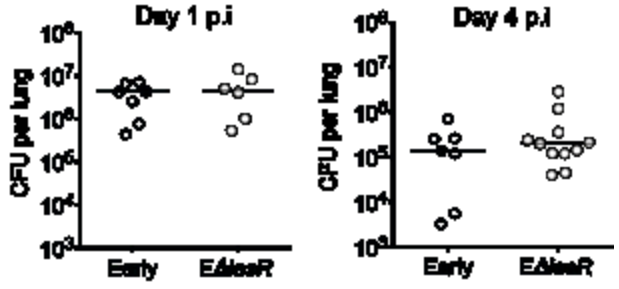
Supp. Fig. S2.2: The Early, Late and EΔ*lasR* isolates grow to similar bacterial density in planktonic and biofilm cultures

(A) Planktonic growth of the Early, Late and EΔ*lasR* isolates in SCFM at 37°C with shaking at 250 r.p.m.
(B) Biofilm bacterial density after 48 hrs growth of the Early, Late and EΔ*lasR* isolates in SCFM at 37°C.
Results are shown as mean +/- SD (n ≥ 3 independent biological replicates).



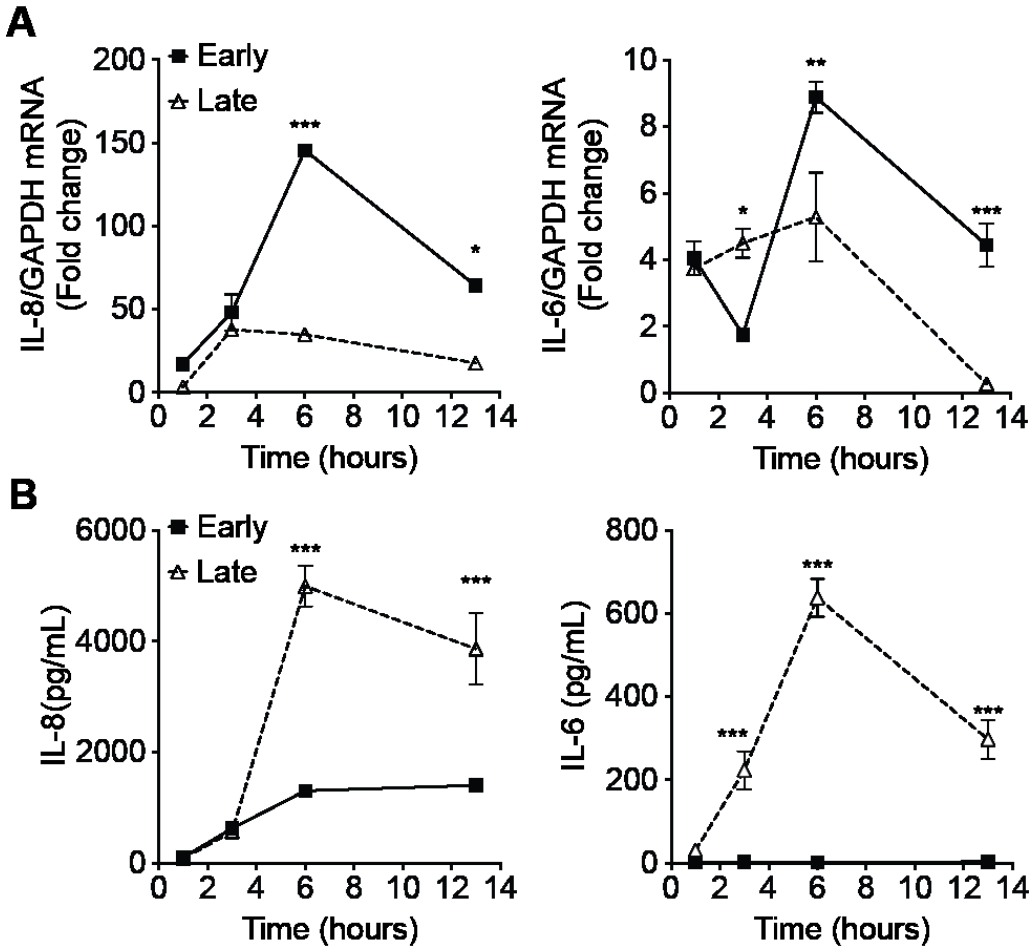
Supp. Fig. S2.3: *P. aeruginosa* cell-free filtrates and biofilms do not cause significant cytotoxicity to AEC cultures

(A) BEAS-2B monolayers were stimulated with 60µL filtrates for 8 hrs.
(B) BEAS-2B monolayers were co-cultured with biofilm aggregates for 18 hrs, as described in the biofilm-AEC co-culture system. For A and B, cytotoxicity was quantified using the LDH release assay, and the positive control is complete cell lysis with Triton X-100. Results are shown as mean +/- SEM (n =3 independent biological replicates).
(C) BEAS-2B monolayers were stimulated with 60 µL filtrates for 24 hrs. Cells were stained with Fixable Viability Dye eFluor780 and the proportions of live and dead cells were determined by flow cytometry at Ex 642/ Em780.
Statistical comparisons were done by 1-way ANOVA with Bonferroni's post-test comparison between the media only control group and each treatment group for panels A and B.



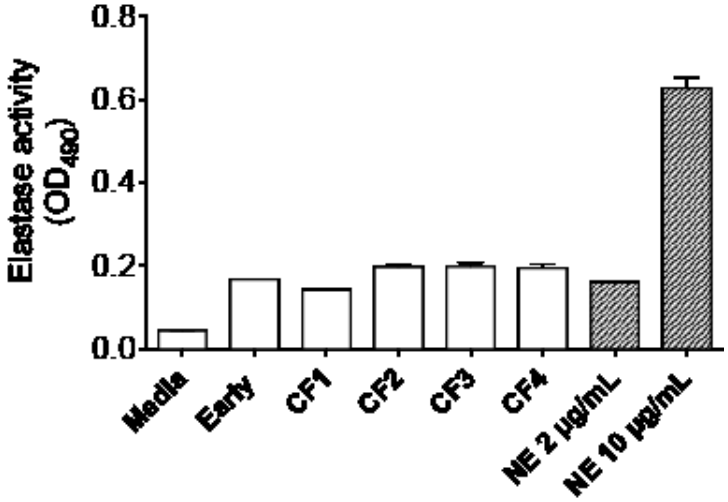
Supp. Fig. S2.4: The pulmonary bacterial loads are similar in Early and EΔ*lasR* infected mice

Whole lungs were recovered at Day 1 and Day 4 p.i. for enumeration of viable CFU counts. Results are shown as median and each data point represents one animal.



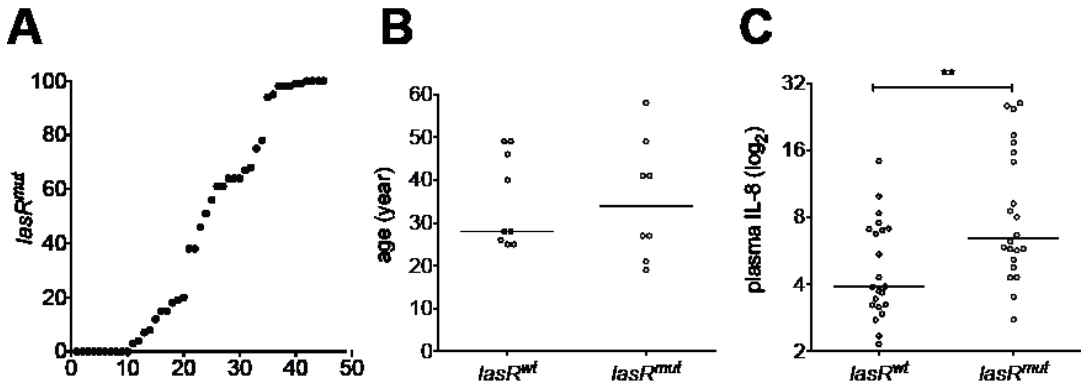
Supp. Fig. S2.5: Relative expression of IL-6 and IL-8 mRNA in BEAS-2B cells treated with Early and Late filtrates

BEAS-2B monolayers stimulated with 60 µL filtrates or medium control (SCFM) for the indicated duration and simultaneously analyzed by qRT- PCR and ELISA.
(A) Total RNA was isolated from cells at indicated time points and relative IL-6 and IL-8 mRNA levels were measured by qRT-PCR. Results shown are the fold change increase of each transcript relative to untreated cells at T=0 hrs, and are normalized to GAPDH.
(B) AEC conditioned culture supernatants were collected at indicated time points and levels of IL-6 and IL-8 were measured by ELISA.
Results are shown as mean +/- SEM (n ≥ 3 independent biological replicates). Statistical comparisons were done by 1-way ANOVA with Bonferroni's post-test comparison between the Early and Late sample groups at each respective time point. *=$p \leq 0.05$; **=$p \leq 0.01$; ***=$p \leq 0.001$.



Supp. Fig. S2.6: Elastase activity of filtrates used to degrade rhIL-8 and rhIL-6

The filtrates from different *P. aeruginosa* clinical strains were titrated to the equivalent elastase activity of 2 µg/mL of purified human NE using the elastin congo red assay.



Supp. Fig. S2.7: Colonization with *lasR* mutant *P. aeruginosa* is associated with higher plasma IL-8 in CF patients

(A) Frequency of *lasR* alleles measured by FREQ-seq in sputum samples collected longitudinally during both stable and exacerbation clinical states in CF patients.
(B) Age of CF patients according to their *lasR* allele status.
(C) Plasma IL-8 levels measured longitudinally during both stable and exacerbation clinical states.in adult CF patients according to their *P. aeruginosa lasR* allele status.
Results in C are shown as median of all samples collected (*lasR* ^{wt} group n=9 patients; *lasR* ^{mut} group n=8 patients). Statistical comparison was done using the Mann Whitney test compared with the with the *lasR* ^{wt} group. **=$p \leq 0.01$.

Supplemental Tables

Supp. Table S2.1: Clonally related early and late isolate pairs from CF patients

Patient	Clinical isolate	RAPD genotype	Timespan between paired E and L isolates (years)	Mucoidy
1	E1	A097	11	Yes
	L1	A097		No
2	E2	A030	15	No
	L2	A030		Yes
3	E3	A136	21	No
	L3	A136		Yes

Supp. Table S2.2: *P. aeruginosa* isolates and strains

Strains	Relevant genotype or phenotype	Reference
AMT0023-30	"Early" clinical isolate from a young cystic fibrosis patient, with wild-type *lasR* allele.	(*301*)
AMT0023-34	"Late" clinical isolate clonally related to "Early" isolate, recovered from the same CF patient 7.5 years later. 1bp deletion in *lasR* at nucleotide 147 leading to a nonsense mutation.	(*301*)
E∆*lasR*	AMT0023-30 with *lasR*::Gm^R	(*217*)
CF1	"CF215" clinical isolate from a young CF patient, with wild-type *lasR* allele	(*217*)
CF1∆*lasR*	CF1 with *lasR*::Gm^R	(*217*)
CF2	"CF3-0.8" clinical strain from a young CF patient, with wild-type *lasR* allele	(*217*)
CF2∆*lasR*	CF2 with l*asR*::Gm^R	(*217*)
CF3	"CF6-1" clinical strain from a young CF patient, with wild-type *lasR* allele	(*217*)
CF3∆*lasR*	CF3 with l*asR*::Gm^R	(*217*)
CF4	"CF716" clinical strain from a young CF patient, with wild-type *lasR* allele	(*217*)
CF4∆*lasR*	CF4 with l*asR*::Gm^R	(*217*)
PAO1-V	Wild-type *P. aeruginosa* variant of PAO1 (serogroup O5)	(*302*)
PAO1-V∆*lasR*	PAO1-V *lasR::*Gm^R	This study
PAO1-V∆*aprA*	PAO1-V*aprA*ΩGm^R	(*302*)
PAO1-V∆*lasA*	PAO1-V*lasA*ΩGm^R	(*302*)
PAO1-V ∆*lasB*	PAO1-V with a Δ*lasB (*Δ59-1477 bp)	This study
E∆*lasB*	AMT0023-30 with Δ*lasB (*Δ59-1477 bp)	This study
L+*lasB*	AMT0023-34 *attCTX::miniCTX2.1-Tc-GW-araC-pBAD::lasB,* Tc^R	This study
E1, L1, E2, L2, E3, L3	Clonally related longitudinal isolate pairs from CF patients (see Supplemental Table S4 for details).	This study

Supp. Table S2.3: Plasmids

Plasmids	Description	Source or reference
pEX18GmGW	pEX18Gm suicide vector containing a Gateway destination cloning site, Gm^R	(*303*)
pSB219.9A	Suicide vector (pRIC380) for allelic replacement of *lasR* with a mutant allele of *lasR::Gm*^{R}	(*304*)
pSL5	pEX18GmGW containing an unmarked *lasB* deletion allele *(*Δ59-1477bp)	This study
pDONR221P5P2	Multisite Gateway donor vector with *attP5* and *attP2* recombination sites, Cm^R, Km^R	Invitrogen
pSL1	pDONR221P5P2 containing the entire *lasB* ORF fragment (1497 bp)	This study
pJJH187	pDONR221P5P2 with an *attL*-flanked, 1192-bp fragment encoding the *araC* repressor and the *pBAD* promoter, Km^R	(*305*)
miniCTX2.1-GW	miniCTX2.1-Tc with a Gateway destination cloning site, Tc^R	J J Harrison
pSL3	miniCTX2.1-Tc-GW-araC-pBAD::*lasB*, Tc^R	This study

Supp. Table S2.4: Primers

Primer name	Sequence
oLSL8_Pa_lasR+120F	5'-GCCTAAGGACAGCCAGGAC-3'
oLSL10_Pa_lasR+740F	5'-CGCCGACCTGAGAGAGGCAAGA-3'
lasB-UPF01-attB1	5'- GGGGACAAGTTTGTACAAAAAAGCAGGCTACGGCGAGCGTCA CCTGAAGC-3'
lasB-UPR01-SOEfrag	5'- CAACGCGCTCGGGCAGGTCCCGGCGAAACACCCATGATCG-3'
lasB-DOWNF01	5'-GACCTGCCCGAGCGCGTT-3'
lasB-DOWNR01-attB2	5'- GGGGACCACTTTGTACAAGAAAGCTGGGTAGGCGTGGGCCAG CTGGAC-3'
lasB_outside_F	5'-TGCAGCAGCGGATCGTCG-3'
lasB_outside_R	5'-GCCAGGTACTCGCCTTGC-3'
lasBF01-GWB5-RBS	5'- GGGGACAACTTTGTATACAAAAGTTCGCAGAGGAGGATATTCA TGAAGAAGGTTTCTACG-3'
lasBR01-GWB2	5'- GGGGACCACTTTGTACAAGAAAGCTGGGTATTACAACGCGCTC GGGC-3'
lasR-F	5'- GAGTGGGCTGACTGGACATCTTC – 3'
lasR-R	5'- GGCTTATCCCGAAGCGGCTC- 3'

Supplemental materials and methods

Bacterial mutant strains and plasmid construction

The markerless *lasB* deletion construct was generated by overlap extension PCR to generate a markerless deletion (Δ59-1477bp) of the *lasB* ORF using primers lasB-UPF01-attB1 / lasB-UPR01-SOEfrag and lasB-DOWNFO1 / lasB-DOWNR01-attB2 respectively. The DNA fragment was then recombined into the destination vector pEX18GmGW using BP clonase II (Invitrogen) to generate the suicide vector pSL5. The *⊿lasB* and *⊿lasR* mutants were generated by allelic replacement with the vectors pSL5 and pSB219.9A using established protocols (*306*). Briefly, the parental strain was transformed with the suicide vector by biparental mating. Gentamicin resistant transformants were further counter-selected for *sacB*^{R} on agar plates with 10% sucrose for a second recombination event. The deletion was confirmed by PCR using primers lasB_outside_F and lasB_outside_R for the *⊿lasB* mutation, and oLSL8_Pa_lasR+120F and oLSL10_Pa_lasR+740F the *⊿lasR* deletion.

To generate an inducible *lasB* expression construct, the PAO1 *lasB* ORF was amplified by PCR using primers lasBF01-GWB5-RBS and lasBR01-GWB2, and recombined into pDONR221P5P2 using BP clonase II to generate the entry vector pSL1. The entry vectors pSL1 and pJJH187 were recombined with the destination vector miniCTX2.1-Tc-GW using LR Clonase II Plus (Invitrogen) to generate the *pBAD::lasB* construct, pSL3. The pSL3 vector was subsequently integrated into the genome of the Late isolate creating L+*lasB* as previously described (*305*). *lasB* expression was induced with 2% (w/v) arabinose added to the growth media. All primer sequences are listed in Supp. Table S2.3.

P. aeruginosa phenotypic assays

Twitching motility assay was carried out as described previously (*307*). Briefly, thin 1% agar LB plates were stab inoculated with bacteria from single colonies and incubated for 72 hrs at 37°C. The diameter of the zone of motility at the agar/Petri dish interface was measured after removal of the agar layer.

Pyocyanin was extracted from overnight cultures grown in SCFM with vigorous aeration by chloroform and HCl extraction as previously described (*308*). Absorbance at 520 nm was measured by spectrophotometer (Genesys™10 UV-Vis, Thermoscientific). Pyoverdine was measured in the culture supernatant of overnight cultures grown in SCFM by absorbance at 405 nm as previously described (*309*).

Planktonic and biofilm growth kinetics

For planktonic cultures, overnight cultures grown in SCFM were diluted to OD_{595}=0.05 in 25mL SCFM (250 mL flask), and incubated at 37°C with shaking at 250 r.p.m. Absorbance at 595 nm was measured with a microplate reader (Bio-Rad 3550). For biofilm cultures, overnight cultures grown in SCFM were diluted in 50% SCFM, 20 mM $NaNO_3$, and 0.8% melted agar to a final concentration of 5000 cell/mL, and incubated at 37°C statically. To measure biofilm bacterial density after 48 hrs of growth, biofilm aggregates were homogenized in sterile PBS (Fisher PowerGen 125) and viable bacteria were enumerated by serial microdilution and CFU counts.

Lactate dehydrogenase (LDH) cytotoxicity assay

LDH levels were measured in BALF or AEC supernatants with the CytoTox 96 Cytoxicity Assay (Promega) according to the manufacturer's instructions by absorbance at 490nm with a microplate reader (Bio-Rad Model 680). The cytotoxicity was calculated as the relative LDH levels compared to the positive control, which represents maximal released LDH levels upon complete cell lysis using the Lysis Solution (9% v/v Triton X-100) according to the manufacturer's instructions.

Live and dead cell enumeration by Flow cytometry

BEAS-2B cells were stimulated with bacterial filtrates (or SCFM control) for 24 hrs, washed twice with PBS and the cell pellet was resuspended in a 1:1000 dilution of Fixable Viability Dye eFluor780 (Affymetrix eBioscience) as per

the manufacturer's instructions. Cells were then fixed (Cytofix, BD) and analyzed using a LSRII flow cytometer (BD Biosciences) and FlowJo 10.0.7 software (Tree Star, Stanford, CA).

IL-6 and IL-8 qRT-PCR

BEAS-2B cells were stimulated with bacterial filtrates (or SCFM control) for the indicated time, then washed and resuspended in RLT Plus lysis buffer (Qiagen) with 10% 2-mercaptoethanol. Cells were lysed using the QIAashredder (Qiagen). Genomic DNA was removed and total RNA was extracted and purified using the RNEasy Plus RNA kit (Qiagen) following the manufacturer's instructions. cDNA was synthesized from purified RNA by reverse transcription using random primers (High Capacity Reverse Transcription Kit, Applied Biosystems). Quantitative PCR was performed with the QuantiFast SYBR Green PCR Kit (Qiagen) using the QuantiTect human primer sets (Qiagen) for IL-8 (QT00000322), IL-6 (QT00083720) and GAPDH (QT00079247) on a 7500 Real Time PCR System (Applied Biosystems). PCR negative controls were performed with purified RNA samples to ensure that there was no carryover genomic DNA contamination. Results were analyzed using V500 Life Technologies software (v2.06), with normalization to the SCFM medium control.

Lung inflammation score

Lung inflammation score = Airway inflammation score + Parenchymal inflammation score

Airway inflammation score = Frequency score x Severity score

Frequency of airway inflammation	
0	No airway inflammation present
1	Rare airways affected (<25% section area affected)
2	Occasional airways affected (25-50% section area affected)
3	Frequent airways affected (50-75% section area affected)
4	Most airways affected (>75% section area affected)
Severity of airway bead-associated inflammation	
0	No airway inflammation present
1	Airway lumen with few inflammatory cells

2	Airway lumen with moderate numbers of inflammatory cells, without plugging
3	Airway lumen plugged with inflammatory cells, with little epithelial injury
4	Airway lumen plugged with inflammatory cells, with frequent necrosis/loss of airway wall
Parenchymal inflammation score	
0	within normal limits with minimal inflammatory cells
1	few small foci of inflammatory cells (< 25% section area affected)
2	occasional small or few moderate foci of inflammatory cells (25-50% section area affected)
3	frequent small and moderate, or few extensive foci of inflammatory cells (50-75% of section area affected)
4	abundant inflammatory cell infiltration, (>75% of section area affected)

2.7. Chapter transition

In chapter 2, we investigated how the loss of LasR quorum sensing in CF-adapted isolates alters inflammatory responses during chronic infections. In particular, we focused on how *lasR* mutants alter AEC IL-6 and IL-8 responses using *in vitro* chronic infection models and *in vivo* using a chronic murine model of *P. aeruginosa* pulmonary infection. We found that *lasR* mutants were associated with increased levels of IL-6 and IL-8 compared to wild-type strains, both *in vitro* and *in vivo,* and that this could lead to increased neutrophil recruitment. Finally, we showed that in CF patients chronically colonized by *P. aeruginosa,* there is a positive correlation between the frequency of *lasR* mutants in the lungs and plasma IL-8, a marker of neutrophil inflammation.

We demonstrated that *lasR* mutants elicited greater AEC IL-6 and IL-8 inflammation *in vitro* due to reduced bacterial protease-dependent degradation of these cytokines. We identified the secreted bacterial elastase LasB, whose production and secretion is under the control of LasR, as the protease responsible for the degradation of IL-6 and IL-8 *in vitro*. In chapter 3, we focused more extensively on the role of LasB on host inflammatory cytokine responses during chronic infection using both *in vitro* and *in vivo* models. We also investigate how the loss of LasB, a prototypical *P. aeruginosa* acute virulence factor, affects *P. aeruginosa* virulence and immunopathology during chronic infections *in vivo.*

Chapter 3: Loss of elastase activity in *Pseudomonas aeruginosa* and its impact on host pulmonary inflammation

Manuscript in preparation

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3.1. Introduction

The ubiquitous gram-negative bacterium *Pseudomonas aeruginosa* is a versatile opportunistic pathogen, capable of infecting hosts as distinct as amoebas, plants and mammals (*310*). In humans, *P. aeruginosa* causes a wide-array of acute infections including pneumonia and sepsis (*129*). Expression of a large arsenal of virulence factors, including many secreted products, contributes to the success of this pathogen in such diverse habitats (*311-316*). Although acute *P. aeruginosa* infections can be associated with severe disseminated and rapidly fatal disease, this bacterium also causes chronic infections that remain localized and persist for years to decades. Most notably, *P. aeruginosa* chronically infects the structurally abnormal airways of hosts such as individuals suffering from cystic fibrosis (CF), leading to progressive lung dysfunction (*316, 317*).

In contrast to *P. aeruginosa* isolates recovered from environmental sources or acute infections, isolates recovered from chronic infections are often deficient in the production of acute virulence factors such as pyocyanin or type III secretion system. Notably, protease-deficient *P. aeruginosa* variants are isolated in up to 65% of chronically infected CF patients (*177, 202*), and are even recovered from the airways of patients with chronic obstructive pulmonary disease (COPD) that are chronically colonized with *P. aeruginosa* (*318*). *P. aeruginosa* produces several secreted proteases, which include LasB, LasA and AprA (*316*). The loss of protease activity in CF-adapted *P. aeruginosa* isolates has been mainly attributed to loss-of function genetic mutations in the *lasR* quorum sensing regulator gene (*177, 319, 320*), although it may also result from other mutations (*71, 201, 320-322*).

LasB, a zinc metalloprotease known as *P. aeruginosa* elastase or pseudolysin, is the most abundantly produced exoprotease whose transcription and secretion are largely under the control of LasR-mediated quorum sensing

(*294, 323-325*). LasB is responsible for the majority of secreted elastolytic activity in *P. aeruginosa* isolates, and this activity is often impaired in clinical isolates from chronically infected CF patients (*255, 326, 327*). LasB has an unusually broad substrate range (*328*) and has been primarily recognized as a major acute virulence factor capable of causing direct host tissue damage by degrading components of connective tissue matrix or epithelial barrier (*329-333*). Such direct injury to the host is consistent with the role of LasB in the pathogenesis of invasive infections (*220, 276, 278*). Studies using experimental models of bacteremia (*276*) or acute pneumonia (*220*) indeed report that *lasB* mutants are attenuated in virulence. However, the prevalence of protease and elastase-deficient *P. aeruginosa* isolates in chronic infections and their association with more severe CF lung disease raises questions about the role of secreted *P. aeruginosa* proteases in the pathogenesis of chronic infections (*226, 327, 334*). While these observations suggest that secreted proteases and LasB may be dispensable during chronic infections, how their loss of function contribute to chronic CF lung disease remains unknown.

Chronic *P. aeruginosa* infections in CF lung disease are characterized by an exuberant neutrophil-dominant inflammation that causes tissue damage and decline in pulmonary function (*129, 335-337*). Since LasB is also capable of degrading different components of the immune system and inflammatory mediators such as immunoglobulins (*338-340*) and cytokines (*280, 341-343*), thus dampening immune and inflammatory host responses, the loss of LasB function could paradoxically lead to more exuberant inflammation. This led us to hypothesize that elastase-deficient *P. aeruginosa* strains may promote greater pulmonary inflammation during chronic infections, leading to greater immunopathology. In this study, we examined the impact of *lasB* mutants on airway inflammation *in vitro* and *in vivo* using airway epithelial cell culture systems and a murine model of chronic airway infection with *P. aeruginosa.*

3.2. Results

P. aeruginosa secretes multiple extracellular proteases, including the LasB elastase, and many CF-adapted clinical isolates are elastase-deficient.

We first characterized the extracellular elastase activities in longitudinal pairs of *P. aeruginosa* clinical isolates collected from 13 CF patients (P1-P13) over time. We compared isolates recovered at early stages of disease to isolates recovered at later stages of disease (CF-adapted isolates), as described in Supp. Table S3.1. We measured the total secreted elastase activities of *P. aeruginosa* cultures grown in synthetic CF sputum media (SCFM) [11], a defined medium that mimics the nutrient environment of the CF lung. Consistent with previous reports, we found that a greater proportion of isolates deficient in secreted elastase production were recovered at later stages of disease (Fig. 3.1A). In eight of thirteen (61.5%) pairs, CF-adapted isolates had reduced secreted elastase activity compared to early infection isolates. Although five of thirteen paired isolates had similar elastase activity, no CF-adapted isolate had greater elastase activity than their paired early infection isolate (Fig. 3.1A). In order to examine the specific contribution of LasB to secreted protease activity in a clinically relevant genetic background, we constructed an engineered *lasB* mutant (Δ*lasB*) in an early infection isolate recovered from a young CF patient (AMT0023-30 (*177*)) at 6 months of age. Compared to the parental early stage isolate (referred to as wild-type or WT), the isogenic Δ*lasB* mutant displayed minimal elastolytic activity (Fig. 3.1A) and caseinolytic activity (Fig. 3.1B), but preserved other secreted proteolytic activities such as collagenase activity (Fig. 3.1C). This confirmed that LasB is the primary protease conferring elastase activity, and that its loss reduces some but not all secreted proteolytic activity in *P. aeruginosa*. This also led us to infer that the loss of elastase activity among CF-adapted *P. aeruginosa* isolates is likely due to a loss of LasB activity.

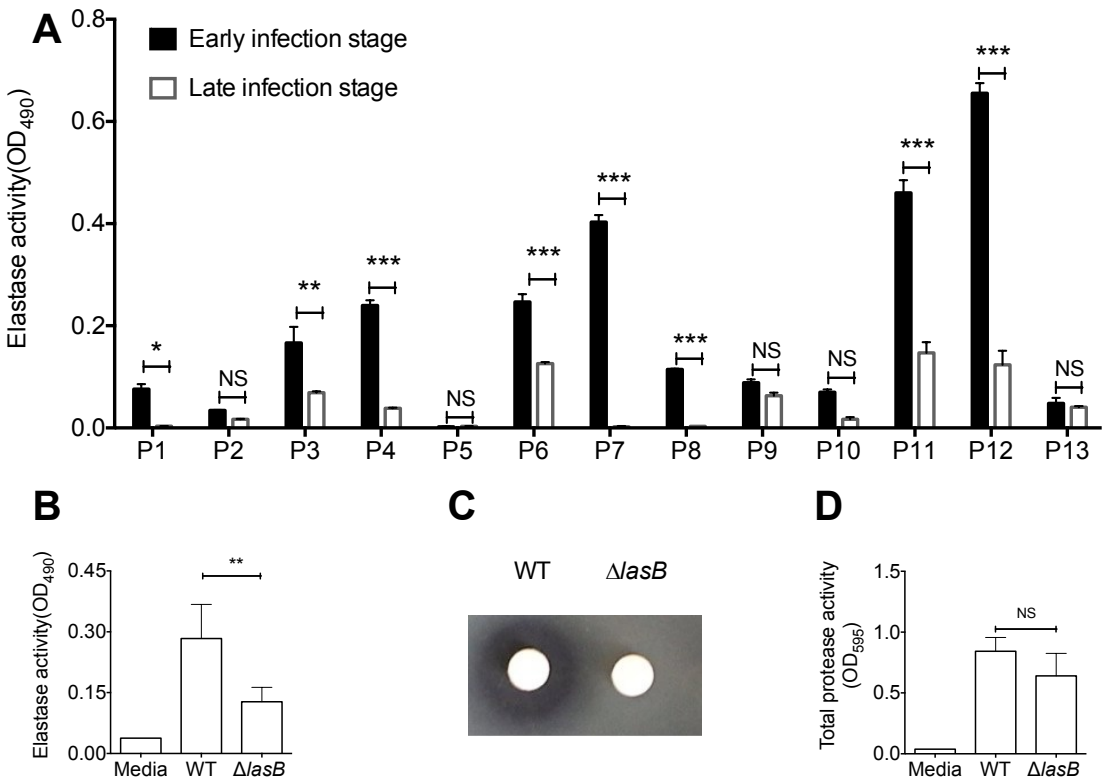


Fig. 3.1: CF-adapted *P. aeruginosa* isolates are deficient in elastase activity

(A) Specific elastolytic activity of longitudinal *P. aeruginosa* isolates recovered from 13 different CF patients (P1-P13) at early and late infection stages.
(B) Specific elastolytic activity of a paired early infection isolate (WT) and its isogenic *lasB* mutants
(C) Secreted proteolytic activity measured by skim milk agar assay
(D) Secreted proteolytic activity measured by Hide-Remazol Brilliant Blue assay.
Results shown are the mean (±SD) of two technical replicates and are representative of n≥2 independent experiments. Statistical comparisons in A were done using 2-way ANOVA analysis followed by Bonferroni's multiple comparison between isolates from the same patients. Statistical comparison in B and D were done using a two-tailed unpaired student t-test. NS= not significant, $p>0.05$;*=$p\leq0.05$;**=$p\leq0.01$;***=$p\leq0.001$

Extracellular LasB induces airway epithelial cell IL-6, IL-8 and IL-1β mRNA expression.

During chronic infections in the CF airways, *P. aeruginosa* grows within the mucus layer overlying the airway epithelium and indirectly interacts with airway epithelial cells (AEC) without invasion (*145, 263*). The AEC response to diffusible bacterial products, such as extracellular proteases, therefore approximates important host-pathogen interactions relevant to chronic CF airway infections. AEC stimulated with cell-free bacterial filtrates also show no significant cytotoxicity even after prolonged co-incubation (Supp. Fig. S3.1A). To examine how the loss of LasB function affects host inflammatory responses, we compared the AEC cytokine responses to secreted products made by the WT strain and Δ*lasB* mutant. Immortalized human BEAS-2B cells were stimulated with cell-free bacterial filtrates containing diffusible bacterial products from WT and Δ*lasB* mutant cultures grown in SCFM. IL-6 and IL-8 mRNA expression increased within 3 hrs following stimulation with WT and Δ*lasB* filtrates, but WT filtrates induced higher IL-6 and IL-8 mRNA levels compared to Δ*lasB* filtrates, with 2.4-fold higher IL-6 ($p \leq 0.001$) and 2.2-fold higher IL-8 ($p \leq 0.001$) by 9 hrs (Fig. 3.2A and 3.2B). IL-1β mRNA expression was induced 2 fold ($p \leq 0.01$) as early as 1 hr after stimulation with WT filtrates and peaked at 6 hrs (4.8 fold increase, $p \leq 0.001$). In contrast, Δ*lasB* filtrates didn't induce IL-1β mRNA levels above baseline expression (Fig. 3.2C). Finally, TNF-α mRNA expression was induced to equivalent levels with WT and Δ*lasB* filtrates (Fig. 3.2D). Together, this indicates that LasB induces IL-6, IL-8 and IL-1β transcriptional responses in AEC.

LasB inactivation enhances G-CSF and GM-CSF mRNA expression in AEC.

AEC are an important source of the growth factors G-CSF and GM-CSF and expression of these factors is induced upon stimulation with *P. aeruginosa* (*344*). Although both WT and Δ*lasB* filtrates initially induced G-CSF and GM-CSF mRNA at equivalent levels (up to 3 hrs for G-CSF and 6 hrs for GM-CSF), G-CSF levels were 3.6 fold higher ($p \leq 0.05$) in Δ*lasB* stimulated AEC by 6 hrs and GM-

CSF levels were 8.3 fold higher ($p \leq 0.001$) by 9 hrs (Fig. 3.2E and 3.2F). In contrast to the IL-6, IL-8 and IL-1β responses, the loss of LasB enhanced the mRNA expression of G-CSF and GM-CSF in AEC.

Stimulation with Δ*lasB*-filtrates lead to increased extracellular cytokine levels in AEC cultures.

Although the mRNA expression of cytokines is an important component of the AEC sensing and response to pathogen-associated molecular patterns (PAMPs), cytokine activity is determined by extracellular protein levels. IL-6. IL-8, TNF-α, IL-1β, G-CSF and GM-CSF mRNAs are all subject to post-transcriptional control by mechanisms that modify mRNA stability (*345*). TNF-α and GM-CSF mRNAs are also subject to translational control (*345*). Additionally, proteolytic inactivation of IL-6, IL-8, IL-1β, TNF-α and G-CSF by host proteases controls the cytokine bioactivity and availability at sites of inflammation (*256, 343, 346-349*). We therefore measured extracellular cytokine protein levels that accumulated in AEC culture supernatants in response to WT and Δ*lasB*-filtrates by ELISA. In contrast to mRNA expression, levels of IL-8 protein levels were initially identical at 1 hour after stimulation with WT and Δ*lasB* filtrates, but steadily increased over time in response to Δ*lasB* filtrates, but not the WT filtrates (Fig. 3.3A). IL-6, G-CSF and GM-CSF protein levels were nearly undetectable in AEC stimulated with WT filtrates, but steadily increased over time upon stimulation with Δ*lasB* filtrates (Fig. 3.3B-3.3D). IL-1β and TNF-α protein levels were undetectable in all conditions (data not shown). Overall, these findings reveal significant differences between the mRNA and extracellular protein patterns in response to WT and Δ*lasB* filtrates. In particular, our results demonstrate that *lasB* mutants induce significantly greater AEC-mediated inflammatory cytokine responses at the extracellular protein level, regardless of the transcriptional response.

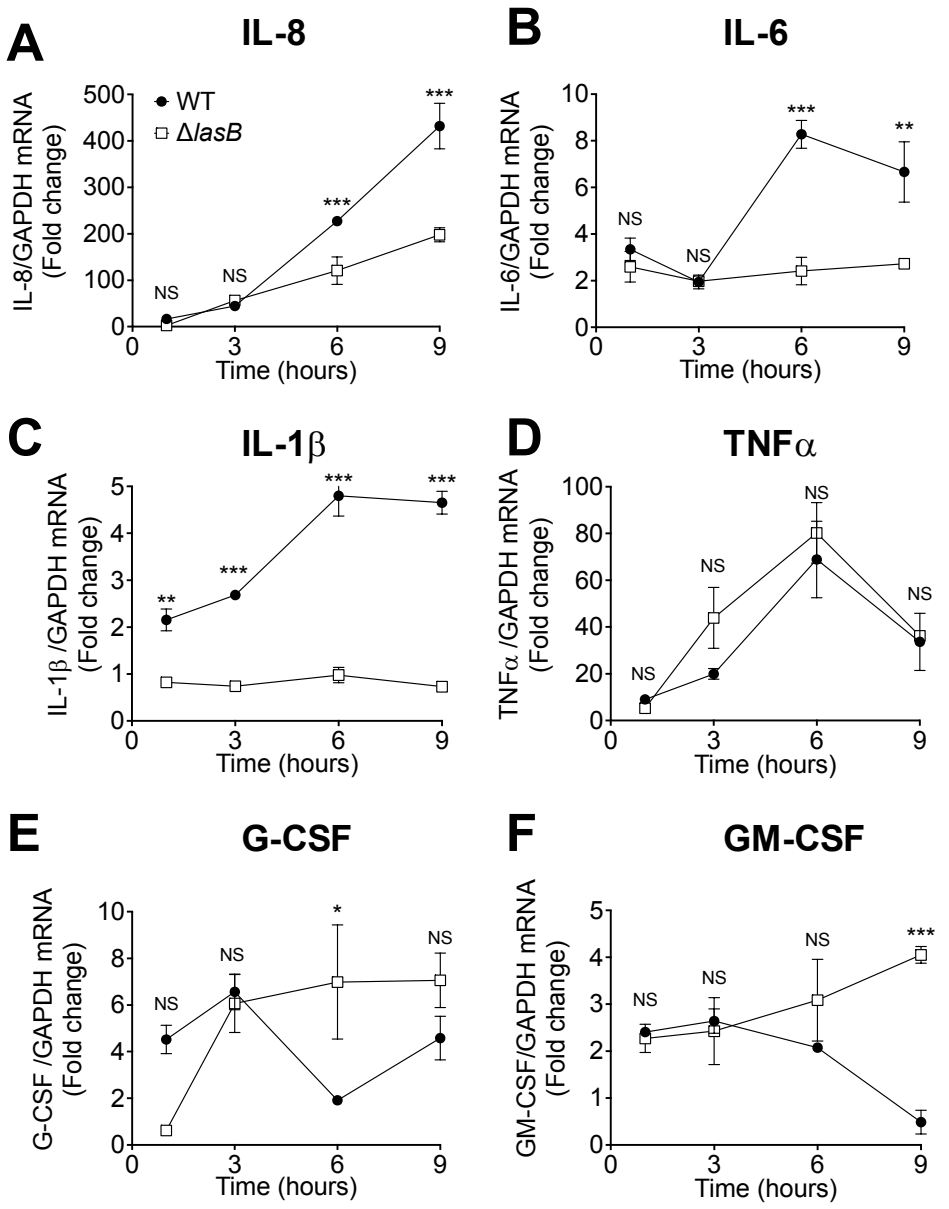


Fig. 3.2: LasB protease modulates AEC pro-inflammatory cytokine transcriptional responses

(A) IL-8 **(B)** IL-6 **(C)** IL-1β **(D)** TNF-α, **(E)** G-CSF and **(F)** GM-CSF mRNA expression were determined by qRT-PCR. BEAS-2B cells were stimulated with 60 µL filtrates or medium control (SCFM) for the indicated duration. Results shown are the mean (±SD) fold change of each transcript relative to control cells and normalized to GAPDH mRNA. Results are from three biological replicates and one independent experiment. NS= not significant, $p>0.05$;*=$p\leq0.05$; ;**=$p\leq0.01$; ;***=$p\leq0.001$

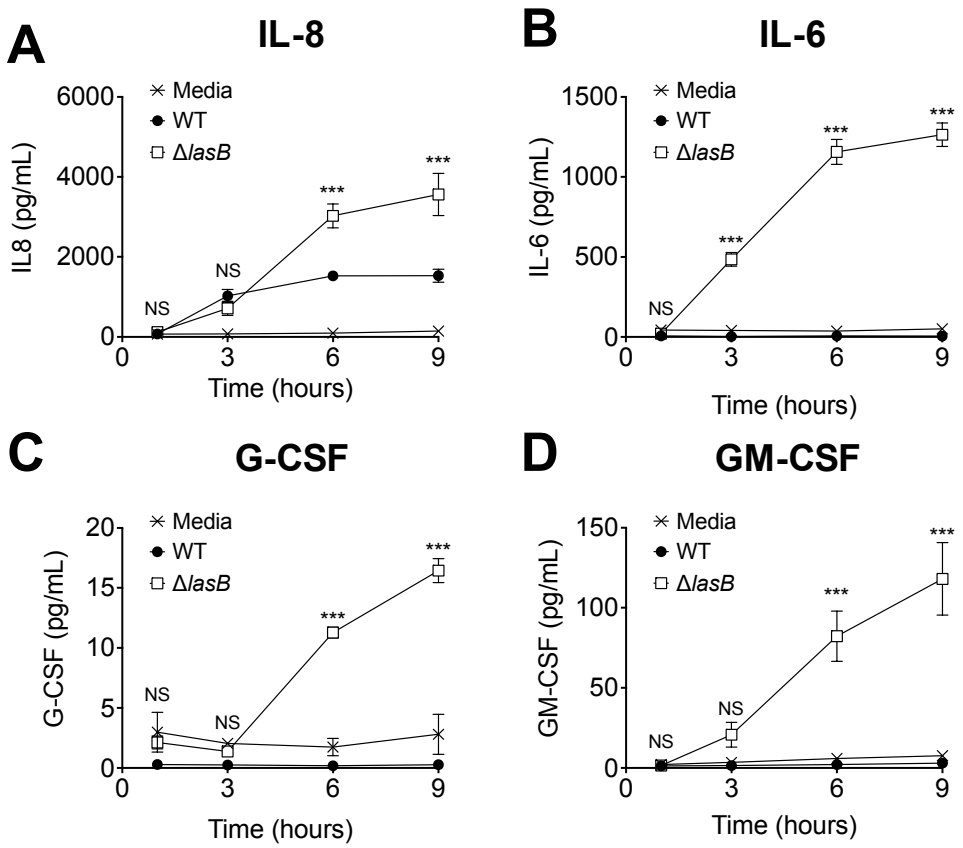


Fig. 3.3: Loss of LasB function leads to higher protein levels of pro-inflammatory cytokines and granulopoeitic cytokines in AEC cultures.

(A) IL-8, **(B)** IL-6, **(C)** G-CSF and **(D)** GM-CSF protein levels in AEC condition supernatant following stimulation with bacterial filtrates. BEAS-2B cells were stimulated with 60µL filtrates or media control for the indicated time. Cytokines were measured by sandwich ELISA. TNF-α and IL-1 protein were undetectable in AEC condition supernatants (data not shown). Results shown are the mean (±SD) pooled from 3 independent experiments that had three biological replicates for each treatment at each time point. NS= not significant, $p>0.05$;*=$p\leq0.05$; **=$p\leq0.01$; ;***=$p\leq0.001$

LasB acts in concert with other bacterial proteases to degrade extracellular cytokines in AEC cultures.

The discrepancy between IL-6 and IL-8 mRNA and secreted protein levels suggested that post-transcriptional processes dampened the IL-6 and IL-8 extracellular protein responses. Based on previous observations that *P. aeruginosa* proteases can degrade IL-6 and IL-8 *in vitro* (*1, 343*), we hypothesized that WT-stimulated AEC cultures do not accumulate high levels of extracellular cytokines due to protease-mediated degradation. To test this, we first examined how inactivation of heat labile factors in bacterial filtrates alter extracellular cytokine levels in WT-filtrate stimulated AEC cultures. As shown in Fig. 3.4, heat-treatment of WT filtrates restored cytokine levels in stimulated AEC cultures to 2.8-fold higher for IL-8 ($p \leq 0.001$) and 833-fold higher for IL-6 ($p \leq 0.001$) compared to AEC cultures stimulated with non-heat treated WT filtrates. Similar results were observed with G-CSF and GM-CSF levels, which were restored 4.5-fold ($p \leq 0.01$) and 8.6-fold ($p \leq 0.001$) respectively by heat-treatment of WT filtrates. To confirm that increased cytokine levels were due to inactivation of heat-labile proteases, we next tested phosphoramidon, a metallo-protease inhibitor of LasB (*294, 350*). Treatment of WT-filtrates with phosphoramidon increased IL-8 levels by 2.4-fold ($p \leq 0.001$), IL-6 levels by 96-fold higher ($p \leq 0.001$), G-CSF by 5.4-fold ($p \leq 0.001$) and GM-CSF by 6.5-fold ($p \leq 0.01$) in AEC cultures compared to WT filtrates without phosphoramidon.

Phosphoramidon, which preferentially targets LasB, did not always restore cytokine levels in WT filtrate-stimulated AEC cultures to the same degree as heat treatment of filtrates, which non-specifically inactivates heat-labile enzymes. This led us to investigate whether other *P. aeruginosa* secreted proteases contribute to the dampening of extracellular cytokines. We stimulated AEC with cell-free filtrates prepared from a laboratory strain (PAO1-V) and derived Δ*aprA*, Δ*lasA*, Δ*lasB* single mutants, as well as a Δ*aprA* Δ*lasA* Δ*lasB* triple mutant that had all three proteases knocked out.

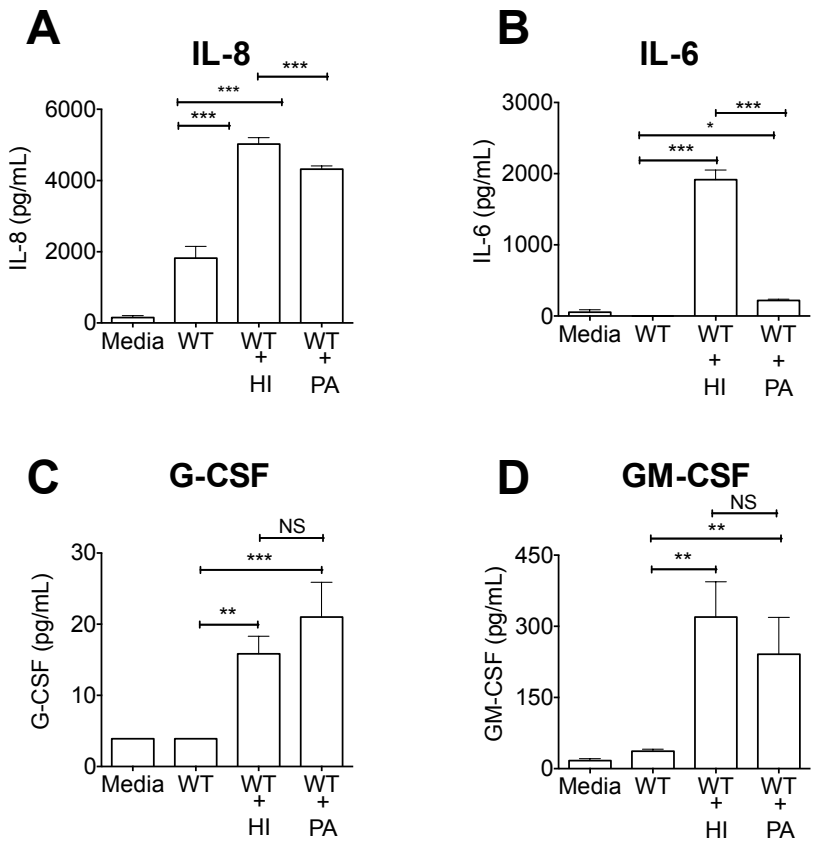


Fig. 3.4: Heat treatment and LasB inhibition increases extracellular cytokine levels in AEC cultures

BEAS-2B cells were stimulated with WT filtrates, heat inactivated WT filtrates (+HI), WT filtrates +250µg/mL phosphoramidon (+PA), or media (SCFM) only for 9 hrs. Levels of **(A)** IL-8 **(B)** IL-6 **(C)** G-CSF and **(D)** GM-CSF protein were measured in the AEC conditioned supernatant by sandwich ELISA. Results shown are the mean (±SD) from three biological replicates and one independent experiment. NS= not significant, $p>0.05$;*=$p\leq0.05$; ;**=$p\leq0.01$; ;***=$p\leq0.001$

Stimulations with the Δ*lasB* single mutant and the Δ*aprA* Δ*lasA* Δ*lasB* triple mutant led to the greatest increase in all cytokine levels compared to treatment with filtrates from the wild-type parental strain PAO1-V (Fig. 3.5 A). Δ*lasB* stimulation led to 3.3-fold higher IL-8 ($p\leq0.001$) 51-fold higher IL-6 ($p\leq0.001$), 151-fold higher G-CSF ($p\leq0.001$), and 61-fold higher GM-CSF ($p\leq0.001$) compared to PAO1-V stimulation (Fig. 3.5B to 3.5D). That the Δ*aprA* Δ*lasA* Δ*lasB* triple mutant was associated with higher cytokines levels than the Δ*lasB* mutant (1.4 fold for IL-8 ($p\leq0.001$), 3.1 fold for G-CSF ($p\leq0.001$), 4.4 fold for GM-

CSF ($p \leq 0.001$), suggests that LasA and/or AprA may have an additive or synergistic effect with LasB in degrading these cytokines (Fig. 3.5A, 3.5C and 3.5D). Interestingly, all three proteases are required for maximal secreted elastase activity (*351*). Together, these results demonstrate that LasB, acting alone or in concert with AprA and LasA, can significantly dampen extracellular cytokine levels in AEC cultures.

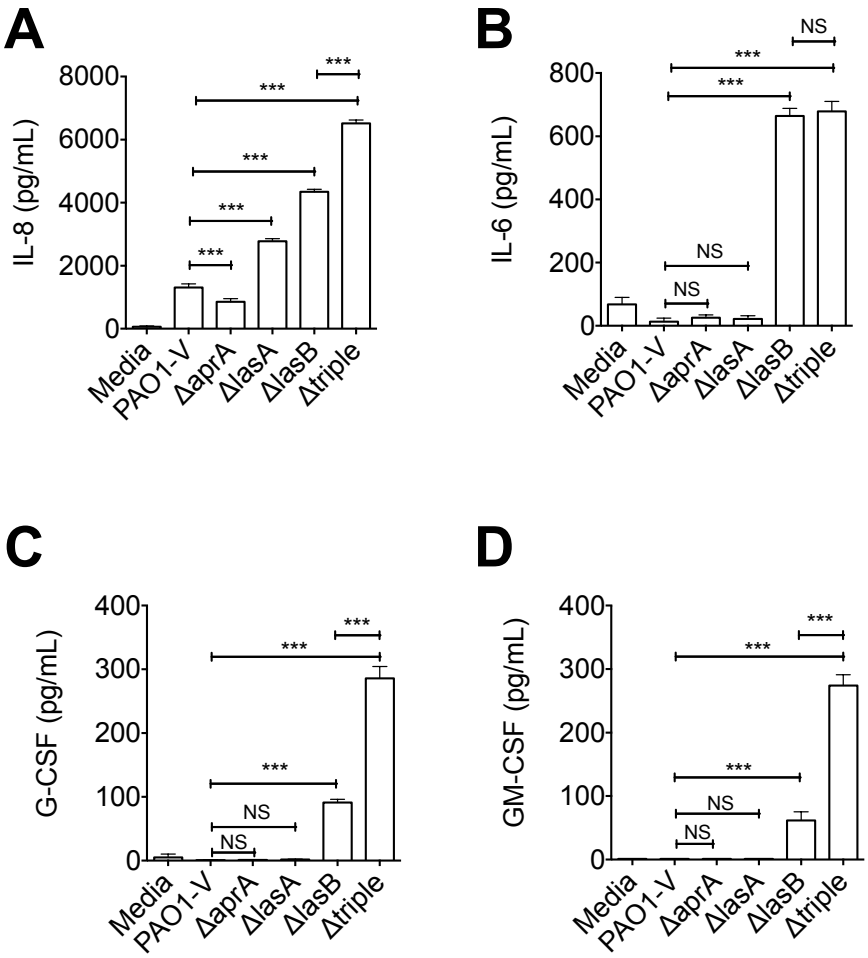


Fig. 3.5: LasB, alone or in concert with LasA and AprA, dampens extracellular cytokine levels in AEC cultures

BEAS-2B cells were stimulated with a laboratory strain (PAO1-V), the isogenic Δ*aprA*, Δ*lasA* and Δ*lasB* single mutants, Δ*aprA lasA lasB* triple mutant or media only control for 9 hrs. Levels of **(A)** IL-8 **(B)** IL-6 **(C)** G-CSF and **(D)** GM-CSF protein were measured in BEAS-2B conditions supernatant by sandwich ELISA. Results shown are the mean (±SD) from three biological replicates and one independent experiment. NS= not significant $p>0.05$;***=$p \leq 0.001$

Chronic pulmonary Δ*lasB* infections induce hyperinflammatory cytokine responses.

Since AEC cytokine responses are key determinants of the host immune response to pulmonary *P. aeruginosa* infections, our *in vitro* results led us to hypothesize that chronic Δ*lasB* pulmonary infections would be associated with hyperinflammatory responses compared to WT infections *in vivo*. We used a chronic murine pulmonary infection model with *P. aeruginosa* embedded in agar beads, a well established model which causes endobronchial obstruction, sub-acute, non-lethal and persistent infections associated with localized areas of inflammation and necrosis (*251*). Male C57BL/6 mice infected with $8.0x10^5$ CFU/mouse of the WT or Δ*lasB* strains showed equivalent bacterial burden at day 1 post-infection (p.i.) (median CFU $3.6x10^6$ (IQR $7x10^5$-$6.6x10^6$) vs. $3.3\ x10^5$ (IQR $1.8x10^5$-$9.7x10^6$), $p>0.05$) and day 4 p.i. (median CFU $7.8x10^4$ (IQR $5.9x10^4$-$2.4x10^5$) vs. $1.25\ x10^5$ (IQR:$6.5x10^3$-$6.3x10^6$) $p>0.05$) (Fig. 3.6A).

To examine the pulmonary inflammatory responses associated with these infections, we measured pro-inflammatory cytokine levels in the BALF of infected and control mice at day 4 p.i. As expected, KC (CXCL1), MIP-2 (CXCL2), LIX (CXCL5), IL-6, IL-1β, TNF-α and G-CSF were all significantly increased in WT-infected mice compared to controls (Fig. 3.6B to 3.6I). Remarkably, the BALF of Δ*lasB* –infected mice showed higher levels of the neutrophil chemokine KC (4.1-fold, $p\leq0.01$), pro-inflammatory cytokines IL-1β (59.3-fold, $p\leq0.05$), TNF-α (5.5-fold, $p\leq0.05$) as well as the granulopoeitic growth factor GM-CSF (9.6- fold, $p\leq0.01$) compared to WT-infected mice (Fig. 3.6B, 3.6E, 3.6F, and 3.6G). Δ*lasB* pulmonary infections were also associated with increased systemic levels of pro-inflammatory cytokines, with higher plasma levels of KC (3.2- fold, $p\leq0.05$) and G-CSF (5.8-fold, $p\leq0.05$) compared to WT-infected mice (Fig. 3.7). No differences were observed with IL-1β, GM-CSF, IL-6, LIX and TNF-α plasma levels between treatment groups, while MIP-2 plasma levels differed between both infection groups but were not significantly increased compared to controls (Supp. Fig. S3.2). These results thus demonstrate that the Δ*lasB* mutant induces

an exuberant local and systemic pro-inflammatory cytokine response in the chronic airway infection model.

Chronic pulmonary Δ*lasB* infections are associated with increased pulmonary neutrophilia.

To determine whether increased levels of cytokines lead to greater pulmonary inflammation in Δ*lasB*-infected mice, we immunophenotyped pulmonary leukocytes (CD45+ cells) and neutrophils ($CD45^+$ $Ly6G^{hi}$ $CD11b^+$) by FACS analysis. At day 4 p.i, the total number of leukocytes ($1.8x10^7$ vs $1.1\ x10^7$, *p≤0.05*) (Fig. 3.8A) and neutrophils ($8.4x10^6$ vs $1.5x10^7$, *p≤0.05*) (Fig. 3.8B) were higher in the lungs of Δ*lasB*-infected mice than WT-infected mice, with neutrophils representing 83% vs. 69.5% (*p*≤0.01) of leukocytes (Fig. 3.8C). The total BALF neutrophil count was 2.5-fold higher (*p*≤ 0.05) in Δ*lasB*-infected mice compared to WT infected mice (Fig. 3.8E), although no significant differences in total leukocytes (Fig. 3.8D) or the proportion of neutrophils (Fig. 3.8F) were observed in the BALF. Manual differential cell counts confirmed that the leukocytes in the BALF from both Δ*lasB* and WT-infected mice were predominantly neutrophils (91% and 74% respectively), whereas control mice mainly had monocytic cells (90%) in the BALF (Fig. S3.3A). Furthermore, the manual counts of BALF neutrophils were higher in the Δ*lasB* group compared to WT ($16x10^5$/mL vs $5x10^5$/mL, $p < 0.05$) (Supp. Fig. S3.3B), but no significant differences were noted with monocytes nor lymphocytes (Supp. Fig. S3.3C and S3.3D). Interestingly, the BALF neutrophilia in Δ*lasB*-infected mice positively correlated with BALF G-CSF (R^2= 0.82, p=0.005) and MIP-2 levels (R^2= 0.89, p= 0.001) (Supp Fig. S3.5E) but not other cytokines, and this correlation not observed in WT-infected mice, suggesting an important contribution of these two mediators in the pulmonary neutrophilia associated with Δ*lasB* chronic lung infection.

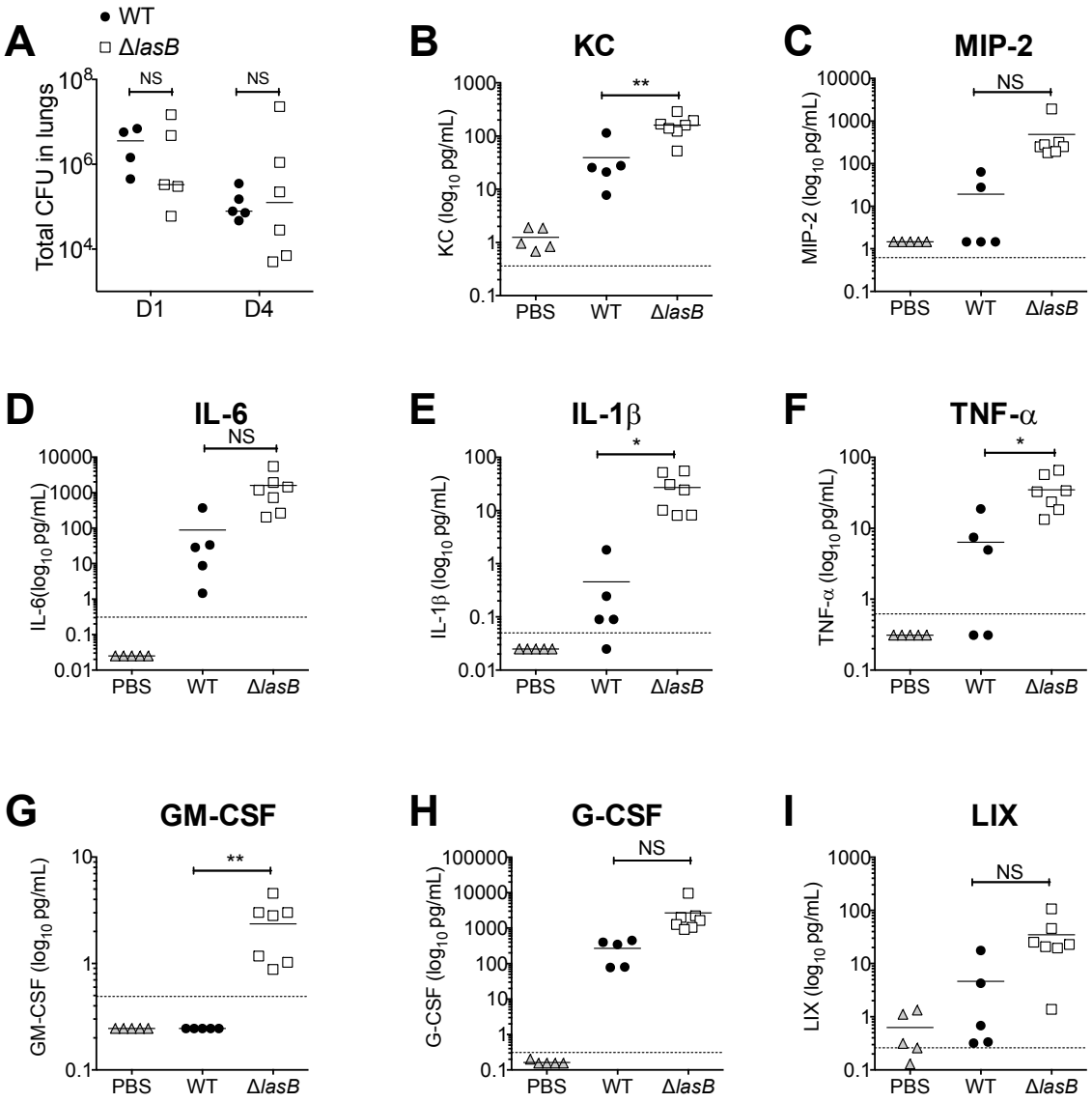


Fig. 3.6: Δ*lasB* infections are associated with increased neutrophil chemokines, pro-inflammatory cytokines and granulopoeitic cytokines

(A) Bacterial burden in whole lungs at days 1 and 4 p.i.
(B) KC, **(C)** MIP-2**, (D)** IL-6**, (E)** IL-1β **(F)** TNF-α, **(G)** GM-CSF,**(H)** G-CSF and **(I)** LIX in BALF at day 4 p.i. measured by multiplex assay. The dotted lines indicate the lower limit of detection (LOD) for each cytokine. Cytokine values below the LOD were arbitrarily assigned a value equivalent to half the LOD. Results are shown as mean. Statistical comparisons were done using two-tailed unpaired student t-test. NS= not significant $p>0.05$; *=$p\leq0.05$;**=$p\leq0.01$

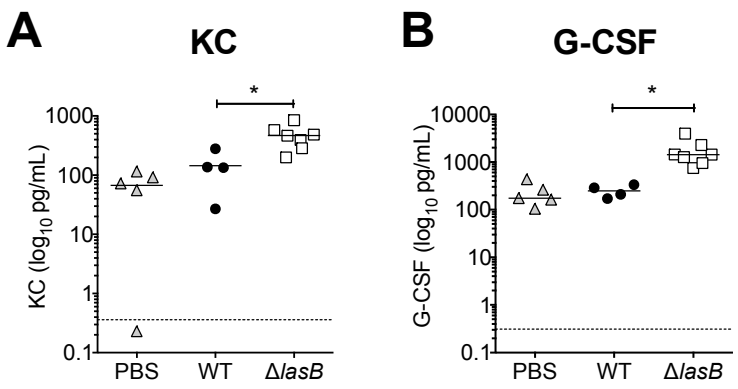


Fig. 3.7: Δ*lasB* infected mice have increased systemic KC and G-CSF

(A) KC and **(B)** G-CSF in plasma at day 4 p.i.were measured by multiplex assay. The dotted lines indicate the lower limit of detection (LOD) for each cytokine. Cytokine values below the LOD were arbitrarily assigned a value equivalent to half the LOD. Results are shown as mean. Statistical comparisons were done using two-tailed unpaired student t-test. *=$p \leq 0.05$;**=$p \leq 0.01$

Δ*lasB* mutants cause greater pulmonary immunopathology and morbidity in chronic infections.

Histological analysis of lung sections recovered at day 4 p.i revealed foci of PMN-dominant inflammation, often associated with endobronchial *P. aeruginosa* agar beads (Fig. 3.9A). Using a histological score for airway and parenchymal inflammation, the lungs of the Δ*lasB* group showed significantly greater inflammation than the WT group (score of 11.6 vs 5.6, $p \leq 0.05$) (Fig. 3.9B), with evidence of alveolar fibrin accumulation, alveolar edema and pleuritis absent in WT-infected lungs, suggesting greater immunopathology. Furthermore, the wet lung/body weight ratios (*352*) and BALF total protein levels, two measures of lung injury, were 2.4 fold higher ($p \leq 0.05$) and 2.3 fold higher ($p \leq 0.01$) respectively in the Δ*lasB* compared to WT group (Fig. 3.9C and 3.9D). Finally, Δ*lasB*-infected mice lost significantly more weight than WT-infected mice by day 4 p.i (25.5% versus 14.9%, $p \leq 0.01$), even though they had lost less weight by day 1 p.i. (14.4% versus 12.2%, $p \leq 0.05$) (Fig. 3.9E). Taken together, these results show that Δ*lasB* chronic airway infections result in more severe lung immunopathology and morbidity than WT infections

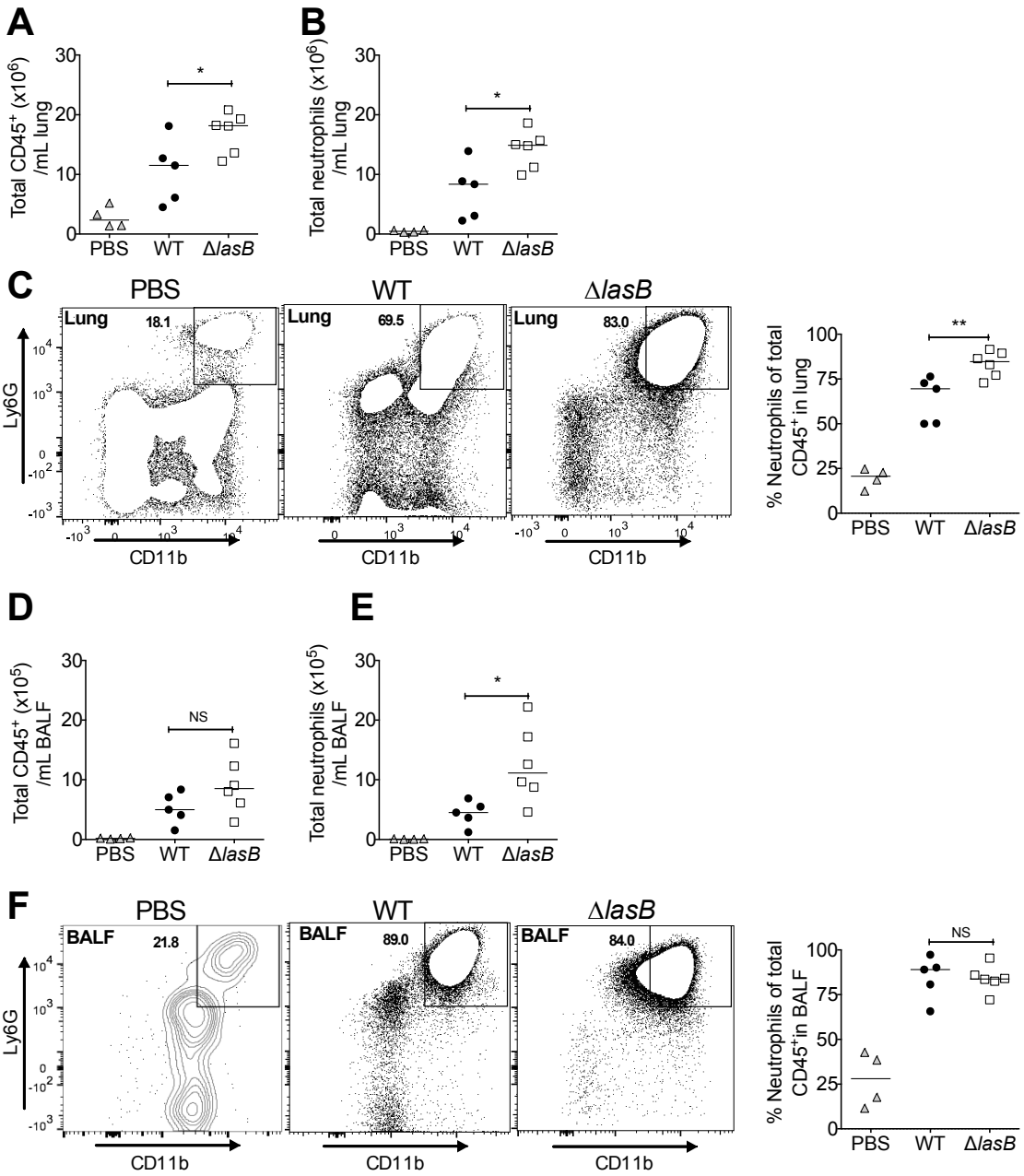


Fig. 3.8: Δ*lasB* infected mice show increased neutrophil-dominant pulmonary inflammation

(A) Total leukocytes ($CD45^+$) counts in lung tissues.
(B) Total neutrophils ($CD45^+$ $Ly6G^{hi}$ $CD11b^+$) counts in lung tissues.
(C) Proportion of neutrophils ($CD45^+$ $Ly6G^{hi}$ $CD11b^+$) among total leukocytes ($CD45^+$) in the lung tissues.
(D) Total BALF leukocytes ($CD45^+$) counts.
(E) Total BALF neutrophil ($CD45^+$ $Ly6G^{hi}$ $CD11b^+$) counts**.**
(F) Proportion of BALF neutrophils ($CD45^+$ $Ly6G^{hi}$ $CD11b^+$) among total leukocytes ($CD45^+$).

The BALF and lung homogenates of infected or control mice at day 4 p.i were analyzed by FACS. Representative FACS scatter plots for each treatment group are shown. Results are shown as median. Statistical comparisons were done using Mann Whitney test. NS= not significant $p>0.05$; *=$p\leq0.05$;**=$p\leq0.01$.

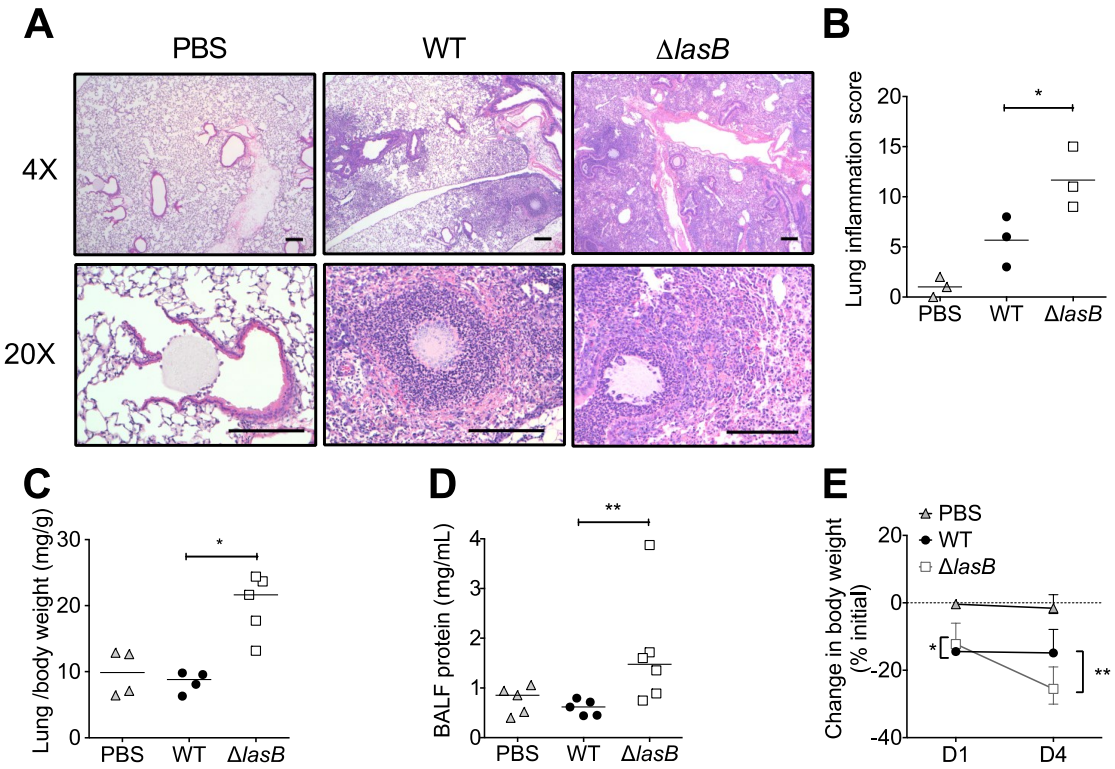


Fig. 3.9: Δ*lasB* strain increased morbidity and lung injury in a murine chronic airway infection model

(A) Representative H&E stained lung sections from mice infected with WT, *ΔlasB* PBS agar beads at day 4 p.i. Scale bar=200μm
(B) Total inflammation score from histologic analysis of lung sections \
(C) Wet lung to body weight ratio on day 1 p.i.
(D) BALF total protein on day 4 p.i.
(E) Body weight change on day 1 and 4 p.i., relative to body weight at the time of infection (day 0).
Results are in B to D shown as median. Results in E are shown as median (± IQR). Statistical comparison for B was done by two-tailed unpaired student t-test. Statistical comparisons for C-E were done using Mann Whitney test.

3.3. Discussion

LasB is a complex virulence factor known to play a pivotal role in *P. aeruginosa* acute infections. This broad-specificity protease can degrade structural components in host connective tissue such as elastin (*329, 333*), disrupt epithelial tight-junctions (*330*) and basement membranes (*353*), and reduce endothelial barrier integrity (*331, 332*), thus causing tissue damage and invasion during acute infections (*16-20*). LasB has also been reported to induce IL-8 levels and neutrophil recruitment in a rat air pouch inflammation model (*354*). Conversely, many studies have also reported that LasB can degrade and inactivate numerous components of the immune system such as surfactant proteins SP-A (*286*) and SP-D (*355*), immunoglobulins IgA (*340*) and IgG (*339*), antimicrobial peptides (e.g. LL-37 (*356*)) and membrane immune receptors (e.g. PAR-2(*357*), uPAR(*358*)). In vitro studies have shown that immune mediators such as cytokines (e.g. RANTES (*280*), INFγ (*341, 342*), IL-6 (*343*)) and chemokines (e.g. IL-8/CXCL8 (*343*), MCP-1 (*280*), CXCL-5(*280*)) can be degraded by LasB activity. It is therefore possible that LasB-mediated proteolytic processing of host immune mediators and components allows *P. aeruginosa* to subvert and evade host responses. While these mechanisms undoubtedly contribute to the pathogenesis of acute and invasive *P. aerguinosa* infections, it is less clear how they impact chronic and non-invasive disease.

Protease-deficient *P. aeruginosa* variants readily emerge and are highly prevalent in chronic CF airway infections (*177, 202, 255, 318*). These variants can also be recovered from the respiratory tract of COPD patients chronically colonized with *P. aeruginosa* (*318, 319*) and from chronic wound infections (*359*). Surprisingly, reduced protease activity in *P. aeruginosa* isolates was found to be strongly associated with more advanced chronic lung disease in CF patients (*226*). In a longitudinal study of young CF patients chronically infected with *P. aeruginosa,* patients harboring high elastase producing strains had better clinical scores than those with low elastase producing strains (*334*). These clinical

observations thus lead us to ask whether the loss of bacterial protease activity was only a marker of more severe disease, or could contribute to the disease progression.

AEC cytokine responses are central in activating the host immune response to bacterial stimuli. During CF chronic lung infections, *P. aeruginosa* typically grows within the viscous mucus layer overlying AEC, and is not invasive. Our *in vitro* and *in vivo* studies were designed to replicate the indirect nature of the *P. aeruginosa*–AEC interactions during chronic infection of the CF lung, which likely occurs through secreted and diffusible factors, rather than direct cell-to-cell interactions. In this study, we stimulated immortalized human AEC with cell-free filtrates from *P. aeruginosa* planktonic cultures and we infected mice with *P. aeruginosa* embedded in agar beads in order to minimize direct contact between bacterial cells and host cells while still allowing contact of host cells with diffusible bacteria products. We demonstrated that the loss of LasB in *P. aeruginosa* amplifies pro-inflammatory cytokine responses in AEC cultures, primarily through a loss of LasB-mediated cytokine degradation. In a murine model of subacute and non-invasive *P. aeruginosa* airway infection, the *ΔlasB* mutant was associated with elevated levels of pro-inflammatory cytokines both locally and systemically compared to a WT strain. Notably, we also found that *ΔlasB*-infected mice had significantly greater pulmonary neutrophilia, lung immunopathology and morbidity compared to WT-infected mice.

Exaggerated pro-inflammatory cytokine production by AEC is a hallmark of *P. aeruginosa* chronic infections in CF (*92, 93, 95, 360, 361*). IL-8, IL-6, TNF-α and IL-1β are elevated in the airways and blood of CF patients and undoubtedly contribute to neutrophil recruitment and activation in the CF lung (*92-94, 360, 362*). The granulopoeitic growth factors G-CSF and GM-CSF, which stimulate neutrophil production and maturation in the bone marrow, can also be elevated in the blood of CF patients (*363, 364*). Previous studies using purified enzyme have suggested that LasB induces IL-8 production *in vitro* (*269*) and *in vivo* (*354*). Our

in vitro results indeed demonstrate that LasB induces a pro-inflammatory cytokine transcriptional response, with increased IL-8, IL-6 and IL-1β mRNA expression in human AEC. That LasB did not induce TNF-α, GM-CSF nor G-CSF mRNA expression suggests that the transcriptional response observed is specific. However, the secreted protein levels in AEC stimulated with bacterial filtrates suggest a completely different picture. Despite the transcriptional up-regulation of IL-8, IL-6, G-CSF and GM-CSF in AEC in response to secreted bacterial factors, the presence of LasB nearly completely abrogated extracellular cytokine levels in conditioned AEC cultures. By extensively altering the extracellular cytokine levels, LasB likely exerts an important modulatory effect in the extracellular cytokine milieu with functional consequences on the inflammatory responses to chronic *P. aeruginosa* infection.

LasB accounts for most of *P. aeruginosa*'s secreted elatase activity and can accumulate to high concentrations in the respiratory tract of CF patients (*273, 365*). Since LasB can degrade cytokines and immune mediators, the low extracellular cytokine levels in the AEC cultures are most likely the result of LasB-mediated proteolytic degradation. Both heat treatment to inactivate heat labile factors (such as LasB) and the use of phosphoramidon, a specific bacterial elastase inhibitor, significantly restored extracellular cytokine levels in AEC stimulated with WT filtrates and support the notion that LasB-mediated degradation has a dominant effect on airway inflammatory cytokine responses. That phosphoramidon treatment did not completely restore cytokine levels is likely due to the residual elastase activity in WT filtrates from incomplete inhibition of LasB (data not shown), in contrast to heat treatment that completely abrogates elastase activity in WT filtrates. It is also possible that other heat-labile but phosphoramidon-resistant factors further dampen IL-6 and IL-8 responses in AEC cultures. The results obtained with the Δ*aprA,* Δ*lasA*, Δ*lasB* single mutants and the triple protease mutant suggest, that while LasB alone is sufficient to degrade all four cytokines, there may be additive and synergistic interactions between this protease and AprA or LasA resulting in greater degradation of IL-8,

G-CSF and GM-CSF. Our studies strongly suggest that both hematopoietic cytokines G-CSF and GM-CSF are sensitive to proteolytic degradation by LasB and other *P. aeruginosa* secreted proteases. While LasB-mediated degradation of IL-8 and IL-6 has been previously reported (*1, 343*), degradation of G-CSF and GM-CSF has not.

In the chronic murine agar bead model of *P. aeruginosa* pulmonary infection, we established a subacute and non-invasive infection by inoculating C57BL/6 mice with $8x10^5$ CFU per mice. In contrast to acute infection models, the Δ*lasB* mutant was not attenuated in virulence in this model. In fact, mice infected with the Δ*lasB* mutant showed greater pulmonary neutrophilia, immunopathology and morbidity than mice infected with the WT strain at equivalent bacterial burdens. The Δ*lasB* infections were also associated with significantly higher levels of airway and systemic chemokines, including the murine IL-8 homologue KC, the pro-inflammatory cytokines TNF-α and IL-1β, and the hematopoietic growth factor GM-CSF. Although we have not yet determined the exact mechanisms that mediate the exaggerated pulmonary inflammation in Δ*lasB* infections, the *in vivo* cytokine profiles suggest increased neutrophil recruitment and homeostasis. The strong similarity between the *in vivo* and the *in vitro* cytokine responses also support the idea that the loss of LasB-mediated proteolytic degradation significantly alters the cytokine milieu, leading to increased pulmonary neutrophilia. Whether this is the primary mechanism involved, and which cytokines play the greatest role *in vivo,* remains to be determined.

By selectively inactivating host immune components, it is possible that LasB dampens innate and adaptive immune responses, thus reducing bacterial clearance by host antimicrobial factors or phagocytes and subverting host immune responses (*21-31*). Additionally, LasB may activate bacterial intracellular pathways that promote biofilm growth (*294, 366, 367*), a growth mode that is paramount for the establishment of chronic infections (*335, 368*). The loss of

protease function in CF-adapted *P. aeruginosa* isolates may be the result of *lasR* mutations, which are positively selected for in the CF airway (*177*), or mutations in other genes. *lasR* mutants may be selected in the CF airway because of broad metabolic changes that confer increased fitness during growth on certain nutrient sources or exposure to certain antibiotics (*217, 290*). *lasR* mutants may also have greater fitness in the CF airways because they are social cheaters who take advantage of quorum sensing products made by other bacteria (*369*). It is less clear why protease mutants that are not *lasR* mutants emerge in the CF lung. One potential explanation may be that the extensive tissue damage and degradation caused by endogenous host factors may obviate the need for LasB-mediated proteolysis by the bacteria in order to gain nutrients from the host. Another possibility is that the loss of secreted proteases like LasB may be the result of pleiotropic mutations that confer selective advantages unrelated to secreted protease activity (*370, 371*).

Taken together, our work suggests that the loss of LasB and elastase function, which commonly occurs in CF-adapted isolates, enhance pulmonary inflammation and may contribute to further disease pathology in the setting of chronic infections. Our results suggest that, paradoxically, LasB is protective to the host during chronic lung infections. This work also highlights the fact that acute and chronic virulence may be mediated by distinct sets of diffusible bacterial products and bacterial cell-associated components. Understanding how the pathogenesis of *P. aeruginosa* differs during chronic infections compared to acute infections is paramount for designing appropriate therapeutic strategies for combatting these infections.

3.4. Materials and methods

Bacterial strains and growth conditions

All bacterial strains and mutants tested are listed in Supplemental Table S3.1. The construction of the Δ*lasB* mutant was previously described (*1*). Bacteria were grown in synthetic cystic fibrosis sputum media (SCFM), a defined culture medium that approximates the nutrient composition of CF sputum (*248*), unless otherwise specified. Planktonic cultures were grown at 37°C in liquid medium with shaking at 250 r.p.m. Antibiotic selection was carried out with gentamicin at 50μg/mL.

P. aeruginosa filtrate preparation

Planktonic bacterial cultures were grown in SCFM for 24 hrs, then centrifuged at 7200 g for 10 min at room temperature. The supernatants were filtered with low-protein binding 0.22 μm cellulose acetate filters (Corning), and aliquots of sterile filtrates were stored at -20°C, with no more than two freeze-thaws before use. Heat inactivated filtrates were heated to 95°C for 10 min then immediately placed on ice before AEC stimulation.

Protease activity assays

Elastase activity in *P. aeruginosa* filtrates was measured using the elastin-congo red elastase assay as previously done (*1*). Total secreted protease/collagenase activity was measured using Hide-Remazol Brilliant Blue R (Sigma-Aldrich) as a substrate as described previously (*372*). Filtrates were incubated with 15 mg of hide azure blue reagent (Sigma-Aldrich) in 0.5 mL 10 mM Tris (pH 7.5) for one hour at 37°C with shaking at 250 r.p.m. The solution was centrifuged at 3000xg for 10 min, and absorbance at 595 nm by spectrophotometer (Genesys™10 UV-Vis, Thermoscientific). Caseinolytic activity of the *P. aeruginosa* filtrates was assessed using 1.5% agar plates containing

1.5% skim milk as previously described (*296*). Briefly, *P. aeruginosa* filtrates was spotted onto sterile 6mm paper discs placed on the agar surface, and plates were incubated for 24 hrs at 37^{o}C.

AEC cultures and stimulation with P. aeruginosa filtrates

Immortalized human bronchial epithelial BEAS-2B cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM, Wisent) containing 4.5 g/L D-glucose and supplemented with 10% heat-inactivated fetal bovine serum (FBS, Wisent), penicillin (100 U/mL) and streptomycin (100µg/mL) at 37^{o}C with 5% CO_2. For stimulation, 2x10^{5} cells were seeded into 12-well polystyrene tissue-culture treated plates (Corning Costar) and grown to confluence before cells were incubated in starvation medium (DMEM supplemented with 0.5% heat-inactivated FBS and antibiotics) for 16 hrs, then stimulated with 60 µL of sterile *P. aeruginosa* filtrates or SCFM control medium in 1mL fresh starvation medium, and incubated at 37^{o}C with 5% CO_2 for the indicated time. Phosphoramidon (Cayman Pharmaceuticals) was diluted to a final concentration of 250µg/mL in 1mL of starvation medium to inhibit elastase acitivity.

qPCR analysis of cytokine mRNA expression

BEAS-2B cells were stimulated with bacterial filtrates (or SCFM control) for the indicated time, then washed and resuspended in RLT Plus lysis buffer (Qiagen) with 10% 2-mercaptoethanol. Total RNA was extracted and purified, and cDNA was synthesized as previously described (*1*). Quantitative PCR was performed with the QuantiFast SYBR Green PCR Kit (Qiagen) using the QuantiTect human primer sets (Qiagen) for IL-8 (QT00000322), IL-6 (QT00083720), IL-1β (QT00021385), TNF-α (QT00029162), G-CSF/CSF3 (QT00001414), GM-CSF/CSF2 (QT00000896) and GAPDH (QT00079247) on a 7500 Real Time PCR System (Applied Biosystems). Relative mRNA levels were normalized to GAPDH mRNA and expressed as fold change compared to the SCFM control conditions. PCR negative controls were performed with purified

RNA samples to ensure that there was no carryover genomic DNA contamination. Results were analyzed using V500 Life Technologies software (v2.06), with normalization to the SCFM medium control.

Cytokine measurements in conditioned AEC supernatants

Conditioned supernatants of AEC cultures were centrifuged at 13000 g to pellet cell debris. Cytokines were measured using OptEIA Human IL-6 and IL-8 ELISA kits, (BD biosciences). Human G-CSF and GM-CSF DuoSet ELISA Kits (R&D Systems) according to the manufacturer's instructions. TNF-α and IL-1β were measured in cell supernatants by MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore) and analyzed using the MILLIPLEX® Analyst software (v.4.2, Millipore). TNF-α and IL-1β were both below the limit of detection in all samples tested.

Chronic murine P. aeruginosa pulmonary infection

The pulmonary infection of mice with agar-embedded *P. aeruginosa* was performed as previously described (*251*). Briefly, planktonic bacterial cultures were grown to OD_{600}=0.5 in LB broth at 37°C shaking at 250 r.p.m. Bacterial cells were pelleted by centrifugation, resuspended in sterile PBS and mixed 1:1 v/v with a solution 2% LB and 3% molten agar. Agar-embedded *P. aeruginosa* beads were generated with continuously stirring heavy mineral oil (Fisher Scientific) as previously described (*251*), and the bacterial density was determined by standard dilution methods after homogenization of beads. Sterile PBS LB agar beads were used in control experiments.

Seven to nine week old, specific pathogen free, male C57BL/6 mice (Charles River Laboratories) were inoculated with 50 µL ($8x10^5$ CFU) of the agar bead suspension using a non-invasive intratracheal method as previously done (*251*). At designated time points, mice were weighed and humanely sacrificed. Whole blood, BALF and lungs (placed in sterile PBS containing protease inhibitors) were collected as previously described (*1*). 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 MUHC.

Lung histopathology

Murine lungs were inflated and fixed overnight with 10% buffered formalin phosphate (Fisher Scientific). Paraffin embedded tissues were sectioned into three 5 µm slices at 50 and 500 µm apart. To assess the lung inflammation, H&E stained sections were evaluated by a veterinary pathologist in a blinded manner using a semi-quantitative score (see Supplemental methods) and the average score of two separate sections was reported. Images were acquired using an Nikon Eclipse N*i* upright microscope with a Nikon Digital Sight DS-Fi2 digital camera.

Bronchoalveolar fluid (BALF), lung homogenates and plasma sample preparation

The BALF was centrifuged to pellet cells and aliquots of the supernatant were frozen at -80°C until analysis. From the BALF cell pellet, RBCs were removed by hypotonic lysis and remaining cells were resuspended in 600µL sterile RPMI 1640 medium and counted by hemocytometer (Hausser scientific). Total protein was measured in the BALF supernatant using the BCA Protein Assay Kit (Pierce). Perfused lungs were minced using razor blades and digested for 1 hour at 37°C in 150 U/mL collagenase (Sigma-Aldrich). For enumeration of viable bacterial counts, digested lungs were further homogenized then serially diluted and plated for CFU counts. For flow cytometry, digested lungs were homogenized to a single-cell suspension by repeatedly passing through a 16G 1” needle (BD) and filtered through a 100 µm pore size cell strainer (BD), with RBCs removed by hypotonic lysis. The cells were then pelleted, resuspended in 1mL sterile RPMI 1640 and enumerated using an automated cell counter (Z1 cell counter, Beckmann-Coulter). Murine plasma was collected from whole blood mixed with 0.5M EDTA, and aliquots were frozen at -80°C until analysis.

Flow cytometry of BALF and lung single cell suspensions

We stained $2x10^6$ cells from lung single cell suspensions, or cells from 400 µL BALF with Fixable Viability Dye eFluor780 (Affymetrix eBioscience), then

blocked with anti-murine CD16/CD32 (Affymetrix eBioscience). Cells were then surface stained with eFluor610-conjugated anti murine-CD45 (30-F11, Affymetrix eBioscience), eFluor710-conjugated anti-murine Ly6G (1A8, Affymetrix eBioscience) and V500-conjugated anti-murine CD11b (M1/70, BD. Finally, cells were fixed (Cytofix, BD) and analyzed using a LSRII flow cytometer (BD Biosciences) and FlowJo 10.0.7 software (Tree Star, CA).

Cytokine measurements by multiplex assay

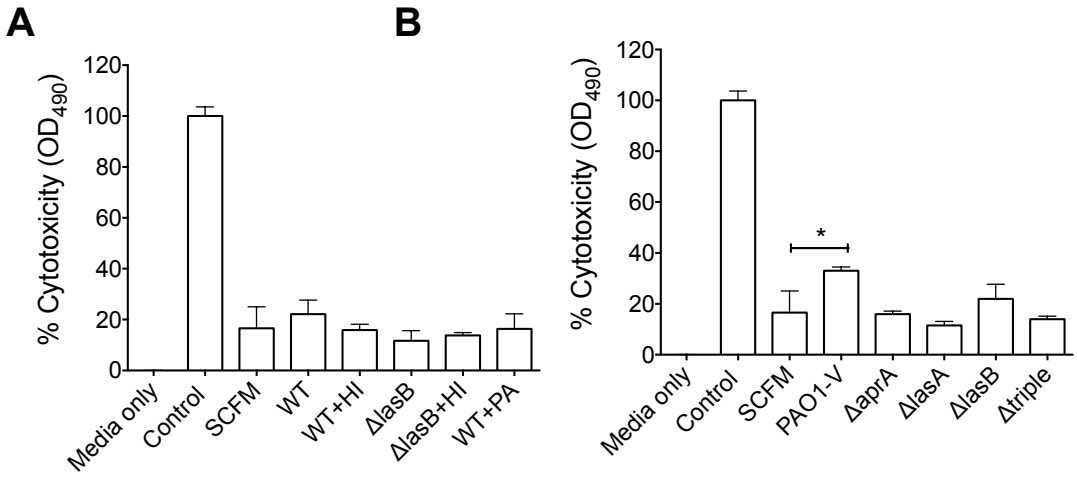
Mouse BALF and plasma cytokines (GM-CSF, G-CSF, IL-1β, KC, MIP-2, TNF-α, LIX and IL-6) were measured using the MILLIPLEX® MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore) and analyzed using the MILLIPLEX® Analyst software (v.4.2, Millipore). Samples with cytokine/chemokine concentrations below the lower limit of detection were assigned a value equivalent to half the concentration of the lower limit of detection.

Statistical analyses

All results are expressed as mean ± SD, or median ± IQR as indicated. Statistical analyses were done using Prism 5 software (GraphPad). Comparisons of the means and medians of two groups were performed using an unpaired two-tailed student's t-test, or the Mann Whitney non-parametric tests respectively. Comparisons between three or more groups were performed using two-way ANOVA with Bonferroni's post test p value of ≤0.05 was considered to be statistically significant. Not significant (NS) $p>0.05$; *$p\leq0.05$; **$p\leq0.01$, ***$p\leq0.001$.

3.5. Supplemental material

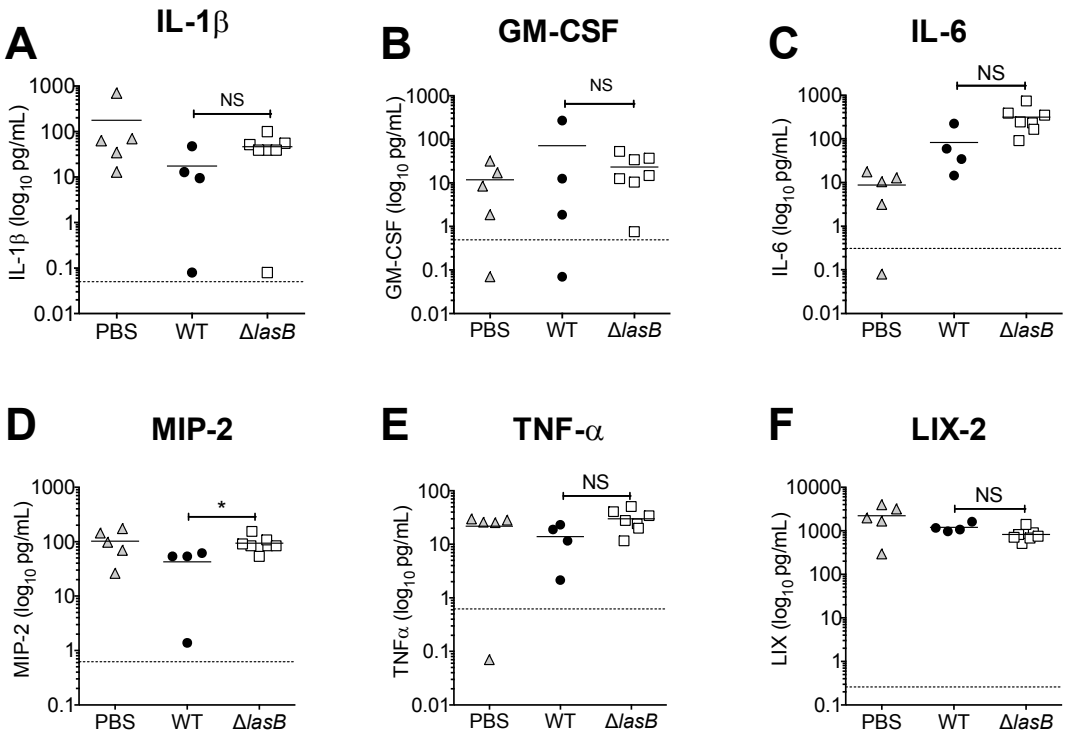
Supplemental Figures



Supp. Fig. S3.1: *P. aeruginosa* cell-free filtrates do not cause significant cytotoxicity to AEC cultures

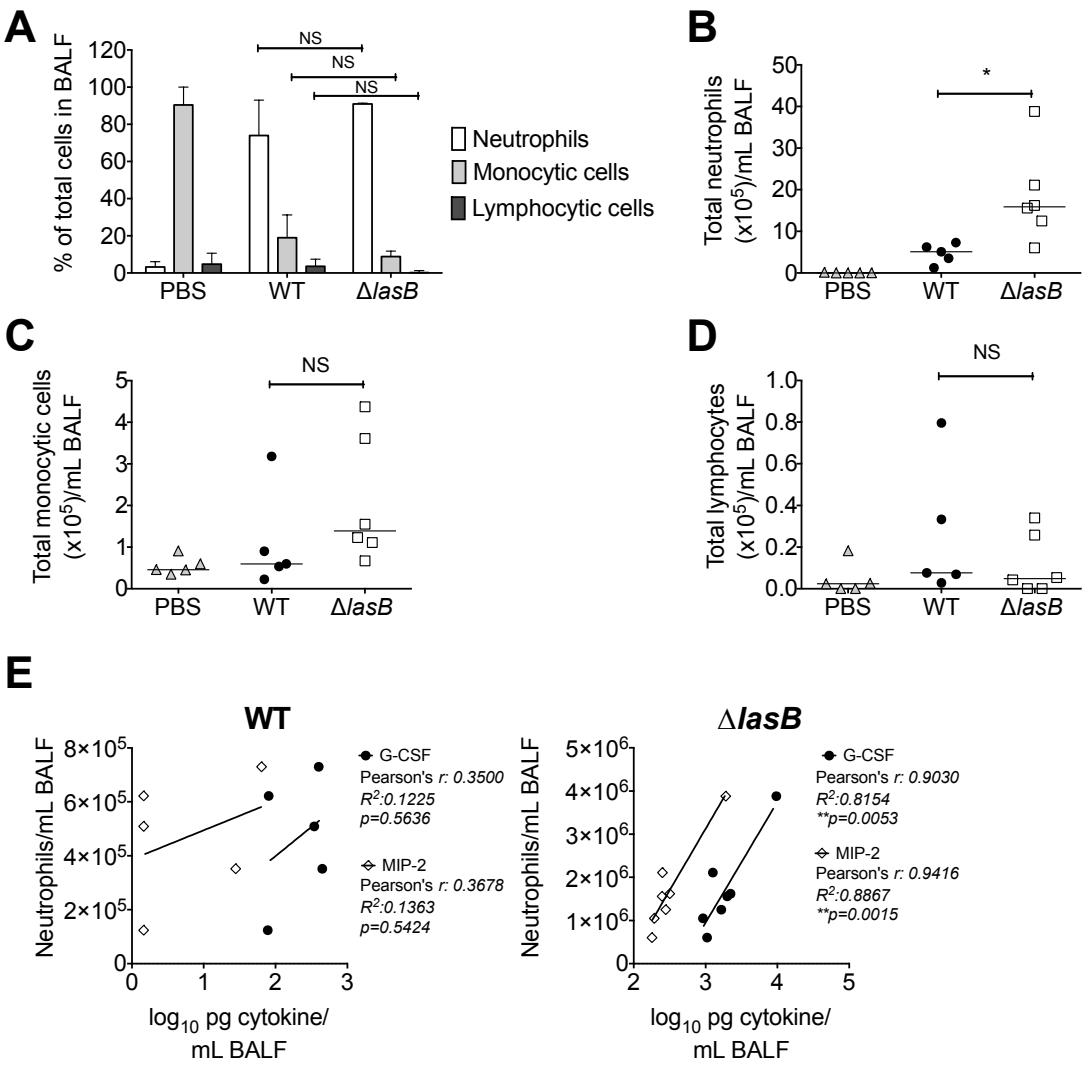
(A) BEAS-2B cells were stimulated with 60 µL of WT and Δ*lasB* filtrates, heat inactivated filtrates (+HI) or filtrates +250 µg/mL phosphoramidon (+PA).
(B) BEAS-2B cells were stimulated with 60 µL filtrates from PAO1-V and its isogenic Δ*aprA,* Δ*lasA,* and Δ*lasB* single mutants and Δ*aprA* Δ*lasA* Δ*lasB* triple mutant.

The cytotoxicity after 9 hrs of stimulation was determined by LDH activity and is expressed as a percentage of maximal cell lysis (control) following Triton treatment. Results are shown as mean (±SD). Statistical comparisons were done using 1-way ANOVA followed by Bonferonni's multiple comparison between SCFM control and treatments.. *$p \leq 0.05$.



Supp. Fig. S3.2: Systemic levels of pro-inflammatory cytokines that are not induced in infected mice

(A) to **(E)** Plasma cytokines were measured on day 4 p.i. by multiplex assay. The dotted lines indicate the lower limit of detection (LOD) for each cytokine. Cytokine values below the LOD were arbitrarily assigned a value equivalent to half the LOD.Results shown are the mean. Statistical comparisons were done using two-tailed unpaired student t-test. NS=Not significant $p>0.05$; *$p\leq0.05$.



Supp. Fig. S3. 3: Manual differential cell counts in BALF of WT and Δ*lasB*-infected mice

(A) Proportions of different leukocyte cell types
(B) Total cell counts
(C) Total counts of different leukocyte cell types
(D) Correlation between total bacterial burden (CFU count) and BALF neutrophils counts at day 4 p.i.
(E) Correlation between BALF G-CSF and MIP-2 levels and BALF neutrophils counts at day 4 p.i.
The BALF leukocytes in mice at day 4 p.i were enumerated by manual differential count. Results in A shown are the median (±IQR). Results in B-D are shown are median. For A-D, statistical comparisons were done using Mann Whitney test.

Supplemental Material and Methods

Lactate dehydrogenase (LDH) cytotoxicity assay

LDH levels were measured in BALF or AEC supernatants with the CytoTox 96 Cytoxicity Assay (Promega) according to the manufacturer's instructions by absorbance at 490nm with a microplate reader (Bio-Rad Model 680). The cytotoxicity was calculated as the relative LDH levels compared to the positive control, which represents maximal released LDH levels upon complete cell lysis using the Lysis Solution (9% v/v Triton X-100) according to the manufacturer's instructions.

Manual enumeration of immune cells in BALF

For manual differential count of BALF immune cells, cells from the BALF were resuspended in RPMI 1640 to ~$5x10^5$ cells/mL and 200 µL was spun onto coated Shandon™ Cytoslides (Thermo Scientific) at 1000rpm for 10 min at room temperature. Slides were dried and stained with Shandon™ Kwik-Diff™ (Thermo Scientific) following the manufacturer's instructions. Stained slides were visualized by light microscopy with a 63X oil objective and immune cells were enumerated in a blinded manner in four different representative frames.

Lung inflammation score

Lung inflammation score = Airway inflammation score + Parenchymal inflammation score

Airway inflammation score = Frequency score x Severity score

Frequency of airway inflammation	
0	No airway inflammation present
1	Rare airways affected (<25% section area affected)
2	Occasional airways affected (25-50% section area affected)
3	Frequent airways affected (50-75% section area affected)
4	Most airways affected (>75% section area affected)
Severity of airway bead-associated inflammation	
0	No airway inflammation present
1	Airway lumen with few inflammatory cells

2	Airway lumen with moderate numbers of inflammatory cells, without plugging
3	Airway lumen plugged with inflammatory cells, with little epithelial injury
4	Airway lumen plugged with inflammatory cells, with frequent necrosis/loss of airway wall
Parenchymal inflammation score	
0	within normal limits with minimal inflammatory cells
1	few small foci of inflammatory cells (< 25% section area affected)
2	occasional small or few moderate foci of inflammatory cells (25-50% section area affected)
3	frequent small and moderate, or few extensive foci of inflammatory cells (50-75% of section area affected)
4	abundant inflammatory cell infiltration, (>75% of section area affected)

Supp. Table S3.1. *P. aeruginosa* isolates and strains

Strains	**Strain information**	**Reference**
Patient 1 WT Δ*lasB*	 AMT0023-30 AMT0023-35	(*177*)
Patient 2 Early isolate CF-adapted isolate	 RAPD typing: A097 RAPD typing: A097	(*1*)
Patient 3 Early isolate CF-adapted isolate	 RAPD typing: A002 RAPD typing: A002	This study
Patient 4 Early isolate CF-adapted isolate	 RAPD typing: A097 RAPD typing: A097	This study
Patient 5 Early isolate CF-adapted isolate	 RAPD typing: A030 RAPD typing: A030	(*1*)
Patient 6 Early isolate CF-adapted isolate	 RAPD typing: A136 RAPD typing: A136	(*1*)
Patient 7 Early isolate CF-adapted isolate	 RAPD typing: A012 RAPD typing: A012	This study
Patient 8		This study

Early isolate CF-adapted isolate	RAPD typing: A012 RAPD typing: A012	
Patient 9 Early isolate CF-adapted isolate	 RAPD typing: A134 RAPD typing: A134	This study
Patient 10 Early isolate CF-adapted isolate	 RAPD typing: A136 RAPD typing: A136	This study
Patient 11 Early isolate CF-adapted isolate	 RAPD typing: A173 RAPD typing: A173	This study
Patient 12 Early isolate CF-adapted isolate	 RAPD typing: A301 RAPD typing: A301	This study
Patient 13 Early isolate CF-adapted isolate	 RAPD typing: A304 RAPD typing: A304	This study
Patient 14 Early isolate CF-adapted isolate	 AMT0005-1 AMT0005-138	This study
Patient 15 Early isolate CF-adapted isolate	 AMT0006-1 AMT0006-66	This study
Patient 16 Early isolate CF-adapted isolate	 AMT002-1 AMT002-84	This study
Patient 17 Early isolate CF-adapted isolate	 AMT0026-1 AMT0026-67	This study
PAO1-V	Wild-type *P. aeruginosa* variant of PAO1 (serogroup O5)	(*302*)
PAO1-V∆*aprA*	PAO1-V*aprA*ΩGm^R	(*302*)
PAO1-V∆*lasA*	PAO1-V*lasA*ΩGm^R	(*302*)
PAO1-V ∆*lasB*	PAO1-V with a Δ*lasB* (Δ59-1477 bp)	(*1*)
PAO1-V triple	PAO1-V*aprA*ΩGm^R*lasA*ΩGm^R*lasB*ΩSm^R	

3.6. Chapter transition

In chapters 2 and 3, we studied how the loss of LasR function and LasB function alone impacted host immune responses to *P. aeruginosa.* However, *P. aeruginosa* isolates recovered from chronically infected CF patients tend to accumulate multiple phenotypic changes overtime. Therefore, in chapter 4, we compared the CF-adapted Late isolate that has 68 mutations leading to several CF-adapted phenotypes, including a loss of LasR quorum sensing and secreted LasB activity, to its clonally-related non-adapted Early isolate in a murine model of chronic airway infection. In particular, we focused on the global impact of the many CF-adapted phenotypic changes occurring in the Late isolate, including the loss of several acute virulence factors and changes to PAMPs, on its virulence and immunogenicity compared to the Early isolate.

Chapter 4: Altered bacterial persistence and host responses to a CF-adapted *Pseudomonas aeruginosa* clinical isolate in vivo.

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Manuscript in preparation

4.1. Introduction

The bacterium *Pseudomonas aeruginosa* is a ubiquitous opportunistic pathogen capable of causing a wide-range of human infections. Although it is a leading cause of acute and rapidly progressive nosocomial infections, it is also the predominant pathogen causing chronic airway infections in individuals with Cystic Fibrosis (CF) (*39, 316, 373, 374*). Such infections can persist for years to decades, and are the leading cause of morbidity and mortality in CF (*139, 140, 142, 143, 375*). Chronic *P. aeruginosa* infections are associated with severe neutrophillic inflammation in CF lungs, causing excessive tissue damage and progressive lung disease (*38*).

Early in life, CF patients are transiently infected with *P. aeruginosa* strains likely acquired from environmental reservoirs (*139, 376*). By early adulthood, most CF patients have developed chronic *P. aeruginosa* infections (*373, 377*). Studies have demonstrated that *P. aeruginosa* undergoes extensive genetic adaptation to the CF lung environment (*177, 218, 378-380*). As a result, *P. aeruginosa* strains isolated early during CF lung infections differ both genetically and phenotypically from strains isolated years later (*139, 175, 177*). The convergent microevolution and adaptation of *P. aeruginosa* to the CF lung environment leads genetically distinct bacterial lineages, both within patients and between patients, to similar “CF-adapted” phenotypes such as the loss of acute virulence determinants like the type III secretion system and secreted proteases, lipases and siderophores (*38, 177, 184, 192*). Changes in pathogen-associated molecular patterns (PAMPs), such as modifications to LPS or the loss of flagella, also commonly occur in CF-adapted strains (*38, 202, 379, 381*). While it might be assumed that the loss of virulence factors and alteration of PAMPs may render CF-adapted *P. aeruginosa* less pathogenic to the host, the emergence of host-adapted isolates is typically associated with chronic phases of infections characterized by progressive inflammation, lung disease progression and worsening patient outcomes (*200, 201, 218, 226, 382*). How the phenotypic

changes occurring in *P. aeruginosa* during host adaption alter host-pathogen interactions and inflammatory responses, and whether such bacterial changes contribute to the pathogenesis of chronic CF lung disease, remain poorly understood. We hypothesize that the genetic adaption of *P. aeruginosa* within the CF lungs may alter its virulence and immunogenicity, having profound consequences on bacterial persistence and host inflammatory responses during chronic infections.

4.2. Results

A CF-adapted Late isolate causes less early mortality and morbidity than its clonally related Early isolate in a murine chronic pulmonary infection model.

For our studies, we exploited a well-characterized longitudinal pair of clinical isolates recovered from the lower airways of a young CF patient. The Early isolate was isolated from the patient at 6 months of age, while a clonally-related Late isolate was isolated when the patient was 8 years old (*177*). The genomes of both strains were fully sequenced and differed by 68 mutations (*177*). Several of these mutations were non-synonymous and resulted in the loss of acute virulence determinants in the Late isolate (*177*) including type III secretion, secreted proteases, LPS O-antigen, pyocyanin, pyoverdine and type IV pili (*177*). A subset of the genes harbouring mutations in the Late isolate and their associated function are summarized in Supp. Table S4.1.

In order to examine the impact of CF-adaptive changes on bacterial persistence and host responses, we compared the Early and Late isolates using *the P.* aeruginosa agar bead chronic murine pulmonary infection model. In this well-established model, bacteria are embedded in agar beads and instilled in the trachea of mice using a non-surgical approach (*251*). The immobilization of *P. aeruginosa* in agar beads leads to a persistent, sub-acute and non-invasive endobronchial infection that shares many features with chronic *P. aeruginosa*

airway infections in the CF lung (*233, 383*). We infected C57BL/6 mice with ~10^6 colony-forming units (CFUs) of the Early or Late isolate, and first compared the pulmonary bacterial burden over time. Overall, the kinetic changes in bacterial burden were similar in both isolates at days 1, 2 and 4 post-infection (p.i) (Fig. 4.1A). The bacterial burden increased during the first two days p.i, peaked at day 2 p.i. (median CFU of $7.4x10^6$ for Early-infected mice and $1.4x10^7$ CFU for Late-infected mice), and subsequently decreased over time in both groups. While the median bacterial burden in the lungs of Late-infected animals remained stable between days 4 and 10 p.i. (median CFU $3.6x10^4$ and $3.3x10^4$ CFU respectively, $p>0.05$), the median bacterial burden in the lungs of E-infected animals decreased significantly (median CFU $7.8x10^4$ at day 4 p.i vs median CFU 0 day 10 p.i $p\leq0.001$). This reduction in bacterial burden among Early-infected mice was primarily attributable to the greater proportion of animals having cleared the infection by day 10 p.i. (73% vs 20 % for Early vs Late infected mice, $p\leq0.05$) (Fig. 4.1B). This suggested that the Late isolate was enhanced for persistence *in vivo* compared to the Early isolate.

To assess the morbidity and mortality associated with the infections, we monitored body weights and survival. As shown in Fig. 4.1C, 25% of Early isolate-infected mice died by day 10 p.i, with most of the mortality occurring within the first 2 days p.i. In contrast, no Late isolate-infected mice died (survival 100% vs 75%, $p<0.05$). Early isolate-infected mice also showed a significantly greater decrease in body weight compared to Late isolate-infected mice on day 1 p.i. (-13.5% versus -7.17%, $p\leq0.001$, Fig. 4.1D). By day 2 p.i., no differences were observed between the two groups (-12% versus -13.4%, $p>0.05$), and mice in both groups regained body weight until day 10 p.i. (Fig. 4.1D). These results suggest that although the pulmonary bacterial burdens are equivalent with both Early and Late isolates in the first 4 days p.i, the Late isolate causes less mortality and morbidity in the early phases of infection compared to the Early isolate.

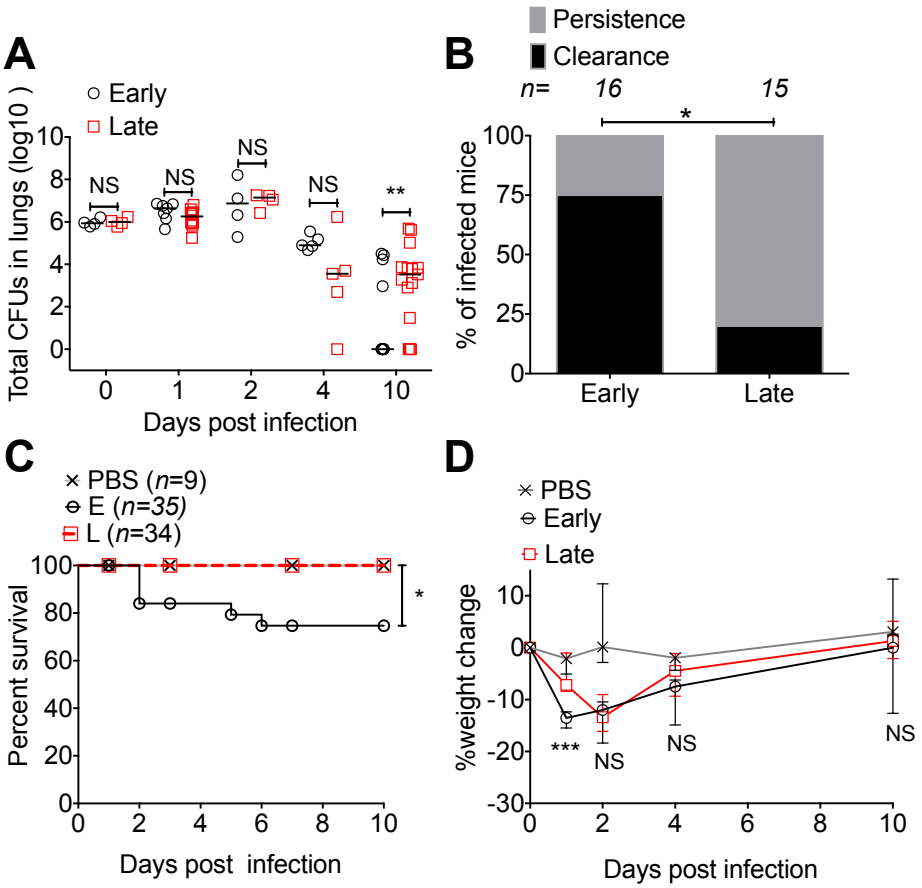


Fig. 4.1: The CF-adapted *P. aeruginosa* Late clinical isolate induced less early mortality and morbidity, but persisted better *in vivo,* compared to the clonally-related Early isolate

(A) The pulmonary bacterial burden in Early and Late infected mice after intratracheal infection with 10^6 agar-embedded CFU per mice. Total colony forming units (CFU) in the harvested lungs were determined at indicated time points by serial dilution plating.
(B) The proportion of mice with persistent infections at day 10 p.i. Persistence was defined as the presence of viable bacteria in the lung homogenates by serial dilution plating (limit of detection = 30 CFU per lung).
(C) Survival of Early (n=34) and Late (n=35) infected and control (n=9) mice in the chronic airway infection model.
(D) Change in weight (%) in Early and Late infected and control mice after inoculation of agar beads (n≥3 at each time point).
Results in A are shown as median and results in D are median (±IQR). For A and D, the medians of the Early and Late infection groups at each time point were compared using the non-parametric Mann-Whitney test. For B, statistical comparison was done using the Fisher's exact test. For C, the survival between the Early and Late groups was compared using the log rank test (Mantel-Cox). Results in A-D are pooled from *n* ≥2 independent experiments. NS=p>0.05; *=$p \leq 0.05$; **=$p \leq 0.01$.

The increased persistence of the Late isolate is not due to the loss of LasR quorum sensing.

The Late isolate harbours 68 mutations compared to the Early isolate, including a loss of function mutation in the quorum sensing regulator *lasR* (*177*). Since *lasR* mutants are recovered from over a third of CF patients and are associated with the chronic stages of *P. aeruginosa* infections, we asked whether the loss of LasR function led to increased bacterial persistence *in vivo* in our chronic airway infection model. We compared the Early isolate with its isogenic genetically engineered *lasR* knock-out mutant (EΔ*lasR*) and observed that both isolates showed similar bacterial clearance at day 10 p.i. (27% vs 33% respectively, p>0.05) (Fig. 4.2). The increased bacterial persistence of the Late isolate is therefore not attributable to the loss of LasR function.

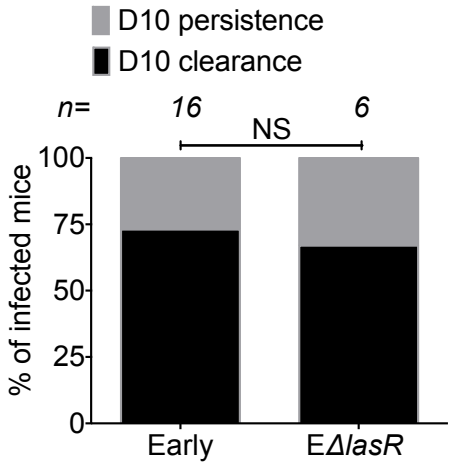


Fig. 4.2: Loss of LasR quorum sensing does not influence bacterial persistence in the murine chronic airway infection model

The Early and EΔ*lasR* infected animals have similar proportions of bacterial persistence at day 10 p.i. Statistical comparison was done using the Fisher's exact test. NS=p>0.05. Statistical comparison was done using the Fisher's exact test. NS= $p>0.05$; *=$p\leq0.05$.

The CF-adapted Late isolate elicits distinct patterns of inflammatory cytokines and pulmonary neutrophilia compared to the Early isolate.

In order to understand why bacterial clearance was impaired in Late

isolate-infected mice, we hypothesized that the Late and Early isolates elicited different cytokine responses in the lung that and that this altered host immune responses and, consequently, bacterial clearance. We examined a panel of cytokines implicated in the immune responses to *P. aeruginosa* murine pulmonary infections (*384-392*) and human CF lung disease (*34, 35, 118*), namely the hematopoietic cytokines (G-CSF, GM-CSF), pro-inflammatory cytokines (interleukin (IL)-1β, IL-17, TNFα), chemokines (MCP-1, KC/CXCL1, MIP-2/CXCL2, LIX/CXCL5) and anti-inflammatory cytokine (IL-10). Cytokine levels were measured by multiplex assay in the bronchoalveolar lavage fluid (BALF) of *P. aeruginosa* infected and control mice on days 2, 4 and 10 p.i. We also assessed the pulmonary immune responses induced in Early and Late infections at the same time points by quantifying total leukocytes, neutrophils, monocytic cells and lymphocytes in the BALF by cytospin and differential staining.

At day 2 p.i., the Early isolate elicited a vigorous pro-inflammatory cytokine response in the BALF. Compared to Late isolate infected animals, Early isolate infections were associated with greater induction of several cytokines, namely G-CSF (1.7-fold, $p \leq 0.01$), IL-1β (42-fold, $p \leq 0.05$), MIP-2 (3.2-fold, $p \leq 0.05$) and MCP-1 (89-fold, $p \leq 0.001$) (Fig. 4.3A, 4.3C to 4.3E). Interestingly, infection with the Late isolate resulted in higher levels of KC compared to Early isolate (3-fold, $p \leq 0.01$, Fig. 4.3D). Levels of GM-CSF, TNF-α, IL-17, LIX and IL-10 did not differ significantly between the two treatment groups at this time point (Fig. 4.3A to 4.3D). Despite the overall more vigorous pro-inflammatory cytokine response mounted by mice infected with the Early isolate compared to the Late isolate, their BALF total leukocyte, neutrophil, monocyte and lymphocyte counts did not differ significantly at day 2 p.i (Fig. 4.3F and Supp. Fig. S4.1).

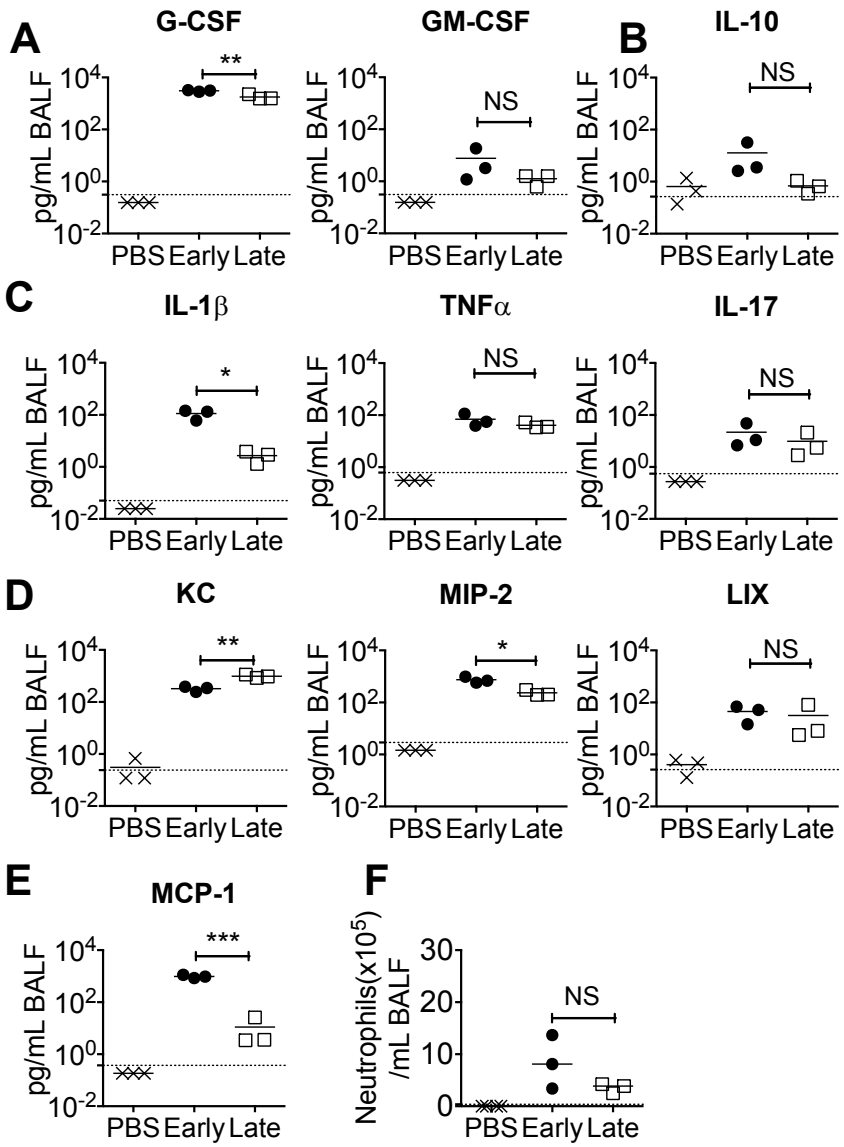


Fig. 4.3: BALF cytokine profiles and neutrophil counts of infected and control mice at day 2 p.i.

Results are shown in A to E as the mean for each group. In F, the median cell counts (total neutrophils) are indicated for each group. The dotted line delineates the limit of detection (LOD) for each cytokine, samples with undetectable cytokine levels were assigned a value of half the LOD. For A-E, statistical comparisons (Early vs. Late) were done using two-tailed unpaired student's T tests. For F, statistical comparison (Early vs. Late) was done the Mann-Whitney test. NS= not significant; $p>0.05$;*=$p\leq0.05$;**=$p\leq0.01$.

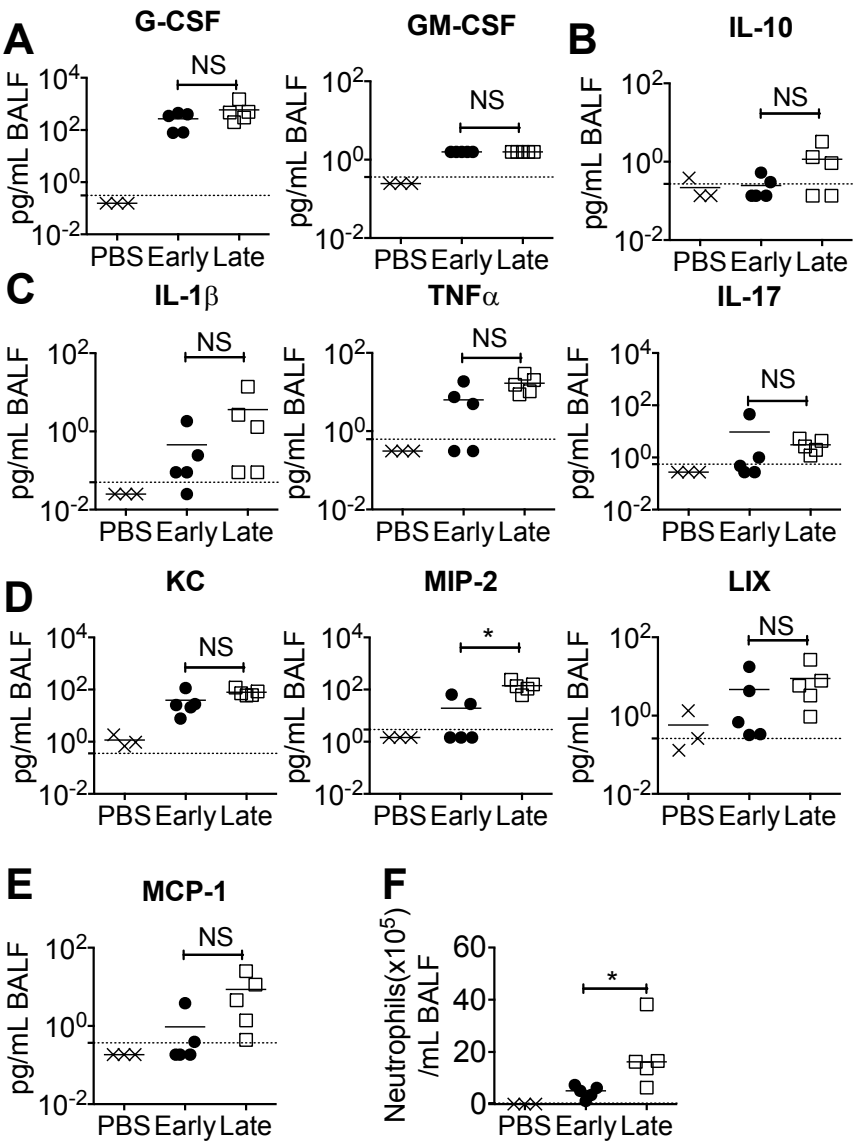


Fig. 4.4: BALF cytokine profile and neutrophil counts of infected and control mice at day 4 p.i.

Results are shown in A to E as the mean for each group. For F the median cell counts (total neutrophils) are indicated for each group. The dotted line delineates the limit of detection (LOD) for each cytokine; samples with undetectable cytokine levels were assigned a value of half the LOD. For A-E, statistical comparisons (Early vs. Late) were done using two-tailed unpaired student's T tests. For F, statistical comparison (Early vs. Late) was done using the Mann-Whitney test. NS= not significant, $p>0.05$;*=$p\leq0.05$.

At day 4 p.i., levels of most cytokines in the BALF of Early-infected mice had decreased dramatically compared to day 2 p.i. Notably, all cytokines were now at equivalent levels in both infection groups at the same time point, except for MIP-2 (7-fold higher in Late vs Early, $p \leq 0.05$) (Fig. 4.4A to 4.4E). Surprisingly, the total leukocyte counts were also 3.2-fold higher ($p \leq 0.05$) in the BALF of Late-infected compared to Early-infected mice (Fig. 4.4F), and this difference was attributable to increased BALF neutrophils (1.62×10^6 vs. 5.1×10^5 cells/mL in Late vs Early, $p \leq 0.05$). No differences in BALF monocyte or lymphocyte counts were observed (Supplemental Fig. S4.1).

At day 10 p.i., the BALF pro-inflammatory cytokine responses were mostly equivalent in both infection groups and had markedly decreased to levels equivalent to control mice for G-CSF, GM-CSF, IL-1β, TNF-α, IL-17, MIP-2, LIX, MCP-1 and IL-10 (Fig. 4.5A to 4.5E). The only exception was KC, which remained 7.3-fold higher in the BALF of Late-infected mice compared to control mice ($p \leq 0.05$, Fig 4.5D). While the BALF total leukocyte counts were similar in both infection group (Supp. Fig. S4.1), the Late-infected mice showed a greater proportion of neutrophils compared to the Early-infected once (12.3% versus 1.15%, $p < 0.05$) (Fig 4.5F). Although there was a trend towards higher BALF neutrophil counts in the Late compared to Early infected mice (median 3.0×10^3vs 1.2×10^3 cells/mL), this difference did not reach statistical significance (Fig. 4.5G). There were also no differences in BALF monocyte or lymphocyte counts (Supp. Fig. S4.1).

In summary, our results showed that the Early isolate stimulated a more vigorous pro-inflammatory response than the Late isolate early after infection, but without eliciting a more pronounced neutrophilic response at any of the time points (Fig. 4.6). In contrast, the cytokine response to the Late isolate resulted in a more pronounced neutrophil chemokine response, with higher KC (at day 2 p.i) and MIP-2 (at day 4 p.i) than the Early isolate (Fig. 4.6) associated with greater pulmonary neutrophilia. This suggests that CF-adapted changes occurring in the Late isolate alter the host cytokine responses without reducing immunogenicity,

and may in fact promote pulmonary neutrophilic inflammation.

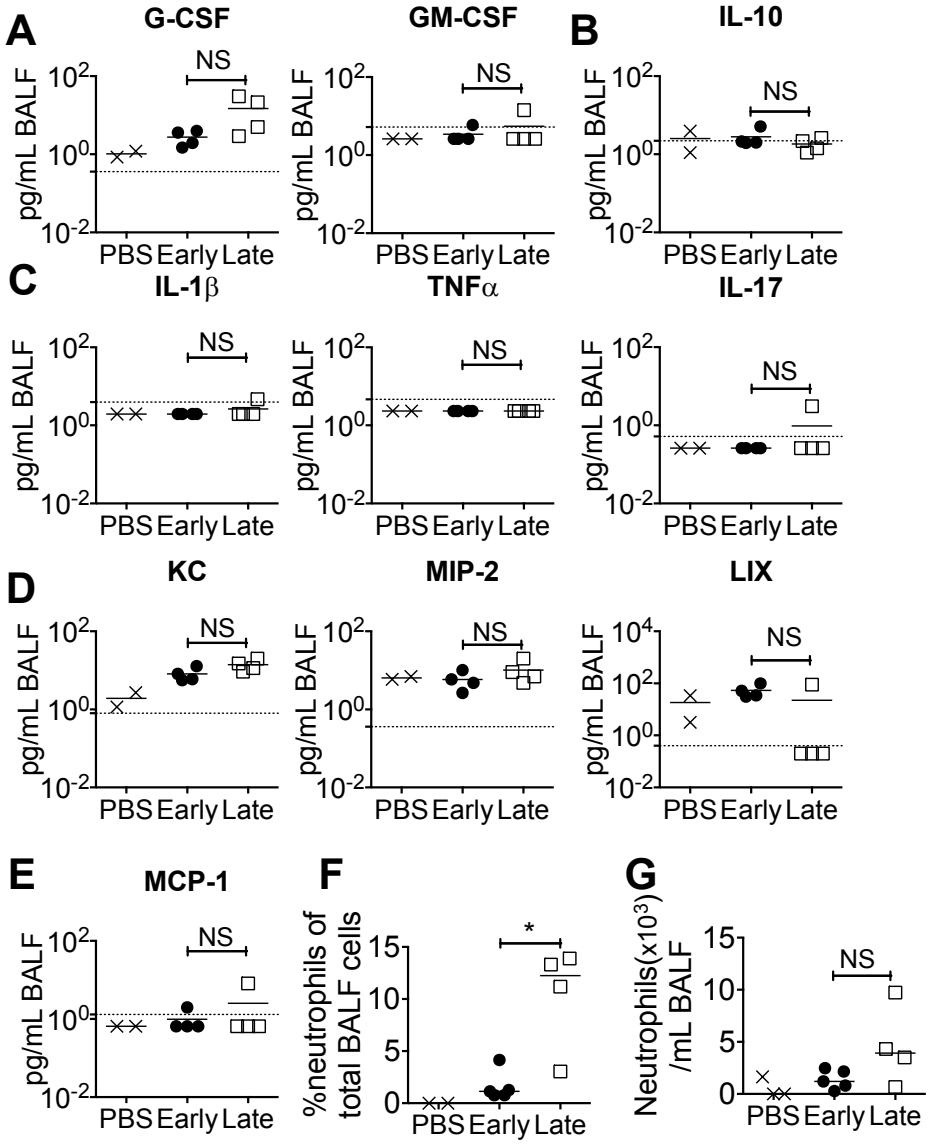


Fig. 4.5: BALF cytokine profile and neutrophil counts of infected and control mice at day 10 p.i.

Results are shown in A to E as the mean for each group. For F the median cell count (total neutrophils) is indicated for each group. The dotted line delineates the limit of detection (LOD) for each cytokine; samples with undetectable cytokine levels were assigned a value of half the LOD. For G the median % of neutrophils of total cells in BALF is indicated for each group. For A-E, statistical comparisons (Early vs. Late) were done using the two-tailed unpaired student's test. For F and G, statistical comparisons (Early vs. Late) were done using the Mann-Whitney test. NS= not significant.

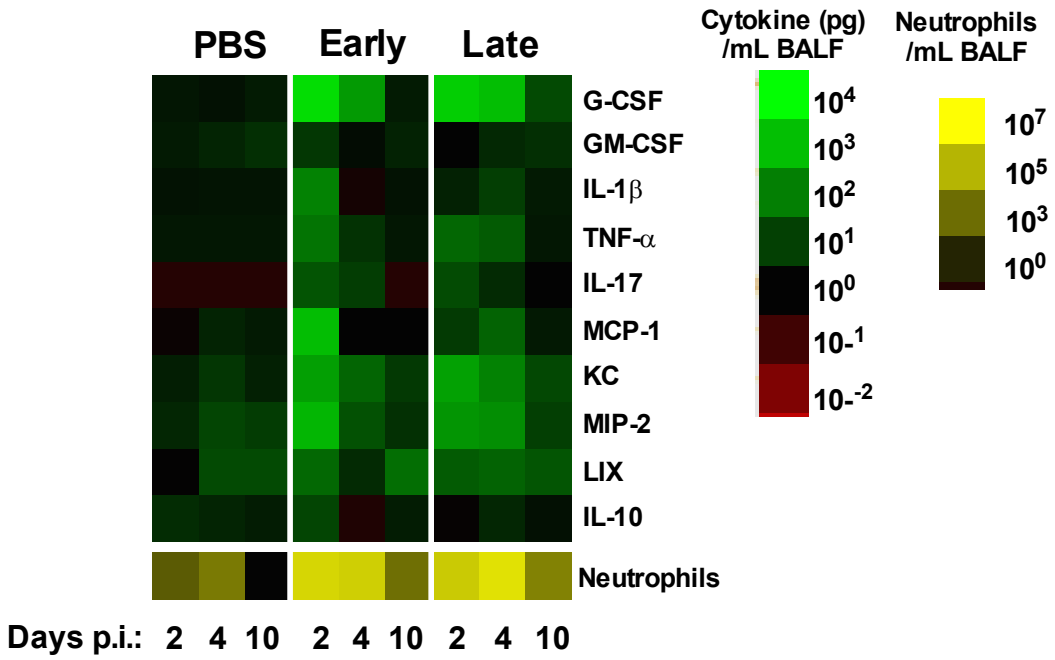


Fig. 4.6: Summary of BALF cytokine levels and neutrophil counts over time

Heat map depicting mean BALF cytokine levels (pg/mL) and median neutrophil counts (number of cells per mL BALF) for each group.

4.3. Discussion

P. aeruginosa isolates from the environment or different clinical origins, including those from early stage CF infections, show considerable conservation of genes encoding known virulence factors and immunogenic molecules (*227, 303, 381, 393, 394*). However, *P. aeruginosa* mutates and adapts to the host during chronic CF lung infections, resulting in reduced expression of many bacterial factors typically implicated in host injury and modulation of immune responses (*38, 143, 175, 177, 183, 319*). This has led to the hypothesis that CF-adapted strains are evolving towards a more symbiotic relationship with the host (*395, 396*). In this study, we investigated the host immune response to and the bacterial persistence of a pair of clonally-related *P. aeruginosa* clinical isolates recovered from a CF patient 96 months apart (*177*). By comparing these Early and Late isolates in a murine chronic airway infection model, we aimed to

understand the impact of host-adapted bacterial changes on the host immune and inflammatory responses.

Our observation that the Late isolate infections were less readily cleared than the Early isolate infections despite a vigorous innate immune response with abundant pulmonary neutrophils, recapitulates important features of chronic CF airway infections (*38, 316*). While we showed that the enhanced persistence of the Late isolate was not attributable to the loss of *lasR* function, several other mutations in this strain are potential candidates. The Late isolate harbours a loss of function in *pilA*, a structural component of the type IV pilus, resulting in the loss of pilus-mediated twitching motility (*177*). Interestingly, an *in vivo* mutagenesis screen that identified bacterial mutations promoting chronic colonization of murine lungs identified several distinct mutations resulting in the loss of twitching motility (*397*). The loss of function in *pilY,* which also impairs twitching motility, renders *P. aeruginosa* more resistant to neutrophil killing and promotes bacterial growth and survival in a murine airway infection model (*398*). The Late isolate also has a loss of function mutation in *wbpA* which results in the loss of the LPS O-antigen and a rough colony phenotype (*177*). O-antigen deficient *P. aeruginosa* strains display a dose-dependent increase in persistence in the airways of mice compared to O-antigen expressing ones (*399*). This study examined one well-characterized longitudinal pair of *P. aeruginosa* clinical isolates from a single CF patient. While several of the host-adapted mutations present in the Late isolate are commonly shared by other CF-adapted *P. aeruginosa* isolates, studies using other CF paired isolates would be important to validate our findings.

Several studies have examined the *in vivo* phenotype of CF-adapted *P. aeruginosa* strains but most have focused on animal mortality and morbidity (*174, 236, 400-402*). Bacterial virulence, or its ability to cause disease, can be defined in several different ways, such as the ability to colonize host tissues, to persist and replicate within the host, or to cause damage to the host (*403*). Lore *et al.*

demonstrated that early *P. aeruginosa* isolates were capable of causing lethality in three non-mammalian hosts and in a murine model of acute respiratory infection, whereas CF-adapted late isolates did not (*236*). However, certain CF isolates, including Liverpool epidemic strains, have enhanced lethality and cause increased lung pathology in a murine acute respiratory infection model (*402, 404*). Thus, the proposed notion that *P. aeruginosa* evolves in the CF airways towards attenuated acute virulence and pathogenicity is not clear-cut. Additionally, because these studies have compared the virulence of CF *P. aeruginosa* isolates using acute models of respiratory infection they are not representative of what is occurring in the chronically infected CF lung. The bacterial factors implicated in virulence during acute and invasive disease likely differ from those that promote virulence during chronic and non-invasive disease.

Work by Bragonzi *et al.* characterized the *in vivo* phenotypes of groups of clonally-related isolates taken from early, intermittent and chronic infections from different CF patients using a murine model of chronic airway infection (*174*). They also found that chronic *P. aeruginosa* isolates caused less mortality and morbidity in mice (*174*). Unlike our study, their study observed that *P. aeruginosa* isolates from chronic infections persisted as well as those isolated from early or intermittent isolates, rather than better (*174*). Since they had no genetic information on those isolates, it is difficult to infer the extent of the genetic differences between the different isolates. In our case, the Early isolate harbours no non-synonymous mutations in its core genome compared to the PAO1 reference strain and can thus be considered representative of a non-adapted isolate (*177*). The Late isolate harbours 68 mutations including mutations in genes commonly mutated in other CF-adapted isolates (Supp. Table S4.1). Interestingly, Bragonzi et al. reported that the PAO1 strain also persisted poorly *in vivo* (with <25% of animals still infected by day 14 p.i.), further suggesting that the enhanced persistence phenotype is attributable to CF-adapted mutations present in the Late isolate and absent in the PAO1 and Early isolate.

The timing and magnitude of cytokine responses are likely important

determinants of the outcome of infections. As far as we know, this is the first extensive comparison of the host inflammatory cytokine response to longitudinal *P. aeruginosa* isolates using the chronic murine *P. aeruginosa* pulmonary infection model. Despite the small number of animals, particularly at the day 2 timepoint, we found significant differences in cytokine levels in the airways of the Early and Late-infected animals. The pro-inflammatory cytokine responses associated with the Early and Late infections primarily differed in their kinetic changes. Infection with the Early isolate led to a strong early pro-inflammatory immune response, which was quickly shut down. In contrast, the Late isolate triggered a less vigorous but more sustained inflammatory response, with a preponderance of neutrophil chemokines KC and MIP-2. Whether these altered cytokine responses are directly responsible for the greater neutrophilic response and enhanced persistence associated with the Late infection is a compelling hypothesis but remains to be determined.

Since neutrophilic inflammation has been extensively associated with progression of chronic CF lung disease (*38, 262, 405*), bacterial adaptive changes that enhance neutrophilic responses in the lungs would likely increase its pathogenicity. While other studies have compared the immune responses to early and CF-adapted *P. aeruginosa* isolates *in vivo*, the analyses were qualitative or semi-quantitative by histological analysis (*392, 400, 406*). In agreement with Bragonzi *et al* (*174*), our study suggests that the bacterial adaptation to the CF lung may mitigate acute virulence determinants but increase chronic pathogenicity by increasing bacterial persistence as well as pulmonary inflammation. Interestingly, infection with the Late isolate led to significantly greater neutrophils in the BALF on day 4 p.i. and a significantly increased proportion of neutrophils in the BALF on day 10 p.i. compared to infection with the Early. This increase in neutrophils is likely due to a *lasR* loss of function mutation harboured by this strain (*1*). Further assessment of the lung injury and pathology associated with the Late isolate infection could better establish its impact on immunopathology during chronic airway infections. In addition, a more

in depth characterization of the host immune responses such as analysis of whole lung immune cells and neutrophil homeostasis may reveal important differences in the recruitment or activation of certain immune cell subsets in response to CF-adapted and non-adapted strains. Additionally, testing host immune responses to clinical isolates that are phylogenetically intermediate between the Early and Late isolates, would allow us to determine how individual mutations and combinations of mutations in *P. aeruginosa* may impact inflammation in the airways. In particular, it would be interesting to determine the Late isolate mutations responsible for dampening the pro-inflammatory cytokine response seen early on in Early isolate infected animals but absent in the Late-infected animals.

In conclusion, this study demonstrates that a CF adapted *P. aeruginosa* isolate, compared to a non-adapted clonally-related isolate, is less readily cleared but is not attenuated in virulence nor in its ability to induce innate immune responses in a murine model of chronic airway infection. Such insights on how host-adapted bacterial phenotypic changes contribute to host immune and inflammatory responses is important to our understanding of the pathogenesis of chronic *P. aeruginosa* pulmonary infections and may guide the rational design of novel antimicrobial and anti-inflammatory therapies for effectively treating these infections.

4.4. Materials and methods

Bacterial strains

The Early (AMT0023-30) and Late (AMT0023-34) clinical isolates were recovered from the same CF patient 96 months apart (*177*). Construction of the EΔ*lasR* has been previously described (*1*).

Chronic murine *P. aeruginosa* pulmonary infection

The chronic pulmonary infection with agar-embedded *P. aeruginosa* was

performed as previously described (*251*). Briefly, planktonic bacterial cultures were grown to OD_{600}=0.5 in LB broth at 37°C shaking at 250 r.p.m. Bacterial cells were pelleted by centrifugation, resuspended in sterile PBS and mixed 1:1 v/v with a solution 2% LB and 3% molten agar. Agar-embedded *P. aeruginosa* beads were generated with continuously stirring heavy mineral oil (Fisher Scientific) as previously described (*251*), and the bacterial density was determined by standard dilution methods after homogenization of beads. Sterile PBS LB agar beads were used in control experiments. Seven to nine week old, specific pathogen free, male C57BL/6 mice (Charles River Laboratories) were inoculated with 50 µL (~$1x10^6$ CFU) of the agar bead suspension using a non-invasive intratracheal method as previously done (*251*). At designated time points, mice were weighed and humanely sacrificed. The BALF was collected as previously described (*1*). 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 MUHC.

Processing of BALF for cytokines and immune cells enumeration

The BALF was centrifuged at 200xg for 10 min at 4°C, and the supernatant was decanted and frozen in aliquots at -80°C. The cell pellet was resuspended in 600 µL of sterile RPMI 1640, and the cell concentration was determined using a hemacytometer (Hausser scientific). The manual differential cell count was determined by cytospin and staining with Kwik-Diff (Fisher scientific). Stained slides were visualized in a blinded manner at 63X under oil-immersion and differential cell counts were enumerated on four different representative frames.

Cytokine measurements by multiplex assay

Mouse BALF cytokines (G-CSF, GM-CSF, IL-1β, TNF-α, IL-17, MCP1 (CCL2), KC (CXCL1), MIP-2 (CXCL2), LIX (CXCL5) and IL-10) were measured using the MILLIPLEX® MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore) and analyzed using MILLIPLEX® Analyst software (v.4.2, Millipore). Samples with cytokine/chemokine concentrations below the lower limit

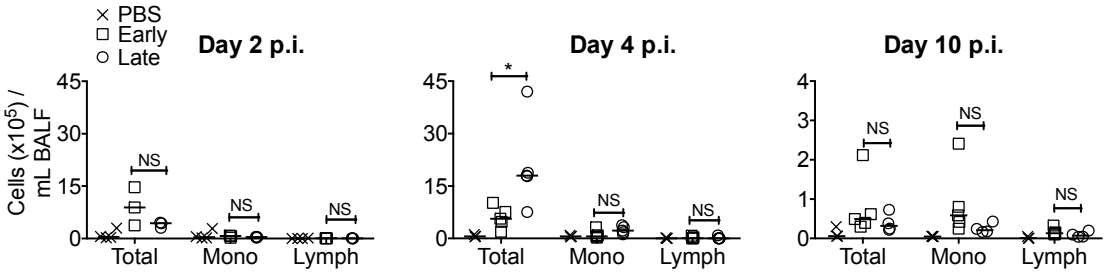
of detection were assigned a value equivalent to half the concentration of the lower limit of detection. Median cytokine values were quantitatively displayed in a heat map with color using the program Java TreeView 1.1.6r4 (http://jtreeview.sourceforge.net).

Statistical analyses

Statistical analyses were done using Prism 5 software (GraphPad). Comparison of the means of two groups was performed by two-tailed unpaired student's t test and comparison of the median was performed by Mann Whitney non-parametric test. Statistical analysis of categorical data was performed using the Fisher's exact test. A *P* value of ≤0.05 was considered to be statistically significant. Not significant (NS) p>0.05, *$p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$.

4.5. Supplemental material

Supplemental Figures



Supp. Fig. S4. 1: BALF monocytic cell and lymphocyte counts over time

Total leukocyte, monocyte and lymphocyte counts in BALF at each indicated time point. Statistical comparisons (Early vs. Late) was done using the Mann-Whitney test. NS= not significant

Supp. Table S4.1. List of acute virulence genes with phenotypically confirmed loss-of-function mutations occurring in the Late isolate

Gene name	Gene annotation	Function
exsA	PA1713	Transcriptional regulator of type III secretion system
wbpA	PA3159	LPS O-antigen biosynthesis
pilA	PA4525	Type 4 fibrial precursor
lasR	PA1430	LasR quorum sensing transcriptional regulator
pvdS	PA2426	Iron uptake regulation
phzS	PA4217	Phenazine biosynthesis

Genetic mutations were identified and, for a subset, phenotypically characterized in (1, 177).

Chapter 5: Discussion

5.1. Summary of major findings

Many CF-adapted *P. aeruginosa* phenotypic variants appear attenuated in acute virulence, yet are associated with worse, not better, lung function and patient outcomes. It is unclear whether these variants contribute to disease progression, or are innocent bystanders that are associated with the later stages of infection. We hypothesized that phenotypes commonly occurring in CF-adapted strains could modulate host innate immune responses, leading to excessive neutrophilic inflammation and impaired bacterial clearance. In this thesis, we demonstrated that the loss of LasR quorum sensing, or the loss of the LasR-regulated secreted protease LasB, increased pro-inflammatory cytokine responses and neutrophil recruitment *in vitro* and *in vivo*. In a small cohort of adult CF patients chronically infected with *P. aeruginosa*, we also observed that the frequency of *lasR* mutants in the sputum of CF patients positively correlated with plasma IL-8, a marker of neutrophil inflammation. Finally, we found that a CF-adapted clinical isolate that is impaired in several acute virulence traits, including *lasR* quorum sensing, displayed enhanced persistence despite eliciting a strong neutrophilic inflammatory response in a chronic murine *P. aeruginosa* pulmonary infection model. Although many studies have demonstrated associations between *P. aeruginosa* chronic infections and excessive inflammation in progressive CF lung disease, our results are novel and important because they demonstrate the contribution of specific *P. aeruginosa* CF-adapted phenotypic variants and suggest possible mechanisms for these altered host-pathogen relationships.

5.2. Modeling *P. aeruginosa* CF chronic lung infections *in vitro* and *in vivo*

In vitro models

P. aeruginosa chronic CF lung infections involve a complex and dynamic interplay between the host and pathogen. In order to clarify the role played by *P. aeruginosa* in CF lung disease progression, there is a need for tractable *in vitro* model systems that closely mimic the CF lung environment. During CF chronic lung infections, *P. aeruginosa* typically grows as biofilm aggregates within the viscous mucus layer overlying the airway epithelial surface, and is not invasive (*145, 263, 407*). Thus *P. aeruginosa*–AEC interactions during chronic infection are likely mediated primarily through secreted and diffusible factors. *In vitro* models of *P. aeruginosa* chronic airways infections should thus approximate these interactions.

For our studies we used synthetic CF sputum media (SCFM), a defined artificial growth media that approximates the average nutritional composition of CF sputum. Bacteria grown in SCFM thus have growth rates, gene expression profiles and cell-cell signaling that may approach those found in the CF airways (*248*). While this media approximates the nutritional content of CF sputum, it lacks other important characteristics such as the viscosity of CF sputum and abundance of host products or factors (e.g. DNA, neutrophil elastase). To overcome these limitations, some groups have developed artificial sputum growth medium that contains mucin from pig stomach mucosa and salmon sperm DNA (*408*). Despite these efforts, it remains nearly impossible to develop *in vitro* growth conditions that incorporate the spatial and temporal heterogeneity of oxygen, nutrient, host factors and antimicrobials of the CF lung environment.

Our group and many others have used cell-free filtrates from planktonic cultures to stimulate AEC cells. This provides a highly tractable system to test the effects of extracellular (secreted or shed) bacterial factors on AEC cytokine

responses using a range of *P. aeruginosa* laboratory strains and clinical isolates. Since *P. aeruginosa* grows as biofilms in the CF airways, rather than planktonic cells, we recognize that the switch to this growth mode may have important consequences on gene expression and cell signaling (*409*). Although many *in vitro* biofilm models exist (*410-414*), biofilms grown in laboratory flow cell chambers or 96-well plastic culture dishes are surface attached and bear no structural resemblance to *P. aeruginosa* biofilm aggregates occurring in CF airways (*247*). We have therefore adapted a recently developed "gel" biofilm model for use in an AEC-biofilm co-culture model in order to validate the effect of diffusible bacterial products made by *P. aerguinosa* biofilms on AEC inflammatory responses (*247*). In contrast to previously developed *P. aeruginosa* biofilm AEC co-culture models where AEC develop cytotoxicity and cell death within a relatively short period of time (~8 hrs) in response to direct contact with live *P. aeruginosa* (*415*), our biofilm AEC model allows for the co-culture of live bacterial cells with AEC for up to 18 hrs without significant AEC cytotoxicity. Limitations of this model include our use of a non-biologically relevant gelling agent (agar) and that it is low thoroughput.

Our *in vitro* studies focused on AEC inflammatory responses to *P. aeruginosa* and were primarily conducted with immortalized BEAS-2B cells, a well-characterized and highly reproducible non-CF bronchial epithelial cell line. While cell immortalization offers many advantages to researchers, the process of immortalization can have significant physiological effects that may alter cellular phenotypes, epigenetics, and signaling pathways (*416*). To validate our findings in a primary human cell culture model, we used nasal polyp explants. Nasal poly explants are fragments of surgically resected tissue that contain primary upper respiratory tract epithelial cells within their extracellular matrix and retain cytokine and mucin secretion function (*417, 418*). These tissues are unfortunately not readily available and show significant variability between different patients.

Many studies have suggested that the loss of CFTR function in AEC may also affect PRR expression and localization, signaling, and cytokine production and thus results in a hyperinflammatory state (*51, 52, 102, 108, 419, 420*). While primary CF bronchial cells are very important tools, they are limited by their availability, patient variability and the high prevalence of bacterial contamination with antibiotic resistant organisms (*421*). As a result, several immortalized CF cell lines have been developed (*421*). In order to validate our main results in CF cells, we used immortalized CFBE41o- CF AEC because they are homozygous for the common ΔF508 mutation, have been well characterized and are widely used (*422-424*). Although we tested the CFBE41o- grown as AEC monolayers, these cells could also be grown as differentiated AEC at the air-liquid interface to increase the physiological relevance of the system.

There is a great need for *in vitro* models that capture the relevant complexity of human tissues to model human disease (*425*). Many fields (ex. cancer, cell biology, infectious disease) are shifting away from traditional cell culture models to three dimensional (3D) models which mimic morphological and functional properties of the organ or tissue from which they were derived (*426*). These models are amenable to multi-cellular co-cultures, can maintain cells in a differentiated state, and are capable of complex 3D architecture (*427*). 3D cell culture models are valuable tools for the study of host-pathogen interactions in infectious disease and may someday bridge the gap between *in vitro* models and *in vivo* models, thus facilitating the translation of basic scientific research to the clinic. The development of human CF 3D organoid models, which is already underway, is an important step towards research and personalized medicine for CF patients. For instance, a CF intestinal organoid model has been developed using intestinal adult stem cell cultures generated from rectal biopsies from CF patients (*428*). Recently, Hannan *et al.* developed an organoid model of distal airways from human foregut stem cells (*429*). Such models using primary human tissues may allow us to assess individual patient's responsiveness to drugs, such as CFTR modulators, as well as expand the tools for drug discovery.

In vivo models

In vitro models in infectious disease are inherently limited in their inability to recapitulate the complex interactions and multiple cell types that mediate effective antimicrobial defense and inflammatory processes. Although several animal models of *P. aeruginosa* chronic lung infection have been developed to date, in both wild-type and CFTR mutant transgenic animals, all present both advantages and limitations, as outlined below.

Several CF mouse models have been developed in the past two decades, including CFTR null mice, mice with mutant forms of CFTR (such as the ΔF508 mutation) with or without gut correction of CFTR expression, and mice with ENaC overexpression (*41, 52, 430, 431*). While these CF mice have provided many important insights to our current understanding of CF, they are not very good models of chronic CF lung disease. Most CF mice do not develop spontaneous lung disease or infections, and lack the mucus plugging characteristic of human CF lung disease (*431*). Some groups have reported that CF mice display some CF lung disease phenotypes such as hypersusceptibility to chronic *P. aeruginosa* oropharyngeal colonization and lung infection, and reduced clearance of *P. aeruginosa* from the respiratory tract (*432-434*). However, these studies involve direct inoculation of the respiratory tract of CF mice with high doses of *P. aeruginosa.* The development of spontaneous lung infections, like those occurring in CF patients, is not commonly observed in CF mice (*52, 431*). Most recently, non-rodent CF models using ferrets and pigs have been developed but these are not widely available (*231, 232*). Furthermore, no models of *P. aeruginosa* lung infections in these animals have been established. While we recognize the importance of using a CF *in vivo* model, we used wild-type (non-CF) mice in our experiments in order to first characterize the effects *of P. aeruginosa* on host responses.

Modeling *P. aeruginosa* chronic lung infections in murine models usually requires embedding bacteria in an immobilizing agent such as agar or alginate (*233*). Alternatively, mucoid *P. aeruginosa* clinical isolates which overexpress alginate have also been used in chronic infection models (*406*). Immobilization of *P. aeruginosa* in a semi-solid matrix promotes the formation of biofilm aggregates analogous to the microcolonies formed by *P. aeruginosa* during growth within the mucus of CF lungs (*145, 247*). Intratracheal infection with *P. aeruginosa* embedded in agar beads causes mechanical endobronchial obstruction of the airways and impairs bacterial clearance, akin to CF lung disease. This causes a sub-acute to chronic infection primarily localized to the airways that is minimally invasive and rarely disseminates. In contrast, infection with planktonic (free floating *P. aeruginosa*) either causes acute and fatal pneumonia (at high doses $>10^7$ CFU per mice) or rapid and complete bacterial clearance (at lower doses <1-5x 10^6 CFU per mice) (*406*). In addition, the agar bead *P. aeruginosa* infection model also induced highly localized inflammation and tissue damage in the mouse lungs, which are reminiscent of the heterogeneous airway damage and inflammation observed in the lungs of CF patients (*435*). Since our studies are focused on comparing host responses to different *P. aeruginosa* strains, the agar bead model provided us with an experimental system that preserved a few key features of CF lung disease while allowing the establishment of a chronic infection using several different *P. aeruginosa* strains.

5.3. The emergence of *lasR* and protease-deficient mutants during CF chronic lung infections.

Why LasR (quorum sensing) or protease deficient *P. aeruginosa* mutants arise so frequently in the lungs of CF patients remains unknown. Studies examining the genetic micro-evolution of *P. aeruginosa* during chronic CF infection clearly suggest convergent evolution with positive selection for these mutants (*177, 192, 193, 202, 236*). What selective advantage such mutants may have in the host remains largely speculative and several different theories have

been proposed. First, it has been suggested that the reduced or deficient expression of virulence factors might allow for immune evasion and thus increase the survival of *P. aeruginosa* in the host (*38*). Attenuation of acute virulence may also promote survival of the host, thus ensuring long-term colonization of *P. aeruginosa* (*436, 437*). Production of exoproducts (such as virulence factors) is a metabolic burden and mutants who do not produce them may have a growth advantage in mixed bacterial populations when exoproducts are being made by others (*219*). Finally, the loss of acute virulence factors may also be secondary to the selection for other traits that are beneficial to *P. aeruginosa* in the CF lung, such as the improved ability to utilize certain nutrient sources that are highly prevalent in the CF lung (ex. aromatic amino acids) (*38, 214, 290*).

Our work, and the work of others, suggests that the loss of LasR quorum sensing and secreted elastase does not dampen recognition of *P. aeruginosa* by the host immune system. Both *lasR* and *lasB* mutants elicited increased AEC pro-inflammatory cytokine responses and greater neutrophil recruitment *in vitro* and *in vivo* compared to the wild-type strain. The frequency of *lasR* mutants in the sputum of CF patients was also positively correlated with increased plasma levels of the neutrophil chemokine IL-8, suggesting that the presence of *lasR* mutants is sensed by the host and triggers increased systemic immune responses. Wu *et al.* reported that the *lasI rhlI* double mutant which lacks both *las* and *rhl* quorum sensing systems was associated with stronger serum antibody response and greater peripheral blood neutrophil activation in the rat alginate bead model of chronic airway infection (*438*). Intranasal infection of the *ΔlasB* mutant or wild-type *P. aeruginosa* strains led to equivalent neutrophil and macrophage recruitment into the lungs, but the *ΔlasB* mutant was more readily phagocytosed by macrophages (*286*). Interestingly, we also observed in our agar bead chronic *P. aeruginosa* infection model that the Δ*lasB* mutant was more readily cleared in the first 2 days p.i, compared to the WT strain, when lower bacterial inoculums were used (Appendix 1). Together with our findings, these studies demonstrate that loss of LasR or LasB function in *P. aeruginosa* does not

hamper the host's early pro-inflammatory response, antibody responses or bacterial clearance, thus suggesting that *lasR* and *lasB* mutants are not impaired in their ability to induce innate immune responses. However, because our studies only focused on a subset of pro-inflammatory cytokines at specific time points, it is possible that the loss of LasR quorum sensing and LasB proteolytic degradation influences other immune and inflammatory mediators such as eicosanoids, complement components, antibodies, surfactants, cell surface receptors, or immune cells themselves.

The attenuation of acute virulence phenotypes in *P. aeruginosa* may be a strategy to ensure successful long-term colonization and thus survival in the host. Importantly, this persistence is not benign but rather is associated with deleterious immunopathology. From our studies, we observed that the CF-adapted Late isolate caused less mortality and morbidity *in vivo* early on but had enhanced persistence compared to the Early isolate, and the EΔ*lasR* and Δ*lasB* strains were associated with greater immunopathology compared to the Early isolate. Our findings are thus in agreement with Bragonzi *et al.* who also showed that CF adapted *P. aeruginosa* isolates had altered rather than attenuated virulence in the chronic agar bead infection model, consisting of increased persistence and inflammation, but decreased mortality and invasiveness (*174*).

lasR mutants possess phenotypes that could also contribute to their emergence and selection within the CF lung environment. For instance, *lasR* mutants have increased resistance to β-lactam antibiotics and may therefore be selected for under antibiotic pressure (*214*). The loss of LasR function causes widespread metabolic changes that confers *lasR* mutants a growth advantage on certain amino acids (phenylalanine, isoleucine and tyrosine) that are abundant in CF secretions (*214*). *lasR* mutants display decreased oxygen consumption, which may be advantageous in low-O_2 microenvironments in CF airways (*290*). While many protease-deficient CF isolates are *lasR* mutants, some are not and have intact LasR quorum sensing (*320*). Since protease-deficient strains are

unlikely to have the same broad metabolic re-wiring and growth advantages as *lasR* mutants, it is possible that the loss of protease secretion confers other unrecognized fitness advantage that are selected for during chronic infections.

In order to understand the microevolution of *P. aeruginosa* and the emergence of *lasR* mutants, several groups have examined this question from an ecological perspective (*213, 240, 369, 439, 440*). Exoproducts, such as LasB and other LasR-regulated secreted products, can benefit an entire bacterial population and are thus considered "public goods" (*369*). Mutants that benefit from but no longer produce the public goods themselves, such as *lasR* mutants, have been termed social cheaters (*216, 240, 369, 439, 440*). Diggle et al showed that the production of the LasR quorum sensing signal molecules and LasR-dependent exoproducts, like LasB, are metabolically costly to the bacteria, leading to a fitness advantage for *lasR* mutants in populations mixed with wild-type bacteria (*369*). The emergence of *lasR* mutants in the CF lung may therefore be the result of a fitness advantage gained by social cheating in mixed populations. The fact that *lasR* mutants are often found co-existing with *lasR* wild-type bacteria in the CF lung, including 9 out of the 17 CF patients in our study, suggests that social cheating could occur in a clinical setting (*218, 441*). Whether *lasR* mutants incur a fitness benefit *in vivo* could be tested experimentally by carrying out mixed infections and comparing the competitive index of wild-type and mutant bacteria. Answering the same question in CF patients, however, would be extremely challenging.

5.4. Future Directions

In Chapter 1, that the frequency of *lasR* mutants in CF sputum was positively correlated with plasma IL-8, a marker of neutrophil inflammation, in adult CF patients suggested that our *in vivo* and *in vitro* findings may be valid in patients. However, this clinical study was limited to a small number of patients from a single CF center. The cross-sectional study design allowed us to demonstrate an association between *lasR* mutant frequency and plasma IL-8, but

not causation. To infer a possible causal relationship in CF patients, we would need to carry out a longitudinal and larger cohort study of chronically infected CF patients to examine whether the emergence of *lasR* mutants is associated with greater pulmonary inflammation, and subsequent progression of lung disease and pulmonary exacerbations.

In Chapters 2 to 4, our *in vitro* studies focused on the effect of *P. aeruginosa* adaptive changes on AEC cytokine responses and neutrophil recruitment. Future work should investigate other aspects of host-pathogen interactions relevant to CF lung disease. For example, studies could investigate how *P. aeruginosa* adaptive changes influence neutrophil antimicrobial functions such as degranulation, NETosis, phagocytosis and apoptosis. In preliminary work, we have observed that the CF-adapted Late isolate induces accelerated neutrophil death compared to the Early isolate (Appendix 2). Follow up studies could dissect which host-adapted changes are responsible for this effect and define the biological consequences of accelerated neutrophil death *in vitro* and *in vivo.*

5.5. Conclusion

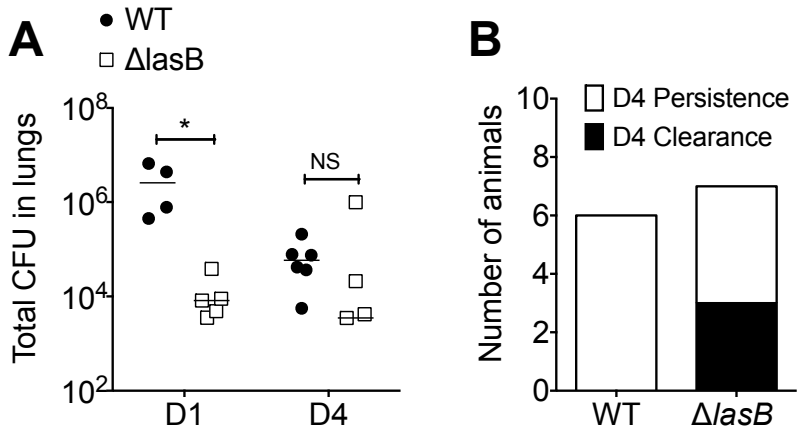
Despite extensive research on the microbiology of CF lung infections, it remains poorly understood how the dynamic interactions between host and pathogens change over time and contribute to the progression of CF lung disease. *P. aeruginosa* is unlikely a passive opportunistic bystander that colonizes abnormal airways. As *P. aeruginosa* evolves within the CF lung, how such phenotypic variants contribute to CF disease pathogenesis remains largely unknown. Our studies provide evidence that the loss of LasR and the secreted elastase LasB, two common CF phenotypic adaptations occurring in *P. aeruginosa*, cause increased pulmonary neutrophilic inflammation. Understanding how CF-adapted phenotypic variants of *P. aeruginosa* modulate host immune responses, leading to changes in inflammation and bacteria

clearance, can provide us insights into the pathogenesis of *P. aeruginosa* during chronic infections and reveal new strategies for combatting these infections.

Chapter 6: Appendices

Appendix 1: Δ*lasB* strain displays attenuated virulence compared to WT strain when administered at a lower dose

In a chronic model of airway infection, we found that infections with the WT and Δ*lasB* strain embedded in agar beads led to similar growth and persistence in the lungs at a high inoculating dose of ~8x10^5 CFU/lung (Chapter 2). However, we also observed that a lower dose (~5x10^5 CFU/lung) led to decreased bacterial burden of the Δ*lasB* strain by day 1 p.i. (Appendix Fig. 6.1A) and increased clearance by day 4 p.i. (Appendix Fig. 6.1B). It has been previously shown that Δ*lasB* mutants are unable to degrade the opsonic surfactant SP-A, and are thus more susceptible to SP-A mediated opsonisation and phagocytosis both *in vitro* and *in vivo* compared to WT strains (*286*). Therefore, differences in the ability of host cells to phagocytize the WT and Δ*lasB* strains may account for the reduced growth and clearance of the Δ*lasB* in the lungs.

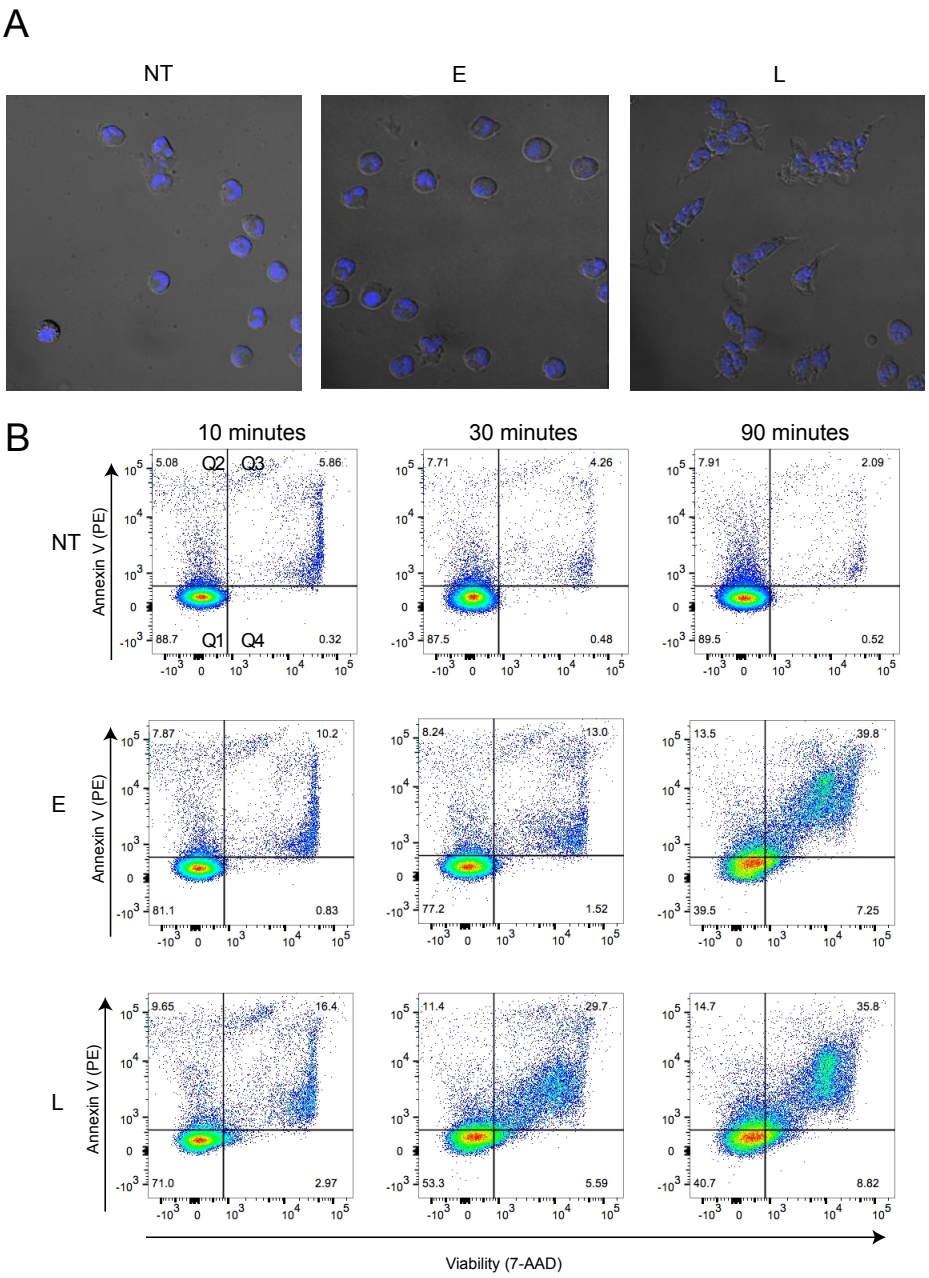


Appendix Fig A1: Δ*lasB* strain displays attenuated virulence compared to WT strain when administered at a lower dose

(A) Bacterial burden in whole lungs at days 1 (D1) and 4 (D4) post infection (p.i.) as determined by enumeration of viable CFU counts.
(B) Enumeration of animals with that had a persistent infection in lungs by D4 p.i. versus strains that cleared the infection (defined as the absence of any detectable CFU in whole lungs by serial dilution plating)

Appendix 2: The Late isolate induces premature neutrophil death *in vitro*

Since we observed that the Late isolate recruited more neutrophils compared to the Early isolate *in vivo*, yet was able to persist significantly better in the host (chapter 4), we hypothesized that neutrophil function against this bacterial strain was impaired. To test this, we co-incubated live Early and Late bacteria at a multiplicity of infection of 10 (MOI 10) with human neutrophils isolated from the peripheral blood of healthy donors and examined the neutrophils for morphological changes by confocal microscopy, and for changes in viability by FACS using markers of cell death. After 30 min, neutrophils co-incubated with the Late isolate lost their normal spherical shape, became elongated and began to clump together (Appendix Fig. 2A). Flow cytometry analysis demonstrated that Late isolate infected neutrophils showed accelerated loss in viability by annexin V and 7-AAD staining compared to Early-infected neutrophils (46.7% vs 22.8% at 30 min) (Appendix Fig. 2B).



Appendix Fig A2: Human neutrophils undergo accelerated death during co-incubation with the Late isolate

(A) Representative images of DAPI stained neutrophils after 30 min of co-incubation with Early and Late isolates (MOI 10), or control cells (NT).
(B) Representative FACS scatter plots of human neutrophil co-incubated with E and L isolates at a MOI = 10, or untreated control (NT). Neutrophils were stained with PE-conjugated annexin V and 7-AAD at the indicated time point. Quadrant 1= viable cells, quadrant 2=early apoptotic cells, quadrant 3= late apoptotic cells (annexin V^+ 7-AAD^+) and quadrant 4= dead cells.

Primary human neutrophils were isolated from the peripheral blood from healthy volunteers by using EasySep ™ Direct Human Neutrophil Isolation Kit (Stem Cell). Results in A and B are representative of n=2 independent experiments.

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