2007

Reducing Lung Disease in Cystic Fibrosis: Model Systems and Anti-Inflammatory Treatment

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Reducing lung disease in cystic fibrosis:

Model systems and anti-inflammatory treatment

by

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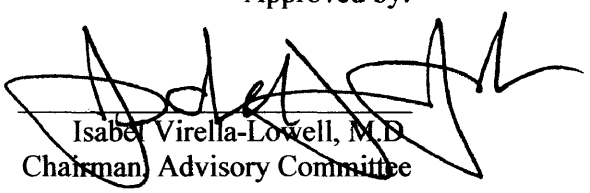
A dissertation submitted to the faculty of the Medical University of South Carolina
in partial fulfillment of the requirement for the degree of Doctor of Philosophy
in the College of Graduate Studies.

Department of Microbiology and Immunology

2007

Approved by:



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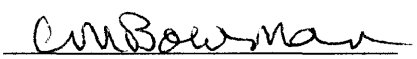

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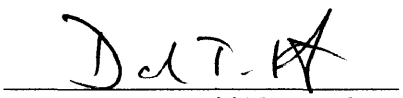

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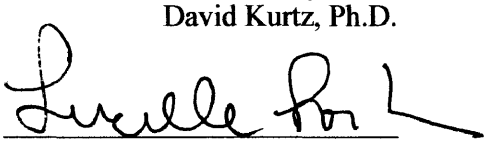

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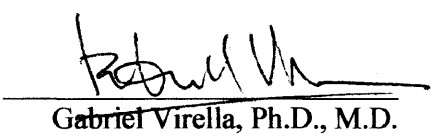


Gabriel Virella, Ph.D., M.D.

To My Parents

Daddy, for teaching me to love science

Mama, for encouraging my "uniqueness"

and

To Grandy,

for inspiring me to enjoy life at every step

Acknowledgements

I would like to thank my mentor, Dr. Isabel Virella-Lowell, for her encouragement and support in my development as a scientist interested in working with patients with cystic fibrosis. Thank you for making sure the lab was a pleasant place to work and for being accessible despite a busy schedule.

Thank you to the members of my committee. Dr. Michael Bowman took me under his wing my first year in graduate school and made me part of the MUSC CF Center. He has always been available, asking pertinent questions and encouraging me to pursue my interests. Dr. Lucille London has been a wonderful asset in my development as a graduate student. Her no-nonsense demeanor with underlying support has helped me tackle obstacles and progress to complete my degree. I have benefited from discussion and advice from Dr. Gabriel Virella. He has asked relevant questions and was complementary of my abilities. I have enjoyed many conversations with Dr. David Kurtz over the years. His eclectic knowledge and interest in the different projects I have pursued in my graduate experience are much appreciated.

My experiments would not have been possible without AAV vector provided by the UF Vector Core. Thank you to Dr. Terence Flotte, Dr. Richard Snyder, Mark, Deena, Tina, and Jason for allowing me to spend an enjoyable month learning to make recombinant AAV. Also, thank you to Dr. Flotte for introducing me to important people at conferences and being interested in my success. Dr. Flotte's lab was helpful with advice and access to reagents; thank you to Chris, Sophi, Ashley, Stuart, Thomas, Travis, Kevin, and Amy. A special thank you goes to Margaret and Sophi for providing a place to stay during my visits.

Another essential component in my experiments was the surfactant phospholipids provided by Dr. John Baatz. Thank you for being available for technical discussions and advice concerning experiments.

Thank you to the MUSC CF Center: Mary, Cheryl, Jen, Chris, and Sarah for helping me understand the many complications a patient with CF faces and the clinical importance of new therapeutics. Thank you to Dustin for becoming a special friend.

My time at MUSC has been enjoyable, mainly the result of important exchanges at any level of interaction. In Dr. John Dong's lab, I began developing my skills as a scientist

both in the laboratory as well as through writing, reading, and technical discussions. Dr. Dong's belief in my abilities as an independent researcher led to contacts with different scientists to establish collaborations, providing initial experience and confidence with professional matters, and I am thankful for the opportunity he afforded me early in graduate school. Dr. Semyon Rubinchik taught me to have a finely-tuned analytical mind, considering all possible angles of an experiment. Thank you for insightful conversations and many experiences shared. Dr. Jan Woraratanadharm and I had an adventure of memories, both inside the laboratory and extra-curricularly. Thank you for your continued encouragement and valued friendship. Dr. Hong Yu had amazing patience in teaching me to work with animals. Thank you for being continuously complementary on the bright future I will have in any activity I pursue. Thank you to Scarlett for your resolute support and a wonderful friendship, and to Scarlett and Ryan both for providing the comic relief I have needed during my last year of graduate school!

Thank you to Dr. Séverine Ouvry-Patat for being a wonderful roommate, involving me in campus life, teaching me to become more balanced, and for your honesty, advice and support. Mostly importantly, thank you for a friendship that will last for many years to come.

Thank you to the Center of Academic Excellence for their time and encouragement. Dr. Shannon Richards-Slaughter gave me essential advice when I especially needed it. A special thank you to Dr. Jennie Ariail for your exhaustive edits, excited interest in my research and aspirations, and all the other important life lessons I learned behind the door to your office. I hope that we will not lose contact with each other.

There were other people who made MUSC a great place to work each day. Thank you to Karen for always being friendly and sending me pictures of her grandsons. To Facilities and Management: John for his pats-on-the-back and a southern touch of home and Hal for his smiles and pleasant demeanor. Public Safety would have made a mint if they had charged for their late-night walks to my car! Thank you to Officers Washington, Scot, Jerimaine, John, Ivory, and all the other campus police officers who picked me up with a smile and kept a steady stream of conversation flowing, so I wouldn't fall asleep on the way to my car. Also, thank you to Burgess for checking under the hood of my car and for his "Every little thing's goin' be alright" attitude as I entered or exited G-lot after-hours and on the weekend.

I had the distinct opportunity to be part of the Presidential Scholars program, opening a new world at my feet. Thank you to Wilmot J. Fraser Elementary, especially Ms. Pamella Gibbs and Ms. LaSheia Oubre, for being interested in the Junior Doctors of Health program and helping make it a success. I have enjoyed the development of our friendship over the years and becoming a part of the Fraser family. Thank you to Dr. Valerie West for supporting the program, believing in my abilities, and for wearing super-crazy socks! A special thank you goes to President Ray Greenberg for funding the program and giving me many opportunities to present it, as well as being mindful of my scientific interests. Also, thank you to Judy, Marcia, Suzanne, and Edie for being a pleasure to work with at any level.

Encouragement and understanding from my friends kept me connected to the also important world outside of graduate school. Thank you to Dianne for being a best friend in all senses of the word; may Dianne Shepherd, CPA and Dr. Scotty Buff meet the world very soon! A special thank you goes to Nicole for having Brie and Leighton leave messages on my phone and for a treasured friendship. Thank you to Ben for catching up every Thanksgiving and to Sean and Chris for re-connecting when too much time had passed. Also, thank you to Michael, Athena, and Taylor for making sure we saw each other every year.

One would be hard-pressed to find a family as encouraging, supportive, excited, and proud as I have been fortunate to have. Thank you to my brother, Mr. Paul, for sharing the ups and downs of our respective graduate experiences; I am glad we have become so close. Thank you to my sister, Mary, for being there when I needed it most, helping me relax, and being my most loyal advocate. Thank you to my sister, Éowyn, for making sure to keep in touch all the time and telling me how great I am☺ Thank you to Sasha for making me laugh and spending extra time with Grandy, and to Aunt Gen for being supportive from Raleigh to Charleston to San Diego on matters from research and relationships to planning weddings. Thank you to Aunt Pat for continuous email up-dates and for being postmistress to Grandy when I was not able to write letters.

My grandmother, Grandy, and my parents are the reason for my determination and natural curiosity. Thank you to Grandy for asking what I do and listening, for sending me letters, pictures, food and visiting, and making me proud of my accomplishments. Mama, thank you for making limited time with family special, and for loving what you do each day

and encouraging me to find it for myself. Daddy, thank you for making me question…everything! And for always being proud of me.

Finally, thank you, Ryan, for loving me from afar and moving to Charleston. Thank you for really being interested in what I do (knowing about the C-F-T-R and A-A-V), coming to seminars and *Student Research Day*, and being patient with many late nights of lab work. Thank you for making it easier for me to write despite our continued time apart. I look forward to the next step in our life together.

Table of Contents

List of Figures

List of Tables

Key to Abbreviations

A549	human lung cancer cell line
AAV	adeno-associated virus
Ad	adenovirus
ATP	adenosine triphosphate
AAT	alpha-1 anti-trypsin
APC	antigen-presenting cell
ABC	ATP-binding cassette
ASL	airway surface liquid
aGM	asialoganglioside
bp	base pair
BUN	blood urea nitrogen
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BALM	bronchoalveolar macrophages
BALT	bronchus-associated lymphoid tissue
BMI	body mass index
CAM	cell adhesion molecule
CsCl	cesium chloride
cfu	colony forming units
CAR	coxackie associated receptor
cDNA	complementary DNA
cAMP	cyclic adenosine 3', 5' monophosphate

CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
ΔF508	CFTR protein with deletion of phenyalanine at position 508
CMV	cytomegalovirus
CMVie	CMV immediate-early
Cβ	CMVie/chicken beta-actin hybrid promoter
ELF	epithelial lining fluid
ENaC	epithelial sodium (Na+) channel
ECM	extracellular matrix
FABP	fatty acid binding promoter
FEV	forced expiratory volume
GSH	glutathione
GMP	good-manufacturing-practice
HeLa	human cervical cancer cell line
HNP	human neutrophil peptide
HSP	heparin sulfate proteoglycan
HSV	herpes simplex virus
HRP	horse radish peroxidase
IB3-1	CF bronchial epithelial cell line
iNOS	inducible nitric oxide
ICS	inhaled coriticosteroids
ICAM	intercellular adhesion molecule
IFN	interferon

IL	interleukin
IL-10R	IL-10 receptor
IL-10T	IL-10-deficient transgenic mice
IPV	intrapulmonary percussive ventilation
ITR	inverted terminal repeat
JAK	Janus kinase
kb	kilobases
KC	keratinocyte chemoattractant
LPS	lipopolysaccharide
MHC	major histocompatability complex
MIP	macrophage inflammatory protein
MSD	membrane spanning domain
MEG	mercaptoethylguanidine
MMP	metalloproteinase
MAPK	mitogen-activated protein kinase
MOI	multiplicity of infection
MPO	myloperoxidase
NK	natural killer cell
NE	neutrophil elastase
NSAID	non-steroidal anti-inflammatory drugs
NFκB	nuclear factor kappa B
NBD	nucleotide binding domain
nt	nucleotide

ORF	open reading frame
ORCC	outwardly rectifying chloride channel
PBS	phosphate buffered saline
pA	polyadenylation
PMN	polymorphonuclear cell
PGE2	prostaglandin E2
PA	*Pseudomonas aeruginosa*
q	long arm of chromosome
rIL-10	recombinant IL-10 protein
RBE	Rep binding element
R/C	rep/cap genes of AAV
S9	CF bronchial epithelial cell line corrected with CFTR
SLPI	secretory leukocyte protease inhibitor
STAT	signal transducers and activators of transcription
SDC	sodium deoxycholate
SOCS	suppressor of cytokine signaling
TBP	TATA binding protein
tg.AAV-CF	AAV vector expressing CFTR from ITR region
trs	terminal resolution site
TLR	toll-like receptor
TA	transactivator
TEPD	transepithelial potential difference
TM	transmembrane domain

TNF	tumor necrosis factor
vg	vector genomes
VP	viral protein

Abstract

SCOTTY MCGLOTHLIN BUFF. Reducing lung disease in cystic fibrosis: Model systems and anti-inflammatory treatment. (Under the direction of ISABEL VIRELLA-LOWELL, M.D.)

Cystic fibrosis (CF), the most common lethal autosomal recessive disease in Caucasians resulting in a median life span of approximately 35 years, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). The primary cause of morbidity and mortality is respiratory failure caused by progressive obstructive lung disease. CF airway disease is characterized by the abundance of proinflammatory cells, chemokines and cytokines with a deficiency of regulatory/anti-inflammatory cytokine interleukin 10 (IL-10). We hypothesized that CF airway obstruction originates from excessive inflammation and gene transfer of IL-10 would decrease inflammation in the airways of mice infected with *Pseudomonas aeruginosa*, a common pathogen in CF.

IL-10 gene transfer was accomplished using an adeno-associated viral vector (AAV) selected for the inherent benefits of long-term gene expression, a proven safety profile, and the ability to elicit a minimal inflammatory response in comparison to other gene transfer agents. Using intratracheal injection, we administered AAV5.Cβ-mIL10 to IL-10 deficient mice, chronically infected the mice with *P. aeruginosa*, and observed high levels of IL-10, decreased lung pathology, and IL-1β and IL-8 (MIP-1α and KC in mice), cytokines frequently seen at increased levels in patients with CF.

To characterize IL-10 gene transfer in CFTR knockout mice, we modified a non-invasive procedure, aspiration challenge, to include surfactant phospholipids as a vehicle for transfer of *P. aeruginosa* and AAV-based vectors. This was important because CFTR knockout mice are sensitive to manipulations such as intratracheal injection, resulting in

mortality from the surgical procedure. We demonstrated aspiration challenge with surfactant phospholipids was similar to intratracheal injection for *P. aeruginosa* and AAV delivery, as measured by percentage weight loss, proinflammatory cytokine production, inflammation-associated histopathology, and bacterial burden for *P. aeruginosa* and vector distribution and transgene expression for AAV.

Then, we characterized AAV5.Cβ-mIL10 in CFTR knockout mice chronically infected with *P. aeruginosa*, observing decreased percentage weight loss, lung pathology, pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, KC, MIP-1α), histopathology, and neutrophil migration with no change in bacterial burden.

These studies support that inflammation is excessive to bacterial infection in CF and demonstrate AAV-based IL-10 gene transfer is a promising anti-inflammatory therapeutic for CF lung disease.

Chapter 1.

Significance and Introduction

1.1. Significance of Research Project

Like most human genetic diseases, cystic fibrosis (CF) requires life-long therapeutic interventions. CF is a complex disease that manifests itself pathologically in several organs with a range of symptoms that vary between individuals, due to the type of CF gene mutation, a host of polymorphisms, and environmental influences [1] and the pleiotropic role of the resulting defective CF protein. Current therapeutic approaches to CF are symptomatic and can be timely and invasive with patients succumbing to early mortality, currently approximately 36 years. Gene transfer has become a promising alternative, as it has the potential for stable maintenance of therapeutic genes inside defective cells, ideally providing long-term treatment within the diseased tissue without serious side effects. The first type of gene transfer investigated for CF used the wild-type gene mutated in CF (the cystic fibrosis transmembrane conductance regulator [CFTR]) because of the success of this approach in correcting the CFTR channel defect in CF lung cells [2].

To date, there have been gene therapy clinical trials for CFTR correction with liposomes, Adenovirus, and AAV. Early studies indicated that lipid-DNA complex delivery to the nasal epithelial showed no treatment-related local or systemic adverse reactions [3], but later studies showed non-specific inflammation is a major obstacle to both intravascular and intratracheal delivery [4, 5]. Additionally, overall gene delivery by liposomes has been of low efficiency, not producing consistent evidence of gene transfer to the nasal epithelium by physiologic or molecular measures [6, 7].

Adenoviral-based vectors can efficiently infect airway epithelia and are capable of producing robust levels of gene expression [8-11]. However, adenovirus vectors also activate the innate immune system and expression of numerous chemokines and cytokines, inducing an acute inflammatory response which severely reduces adenovirus gene transfer efficiency [11-15].

To date, the most promising gene transfer agent in CF is an AAV vector expressing CFTR (tg-AAV.CF). Phase I/II clinical trials have been conducted in greater than 100 CF patients without evidence of significant toxicity. The safety profile of this vector allows patients as young as 12 years old to participate in clinical trials [16]. Tg-AAV.CF has been instilled into the nose, maxillary sinus, and single lung lobe, and delivered through aerosol by oral inhalation to the entire lung. In phase I maxillary sinus studies, dose dependent gene transfer and changes in sinus transepithelial potential difference (TEPD) were observed, consistent with production of wild-type CFTR [17]; however, the lack of sinus TEPD measurements in patients without CF makes interpretation of these data difficult (I. Virella-Lowell, personal communication). These studies demonstrate that tg-AAV.CF is safe with minimal viral shedding in the sputum and no change in baseline inflammation in nasal epithelium as measured by the lack of increase in proinflammatory cytokines in serial bronchial wash fluids [16, 18]. Interestingly, follow-up studies with tg-AAV.CF demonstrated a decrease in sinus concentrations of proinflammatory cytokine, IL-8, at day 14 post-treatment from the vector-treated side as compared to the placebo-treated side, which correlated with absolute sinus neutrophil count [19].

A phase II double-blinded, placebo-controlled study of multiple doses of tg-AAV.CF delivered by oral aerosol inhalation suggested that the aerosolized product, administered via

nebulizer to the lung, was safe and well-tolerated by patients [20]. At day 14, levels of IL-8 were lower in patients receiving tg-AAV.CF, and at day 30, lung function improved ($p=0.04$), both measurements compared to placebo. While a local and systemic AAV neutralizing antibody response was observed, excellent gene transfer, as measured by DNA PCR on cells removed by bronchoscopy, was found in all patients tested. This is the first time in any trial for gene transfer of cystic fibrosis that clinical outcomes measured in the treatment group were significantly improved over placebo.

While tg-AAV.CF has repeatedly been demonstrated to be safe and result in a stable maintenance (weeks to months) of CFTR expression, it does not result in high enough levels of gene expression to significantly change the clinical course of the disease. In addition, it has been difficult to assess the relationship between its molecular action and the observed clinical improvements, because AAV-specific mRNA has not been detected [21]. To address this problem, Flotte et al studied primary nasal cells obtained from patients receiving tg-AAV.CF [22]. The cells were collected at different time points after gene transfer and expanded *in vitro* with the purpose of detecting CFTR mRNA expression to correlate AAV-CFTR transfer and cystic fibrosis airway epithelial cell correction. Vector-specific mRNA was detected in three out of five subjects and, of these three subjects, two displayed an increase in chloride transport, suggesting a correlation between the presence of AAV-CFTR vector genomes, CFTR mRNA expression, and cAMP-activated chloride channel function detectable only after *ex vivo* expansion.

CFTR gene transfer relies on targeting relevant cells for CFTR function (believed to be the submucosal glands) and generating high levels of CFTR expression, shown to be more efficient in correct ion transport abnormalities in CF cells [23]. The limited success of AAV-

mediated CFTR gene transfer and the realization that targeting the CFTR may not be beneficial for the majority of patients with CF due to their extensive lung destruction, has led researchers to evaluate therapeutics targeting the inflammatory cascade and slowing disease progression. CFTR defects are related to immune regulation and the predisposition of affected individuals to the persistent chronic endobronchial infections. In the CF airway inflammation is excessive relative to the amount of infection in the CF airway, and patients have increased stimulation of Nuclear Factor (NF)κB and heightened production of the pro-inflammatory cytokines Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-1β, IL-6, and IL-8 [24-29]. They also have reduced amounts of IL-10, a regulator of inflammation, and IκBα, a regulator of NFκB [26, 28-37]. Aberrant regulation of inflammation results in recruitment of large amounts of neutrophils to the airway where they release high levels of substances that directly and indirectly damage the airways. The imbalance of pro- to anti-inflammatory mediators is well established early in the life of a patient with CF, resulting in many years of lung destruction and progressive airway obstruction.

Therapies for inflammation in CF have been mildly effective and limited in usage by their side-effect profiles. Use of gene transfer to target inflammation could prevent further lung destruction and alleviate obstruction in adolescents and adults with CF. If given in very young patients, lung destruction due to inflammation could potentially be prevented. Pairing the anti-inflammatory gene IL-10, expressed at reduced levels in the CF lung, with AAV delivery is an attractive approach because it has great potential to not only decrease inflammation, but to increase patient adherence through long-term persistence of gene expression. Use of IL-10 is supported by a previous study in our laboratory, Virella-Lowell et al used microarrays to investigate the gene expression profile in a CF bronchial epithelial cell

line (IB3-1) as it was altered by the addition of the wild-type CFTR gene, IL-10, and/or infection with *Pseudomonas aeruginosa* and demonstrated that ***IL-10 is able to reverse some of the phenotypic changes in CF cells that are not observed in non-CF cells*** [38].

This dissertation investigates the therapeutic potential of modifying the abnormal immune response in CF by (1) using AAV-IL-10 delivery and (2) optimizing a non-invasive administration technique to measure attenuation, *in vivo*, of CF lung disease. ***We hypothesized that AAV-IL10 gene transfer would significantly reduce inflammation in* P. aeruginosa*-infected mice, while not causing systemic immunosuppression***. In developing a non-invasive method for *in vivo* analysis, ***we hypothesized that aspiration challenge of* P. aeruginosa*-laden beads and AAV-IL10 vector with surfactant phospholipids would result in a similar infection and gene transfer, respectively, to intratracheal injection while being non-invasive and less difficult to perform.*** To characterize IL-10 gene transfer and optimize the *in vivo* method of infection and therapeutic delivery, the following aims were designed.

Aim 1: To characterize AAV-IL10 as an anti-inflammatory therapeutic in a *P. aeruginosa* chronic infection model in IL-10 deficient mice.

Aim 2: To develop an aspiration challenge with surfactant phospholipids method for administration of *P. aeruginosa*-laden beads.

Aim 3: To develop an aspiration challenge with surfactant phospholipids method for administration of AAV-based vectors.

Aim 4: To use aspiration challenge with surfactant phospholipids for *P. aeruginosa*-laden bead and AAV-IL10 vector administration, and characterize AAV-IL10 as an anti-inflammatory therapeutic in a *P. aeruginosa* chronic infection model in gut-corrected CFTR knockout mice.

1.2. Cystic Fibrosis

Cystic fibrosis, a complex autosomal recessive disease that is primarily diagnosed in children and young adults, is the most common autosomal recessive disease in the Caucasian population. Heterozygotes, who obtain one mutant CF allele and one normal allele in this region, are denoted carriers and are entirely asymptomatic; whereas the child of two carriers has a one in four chance of inheriting the CF mutation on both alleles and being affected with the disease. The prevalence of CF varies by ethnic origin, with the highest predominance in the Caucasian population (1/3000, with 1/20 carriers for the disease), followed by the Hispanic (1/8000), Black (1/17000), and Asian (1/90000) populations [1].

The first comprehensive description of CF was published by Anderson in 1938, where the phrase "cystic fibrosis of the pancreas" was used to describe the extreme pathology that resulted from the lack of pancreatic exocrine function [39]. Although the association between salty skin and early death has been recognized since medieval times [40], DiSant' Agnese was the first to demonstrate that children with CF exhibit excessive salt loss in their sweat [41], a discovery that led to the development of a diagnostic tool whereby the measurement of chloride and sodium in the induced sweat of children could be used identify children with CF [42]. This test remains the gold standard for CF diagnosis today.

Early laboratory studies further defined the molecular characteristics of CF through analysis of patient samples. Paul Quinton demonstrated poor re-absorption of NaCl in isolated sweat ducts from CF patients and hypothesized this was the result of abnormally low chloride permeability, the reason for the high concentration of NaCl in the sweat of CF patients [43]. A similar abnormality was also demonstrated in the respiratory epithelia [44, 45]. Sato et al found that cells derived from patients with CF do not exhibit normal chloride

efflux by activation of a cyclic adenosine 3', 5'-monophosphate (cAMP) –dependent protein kinase (PKA) in response to elevated cAMP levels [46]. In CF cells, activation of PKA was found to be intact, but did not result in chloride efflux [47]. Even though these early studies provided increased knowledge into the underlying defect in CF, the results were inadequate because they could not identify the specific gene which caused the chloride efflux deficiency.

In 1989, the CF gene was identified through positional cloning [48] and named the cystic fibrosis transmembrane conductance regulator or the CFTR gene, reflecting the initial findings of the protein's function. The direct association of the gene and CF was supported by finding mutations in a gene located on the long (q) arm of chromosome at 7 position 31.2 in patients with CF, but not in the normal population.

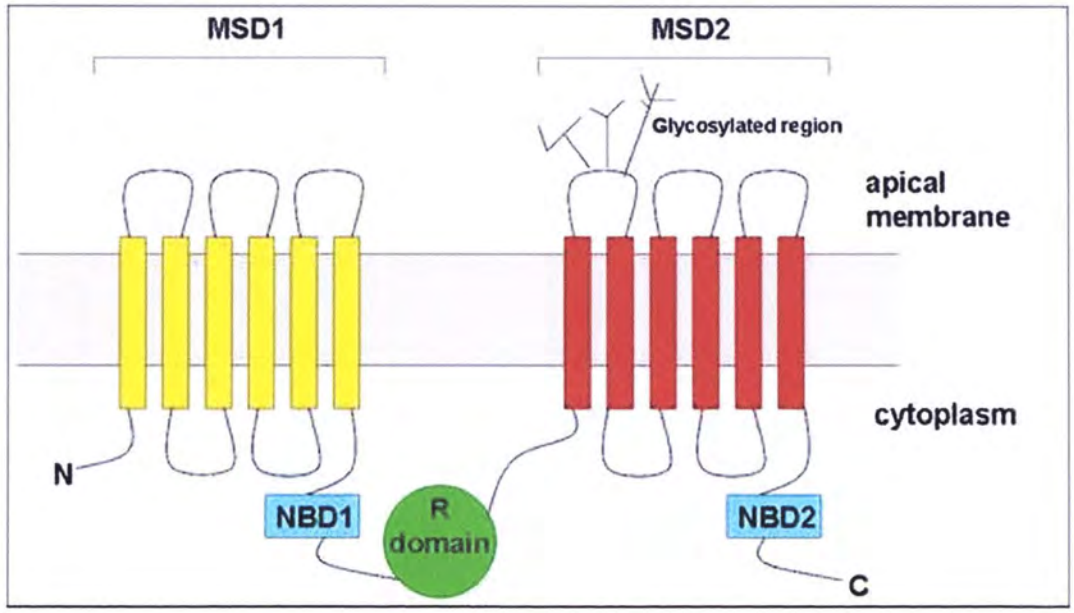
a. Cystic Fibrosis Transmembrane conductance Regulator (CFTR)

The CFTR gene encodes a transmembrane protein of 1480 amino acids, with a structure that has been compared to members of the ATPase or ATP binding cassette (ABC) transporter family. This family and the CFTR share a homologous structure of two membrane-spanning domains (MSD1 and MSD2) and two Nuclear Binding Domains (NBD1 and NBD2) [49] (**Figure 1.1A**).

MSD1 and MSD2 each contain 6 transmembrane (TM) domains which contribute to the formation of the chloride-selective pore [50], presumed to be about 3 Angstroms wide (**Figure 1.1B**).

Figure 1.1

A.


MSD1
MSD2
Glycosylated region
apical membrane
cytoplasm
N
NBD1
R domain
NBD2
C

B.

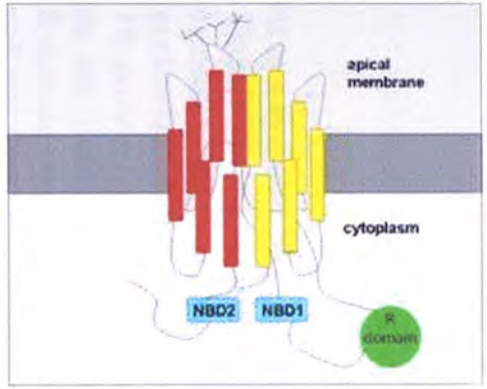

apical membrane
cytoplasm
NBD2
NBD1
R domain

Figure 1.1 The CFTR is a symmetrical protein that forms a transmembrane channel in epithelial cells. (A) The CFTR is a membrane spanning channel protein composed of two membrane spanning domains (MSD1 and MSD2), each of which contains six transmembrane domains (TM), two nucleotide binding domains (NBD1 and NBD2), and a regulatory (R) domain. **(B)** The CFTR protein forms a pore lined with six positively charged amino acids and regulates chloride ion translocation. NBD1 and NBD2 hydrolyze ATP to regulate channel gating. The R-domain contains phosphorylation sites which regulates interaction of ATP with the NBDs and may control ATP hydrolysis and channel gating. Adapted from [49].

The CFTR protein is predominantly expressed in epithelial cells, where it is transcribed at relatively low levels [51]. Studies of CFTR in the human lungs have localized the expression to the respiratory epithelium, which expresses very low levels, and the submucosal gland (**Figure 1.2**), which show considerably higher levels [52, 53], suggesting the submucosal glands might be an important site for CFTR function. In addition to being expressed in the apical membrane, the CFTR has been localized to subcellular regions where it may be involved in vesicle trafficking, including participation in endocytosis, exocytosis, and endosome fusion, and intracellular chloride activity by importing chloride as a counter-ion to permit the organelle's acidification and regulate vacuolar pH while maintaining the internal electrical neutrality [1, 54]

Figure 1.2

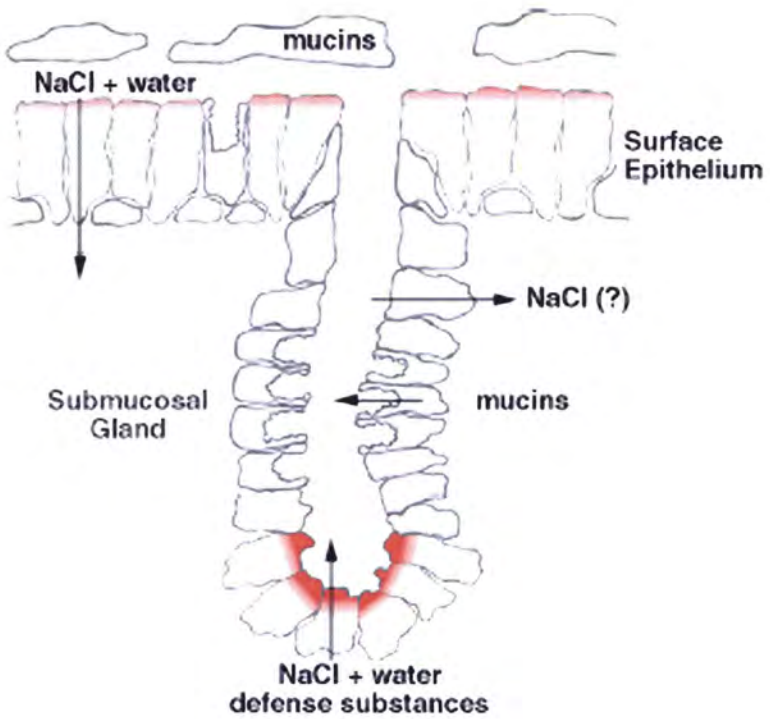

mucins
NaCl + water
Surface
Epithelium
NaCl (?)
Submucosal
Gland
mucins
NaCl + water
defense substances

Figure 1.2 Schematic of proximal airway shows sites of salt and water transport and mucin secretion that lead to formation of airway surface liquid (ASL). Shading indicates relative level of CFTR expression [53].

The primary role of the CFTR protein is as a cAMP-activated chloride channel that plays a key role in fluid and electrolyte secretion by contributing to the rate of transepithelial salt and fluid transport. The CFTR provides a pathway for chloride movement across epithelia as well as regulating the rate of flow. Its functions are pleiotropic, depending upon on the tissue in which it is expressed. In the intestine, pancreas, and sweat gland secretory coil, the CFTR regulates fluid and electrolyte *secretion*, while in the sweat gland duct and airway epithelia, it participates in fluid and electrolyte *absorption.*

The CFTR may also regulate other ion channels, including outwardly rectifying chloride channels (ORCC) and amiloride-sensitive epithelial sodium channels (ENaC). It appears to have a stimulatory influence on the former and, conversely, an inhibitory role on the latter. The ORCC acts as a floodgate with a resulting chloride conductance much higher than that of the CFTR and its dysfunction may have a role in creating the CF phenotype, secondary only to the CFTR [1, 55]. ENaC, which has heightened sodium absorption in the absence of regulation by the CFTR and, with the development of an ENaC overexpressing mouse model that seems to have spontaneous lung disease, is also being considered a prime contributor to CF pathology [56].

Absence, mutations, or low abundance of the CFTR protein results in an ionic imbalance and the dehydration of secretions along epithelial membranes. While a deletion of phenylalanine in position 508 (ΔF508) of the CFTR protein accounts for approximately 70% of the mutant alleles in CF [57], over 1,300 mutations have been identified that cause CF and are grouped in five different classes according to their affect on CFTR transcription, trafficking, or abundance of expression (**Figure 1.3**) [1]. Class 1 mutations cause premature termination signals and result in little or no protein production. Class 2 mutations, which

include ΔF508, result in abnormal folding of the protein product and do not lead to normal trafficking to the apical cell membrane. Class 3 contains mutations in the nucleotide binding domains (NBDs) and result in normal protein production and trafficking, but defective regulation of the protein. Class 4 have mutations in the amino acids lining the channel pore, resulting in a chloride channel with normal regulation, but reduced single channel current. Class 5 mutations target splicing of CFTR transcripts and lead to significantly decreased production of normal CFTR. Some mutations may disrupt CFTR function in more than one way and are categorized in multiple classes [58].

Figure 1.3

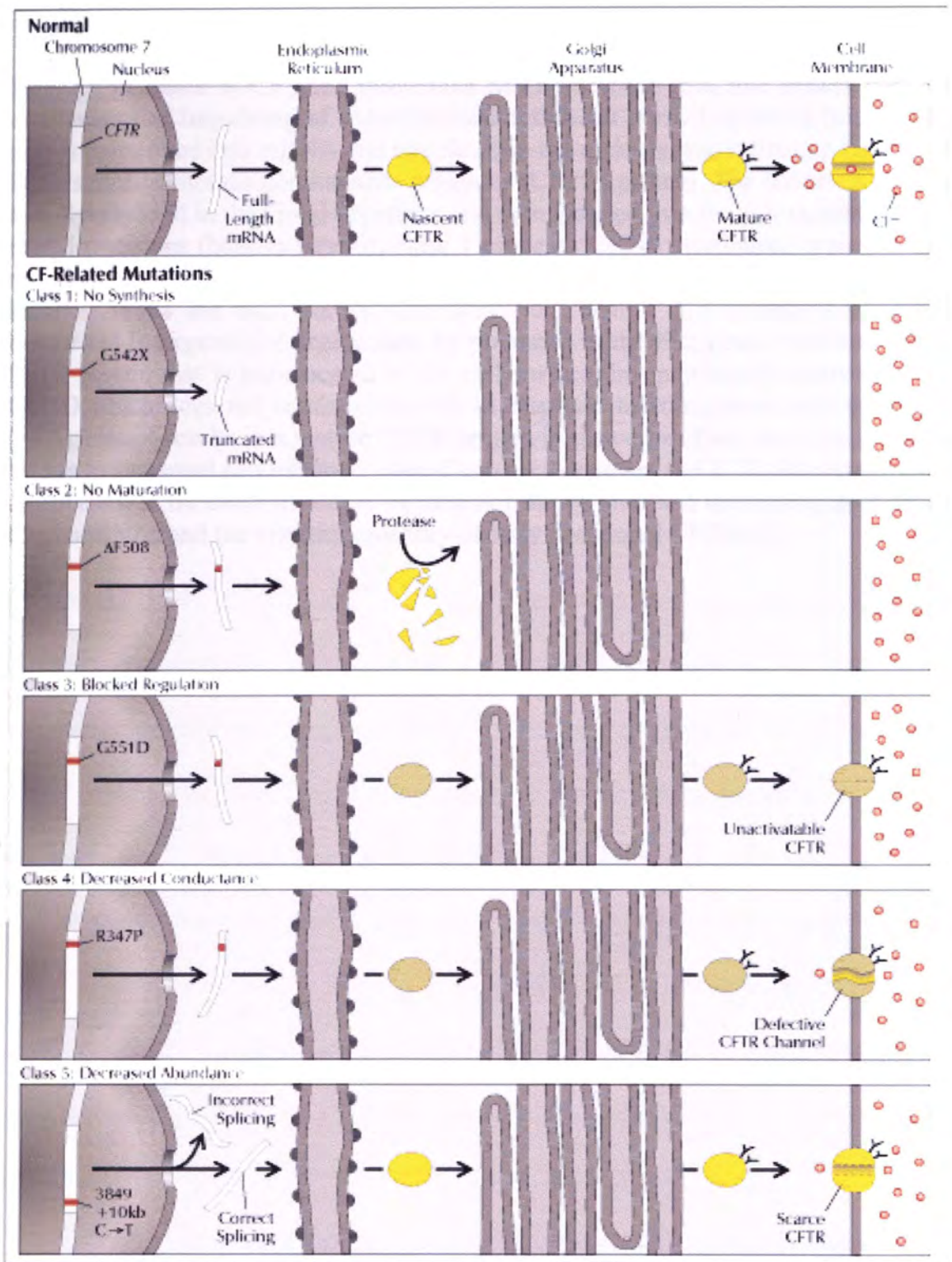

Normal
Chromosome 7
Nucleus
CFTR
Endoplasmic Reticulum
Golgi Apparatus
Cell Membrane
Full-Length mRNA
Nascent CFTR
Mature CFTR
Cl⁻
CF-Related Mutations
Class 1: No Synthesis
G542X
Truncated mRNA
Class 2: No Maturation
ΔF508
Protease
Class 3: Blocked Regulation
G551D
Unactivatable CFTR
Class 4: Decreased Conductance
R347P
Defective CFTR Channel
Class 5: Decreased Abundance
Incorrect Splicing
3849 +10kb C→T
Correct Splicing
Scarce CFTR

Figure 1.3 Classes of CF gene mutations can be divided into five classes that define the mechanisms of impairing of chloride conduction. In normal epithelia (top panel), the CF gene is transcribed into mRNA and translated in the endoplasmic reticulum (ER) to the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The nascent CFTR protein is then glycosylated in the Golgi apparatus and then inserted into the cell membrane. In disease-related mutations (bottom panels), class 1 prevents CFTR translation, typically due to stop mutations such as in G542X, which produces a truncated, non-functional mRNA; class 2, includes ΔF508 the most common CF mutation, results in a translated protein does not mature and is targeted for degradation by proteases in the ER; class 3 produces fully mature CFTR protein that is translocated to the cell surface, but prevents its activation, such as in G411D which does not conduct chloride in response to stimulation with protein kinase A (PKA); class 4 results in a mature CFTR protein in the cell surface that is activated normally, but has low channel conductance; class 5 causes a decrease in CFTR abundance and chloride conductance at the cell membrane by targeting splice sites and decreasing abundance of full-length mRNAs and the resulting quantity of fully functional CFTR [1].

b. Clinical Symptoms and Treatment

Current therapy for CF is palliative, with the combination of current therapeutics extending the life expectancy of patients to 32-35 years (from <1 year in 1940). Although CF is believed to be monogenetic, the symptoms vary between individuals. This difference is somewhat explained by mutation type, but genotype and disease outcome do not completely correlate. As a result, patients can experience multi-organ or unilateral clinical symptoms from the build-up of thick dehydrated mucus along the epithelial lining of the pancreas, reproductive organs, and the lungs.

Eighty percent of patients with CF experience pancreatic deficiency due to blockage of pancreatic enzymes in their transport through the pancreatic ducts to the intestine, which are clogged by the thick mucus build-up [1] and lead to malabsorption and malnutrition. Currently, multivitamin and enzyme supplements are used in the treatment of the nutrition/gastrointestinal complications; however, some patients still require the insertion of a gastric feeding tube implanted to the stomach. Even with these treatments, patients with CF generally have a low body mass index (BMI) when compared to children of comparable age. Also, destruction of pancreatic cells results in diabetes in 30-50% of the patients.

In the reproductive organs, azoospermia, with consequent sterility, occurs in approximately 99% of male patients with CF because of clogging of the vas deferens, which stops the sperm in the testes [1]. There is currently no effective therapy available to combat male infertility.

Chronic lung infection occurs in about 80% of patients with CF [59]. The accumulation of mucus in the airway causes difficulty breathing and fosters an environment prone to bacterial infection. Dehydration of mucus causes airway secretions to become more

viscous and results in trapping microorganisms making infections with *Pseudomonas aeruginosa* and *Staphylococcus aureus* inevitable, promoting inflammation and lung damage. Airway clearance techniques are conducted with either traditional chest physiotherapy ("hand clapping") or with the aid of oscillatory devices (the vest, intrapulmonary percussive ventilation [IPV], and flutter devices) which create vibrations along the airway to dislodge the mucus, move it toward the central airway, and help to thin secretions so they can be removed by coughing or spitting. In coordination with airway clearance, patients take aerosolized medications to open the airway and liquefy the mucus; these include bronchodilators, anti-inflammatory therapeutics, and mucolytic agents such as dornase alpha (Pulmozyme®, Genentech) which contains DNase. Even with the most rigorous airway clearance regiment, persistent bacterial infections result, and most patients with CF remain on a continuous dosage of antibiotics orally or through aerosol delivery [colistin, Tobramycin (TOBI®, Chiron)].

However, the antibiotics only suppress an exacerbation of bacterial infection. Embedded in the mucus, the microorganisms are protected from the body's airway defenses as well as pharmaceutical agents, neither of which easily penetrates the biofilm the bacteria form. Obstructive airway disease as a consequence of airway infection and inflammation is the most frequent cause of morbidity in CF, with mortality caused by respiratory failure.

c. Molecular Mechanisms of CF Lung Disease

A number of additional functions for the CFTR are not well understood, including the mechanism by which CFTR defects are related to immune regulation and predispose affected individuals to chronic endobronchial infections. A model has been developed (**Figure 1.4**),

where the CFTR mutation leads independently to three sets of aberrations: (1) abnormally viscous, salty and/or under-hydrated airway secretions, (2) exaggerated release of inflammatory mediators, such as interleukin (IL)-8, in the presence or absence of infection, and (3) increased *Pseudomonas aeruginosa* retention in the lower respiratory tract. The combination of an *inherent* pro-inflammatory environment in the lungs (all pathways) and an *excessive* response (pathways 2 and 3) lead to inflammation, infection, and persistence of both.

Figure 1.4

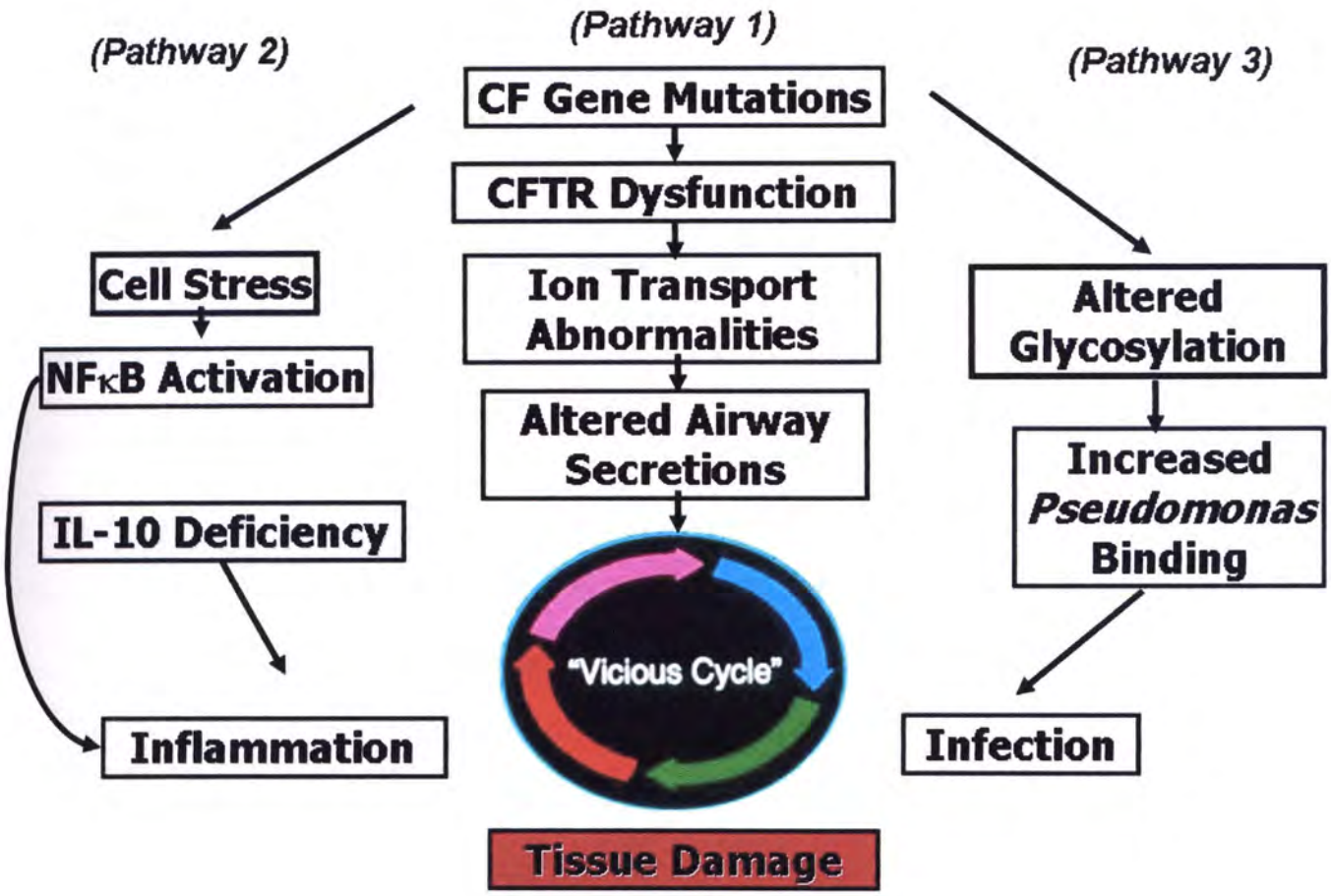

(Pathway 2)
(Pathway 1)
(Pathway 3)
CF Gene Mutations
CFTR Dysfunction
Cell Stress
Ion Transport Abnormalities
Altered Glycosylation
NFκB Activation
Altered Airway Secretions
Increased Pseudomonas Binding
IL-10 Deficiency
"Vicious Cycle"
Inflammation
Infection
Tissue Damage

Figure 1.4 The vicious cycle of inflammation and infection is the major cause of pathogenesis in CF lung disease. Schematic shows probable pathways whereby mutations in the CFTR lead to three independent, though not mutually exclusive, sets of aberrations. In pathway 1, abnormally viscous, salty and/or under-hydrated airway secretions predispose the CF airway to bacterial infection and inflammation. Pathway 2 shows increased NFκB activation results in exaggerated release of inflammatory mediators, such as interleukin (IL)-8, both constitutively and during infection. In pathway 3, altered glycosylation of CF epithelia increases *Pseudomonas aeruginosa* retention in the lower respiratory tract, promoting inflammation and infection. Adapted from I. Virella-Lowell, unpublished.

i. Defective CFTR and Initiation of Disease

Two different hypotheses have been formulated to explain the role of the CFTR in CF lung pathogenesis, both of which focus on the role of salt absorption [60] (**Figure 1.5**). The classical hypothesis is the "low volume hypothesis" in which the lack of CFTR function causes a defect in chloride secretion, resulting in sodium and water hyperabsorption (through ENaC) and severe pathology in the pancreas, intestine, and airways. According to this model, CF and normal individuals both have comparable levels of salt, but patients with CF have decreased surface liquid (ASL) volume [61]. The airway epithelia regulate ASL salt concentration, specifically, in the lungs, leading to a decrease in the ASL covering the epithelia and impaired mucociliary clearance due to increased thickening of airway secretions, promoting infections in the lung. Studies found no evidence that the liquids lining airway surfaces were hypotonic or that salt concentrations differed between CF and normal cultures; however, CF airway epithelia had high rates of ASL absorption and abolished mucus clearance [61]. According to this model, therapeutics should target increasing ASL volume with salt and water, not modulating the ionic composition. The role of ENaC as a primary defect for pathogenesis is supported by animal studies and the clinical effects of hypertonic saline [62].

The alternative hypothesis, the "high salt hypothesis," proposes the increase in sodium and chloride in the ASL is due to poor absorption of salt, not sodium hyperabsorption, leading to an excessively salty ASL because CFTR dysfunction causes reduced transepithelial chloride conductance. The high salt environment of the ASL interferes with the bacterial killing of natural antibiotics such as defenses and lysozyme. Studies were conducted in normal and CF airway epithelia, and CF epithelia had reduced

ability to kill *Pseudomonas aeruginosa* and *Staphylococcus aureus* which was corrected by reducing the NaCl concentration on CF epithelia [63]. In this model, ENaC plays only a minor role to CFTR activity.

Figure 1.5

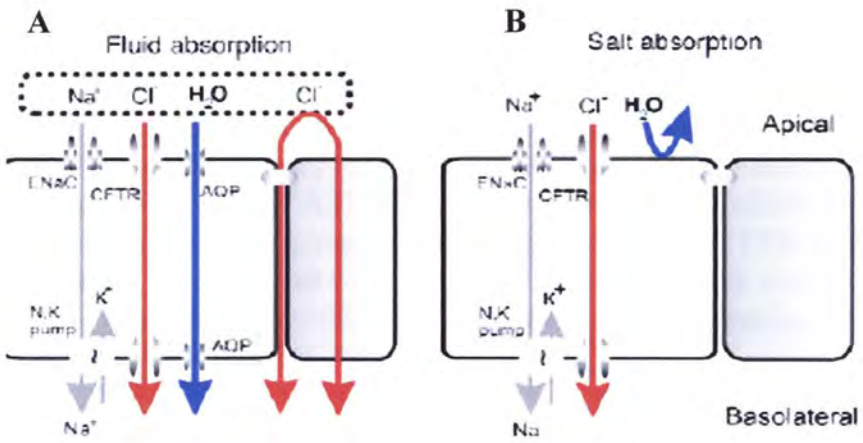

A
Fluid absorption
Na+
Cl-
H2O
Cl-
ENaC
CFTR
AQP
N-K pump
K+
AQP
Na+
B
Salt absorption
Na+
Cl-
H2O
Apical
ENaC
CFTR
N-K pump
K+
Na+
Basolateral

Figure 1.5 The multiple roles of CFTR in salt and fluid absorption. (A) In the low volume model, the primary defect is overactive ENaC activity due to lack of inhibition by CFTR. Normal and CF cells have the same ASL salt concentration. Inhibition of ENaC occurs in normal cells while CF cells have de-inhibition of ENaC, leading to elevated fluid absorption, depleting the volume of ASL, and reducing mucociliary clearance due to viscous build-up. **(B)** In the high salt model, the primary defect caused by CFTR dysfunction is lack of chloride conductance. Normal and CF cells differ in the ASL salt concentration. Normal cells absorb salt in excess of water. CF cells poorly absorb salt, leading to salty ASL and inactivation of antimicrobial peptides. Adapted from [60].

ii. Inflammation

The persistent inflammation in the CF lung is the major cause of progressive lung injury and damage leading to decreased pulmonary function and death. Studies have shown that patients with CF have an *inherent* pro-inflammatory environment in the lungs [27, 64-68] and an *excessive* response to bacterial infection [69].

Shortly after birth, excessive endobronchial inflammation can be detected in the bronchoalveolar lavage (BAL) of patients in the absence or presence of bacterial infection also measured in the BAL. Specifically, BAL samples contain elevated levels of neutrophils, IL-8, and neutrophil elastase (NE) when compared to subject controls [64-67]. Constitutive activation of NFκB with a subsequent increase in IL-8 expression can result from endogenous stimulation as a consequence of cell stress due to the accumulation of mutant CFTR in the endoplasmic reticulum (ER) [70, 71] and may explain the presence of large quantities of inflammatory cells and cytokines in the airways of infants and small children with CF prior to any known infection or colonization [27, 64, 68]. Once, NFκB is activated, a massive release of proinflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8, and TNF-α occurs [24, 25, 27-30, 72, 73]. Bronchoalveolar macrophages (BALMs) from uninfected patients with CF produce elevated levels of proinflammatory cytokines compared to healthy controls [26, 29].

The CF chronic inflammatory response, pathologically, is marked by the massive influx of polymorphonuclear neutrophilic leukocytes (PMNs) into the airways. While the role of neutrophils in host defense is very important, their function is also potentially harmful if turned against the host tissue where neutrophils accumulate and are linked to the long-term

course and exacerbations of several acute and chronic lung disorders, including cystic fibrosis.

Neutrophils are recruited by chemokines and release considerable quantities of NE, which is thought to be the most important protease that damages the CF lung. NE degrades elastin, a key structural lung component that prevents small airways from collapsing, and leads to emphysema-like conditions in the lungs and possibly remodeling of airway tissue. Interestingly, NE is present in increased concentration in uninfected CF patients when compared to uninfected controls and these levels have been found to inversely correlate to lung function [64]. NE can adversely affect the airways by enhancing mucus secretions [74-77], amplifying the signal from inflammatory mediators [77, 78], directly injuring airway tissue [79], and disrupting opsonization and elimination of bacterial pathogens [79-81].

In lungs of patients with CF, the normal levels of anti-protease defenses such as α-1 antitrypsin (AAT) are outmatched, exacerbating the already exaggerated inflammatory response. Additionally, there is significant evidence that the key regulatory factors such as IL-10 [26, 28-31] and IκBα [32-37], an inhibitor of NFκB activation, are down-regulated or rapidly degraded in CF; endogenous lung anti-proteases are repressed as well. The resulting imbalance of pro- to anti-inflammatory mediators is well-established early in life, allowing for many years of lung destruction and progressive airway obstruction.

iii. Bacterial Infection

A direct correlation exists between bacteria colony forming units (cfu) and intensity of airway response in the BAL of patients with CF, including significant increase in inflammatory cells and increased IL-8 concentration [82]. In addition, upon infection with *P.*

aeruginosa CF respiratory epithelial cells produce IL-8 at four times the level observed in normal cells [69].

While initiation of chronic bacterial infection can be explained by the change in ASL volume or salt composition and/or by the early onset of an innate proinflammatory airway environment, these models do not explain why patients with CF are consistently colonized by specific pathogens. Early infections in the CF lung are mainly caused by *Staphylococcus aureus* and *Haemophilus influenza*, but chronic infection with *Pseudomonas aeruginosa* is more significantly correlated with clinical outcomes in CF [83, 84]. Infection with *P. aeruginosa* occurs at a very young age ($\leq$ 3 yrs) and is detected by a positive antibody response and culture growth from samples taken by bronchoscopy [85] or throat swabs from young, non-sputum producing children [86]. Prevalence of *P. aeruginosa* infection increases with age in patients with CF and is accompanied by a corresponding decrease in prevalence *of S. aureus* and *H. influenza*.

P. aeruginosa is an opportunistic pathogen naturally resistant to many antibiotics and significantly causes nosocomial infections. Because patients with CF are specifically susceptible to chronic infections with *P. aeruginosa*, a number of theories have been proposed for the predisposed risk in this population. Most theories describe factors such as alterations in the ASL, decreasing antimicrobial peptide activity, or increased viscosity of secretions, impeding mucociliary clearance, but would not explain the overwhelming presence of *P. aeruginosa* in respiratory tract infections. For this reason, the CFTR protein has been implicated in recognition and elimination of *P. aeruginosa*, potentially acting directly as a receptor for *P. aeruginosa* and/or indirectly by influencing bacterial adherence, endocytosis, and clearance of bacteria from the airway (**Figure 1.6**).

Figure 1.6

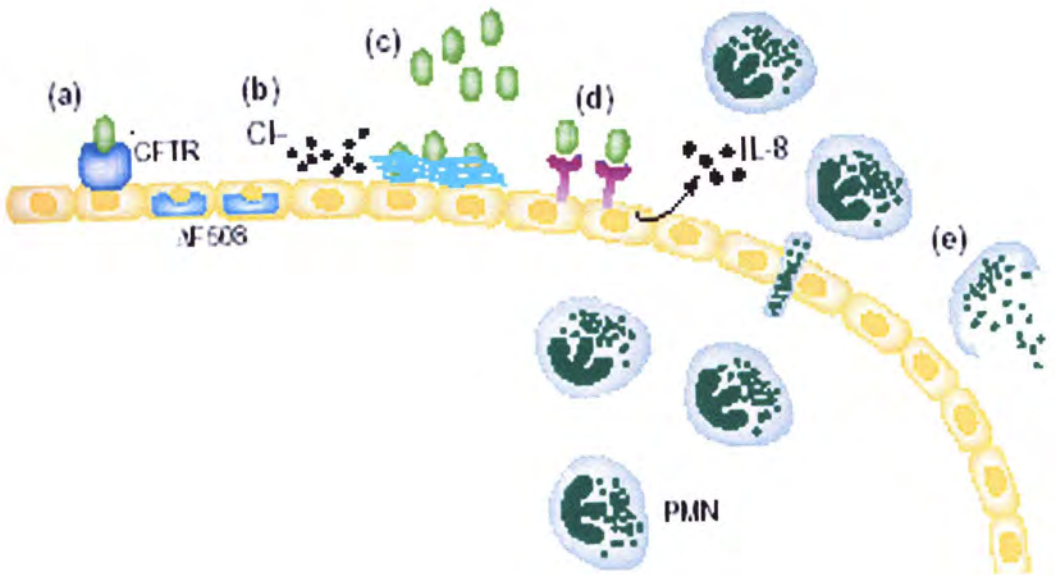


Figure 1.6 Schematic outlines potential factors causing increased susceptibility to *P. aeruginosa* infection in patients with CF. (A) CFTR and *P. aeruginosa* interaction result in internalization of the bacteria by epithelial cells. **(B)** CFTR mutations cause defective chloride transport across epithelial cell surfaces, altering the viscosity of ASL and decreasing bacterial killing. **(C)** Inactivation of innate antimicrobial features allows bacteria to survive in the airway. **(D)** In the CF epithelia, asialo-GM1 receptors are increased and bind *P. aeruginosa* stimulating IL-8 production and recruitment of PMNs to the airway. **(E)** Dysregulated inflammatory response leads to PMN degranulation and release of toxic factors promoting tissue injury [87].

Observations have been made by several groups that *P. aeruginosa* adheres to CF epithelial airway cells more efficiently than non-CF cells [88, 89]. Polarized CF bronchial cells bind *P. aeruginosa* in a reversible and dose-dependent manner, significantly greater than compared matched pairs rescued with the CFTR [88]. Adherence of *P. aeruginosa* to nasal epithelial cells from patients with CF was significantly greater than to cells from normal subjects [89]. Despite increased adherence, CF cells do not appear to internalize the bacteria. Compared to cells expressing wild-type CFTR, cells expressing the ΔF508 CFTR were defective in uptake of *P. aeruginosa* [90]. Murine cells expressing wild-type CFTR ingested *P. aeruginosa* at rates from 30-100 higher than cells lacked CFTR or expressing the ΔF508 CFTR [91]. Adherence and internalization have been directly linked in one laboratory; demonstrating human airway epithelial cells efficiently ingest *P. aeruginosa*, an effect which was reversed by addition of purified CFTR [91]. In addition, this laboratory showed *P. aeruginosa* specifically binds the first extracellular domain of CFTR [91]. This strongly suggests the CFTR may contribute to a host-defense mechanism that is important for elimination of *P. aeruginosa* and its absence in patients with CF results in persistence of the organism in the respiratory tract.

In addition or despite the fact that CFTR may be a receptor for *P. aeruginosa*, there is also evidence that links the inability of CF cells to ingest *P. aeruginosa* to the tetrasaccharide of an asialoganglioside 1 (aGM1) moiety (asialyated glycolipids), located in abundance in the apical membrane of CF epithelia [88, 92], but not normal epithelia. In CF polarized bronchial cells, there was undersialylation of apical proteins and a higher concentration of asialoganglioside 1 (aGM1) in apical membranes of CF compared with rescued epithelia [88]. This was supported by studies in CF regenerating respiratory epithelial cells obtained

from nasal polyps showing increased aGM1 correlated to the increased *P. aeruginosa* affinity for CF cells [92]. In addition, there were increased numbers of asialoGM1 residues available on the surface of the epithelial cells with altered CFTR function which also correlated with higher *P. aeruginosa* adherence in CF epithelia the [93].

Specific *P. aeruginosa* adhesions (pilin, flagellin) are necessary for initial infection and stimulate IL-8 production from respiratory epithelial cells [69]. The inflammatory response is perpetuated by a *P. aeruginosa* autoinducer (PAI), an exoproduct secreted during chronic infection that stimulates IL-8 production in a dose-dependent manner [69].

Mucoid *P. aeruginosa* is associated with the worst clinical outcome in CF [83, 84]. Transition of *P. aeruginosa* from non-mucoid to mucoid strain generally is the result of initial colonization not being efficiently treated and is the result of an overproduction of exopolysaccharide alginate. Chronic infections of alginate-coated mucoid strains of *P. aeruginosa* are almost impossible to eradicate, probably due to biofilm formation and the poor penetration of antibiotics even with intensive regimens [94, 95].

1.3. Anti-inflammatory Therapies in CF

A growing body of evidence emphasizing the role of inflammatory mediators in CF pathogenesis has led to a recent surge in the development and assessment of anti-inflammatory therapy for CF lung disease. Importantly, treatment of patients with CF using anti-inflammatory therapeutics slows the progression of lung disease, but has not been found to exacerbate infection [96-100].

Corticosteroids are potent anti-inflammatory medications. They inhibit neutrophil chemotaxis, adhesion, and tissue infiltration by reducing expression of cell adhesion

molecules (CAMs) on the vessel wall, and suppress proinflammatory cytokine release, but their specific mechanism of action in CF is still unclear [101, 102]. Although glucocorticoids, such as prednisone, have been found to decrease moderately the rate of decline in pulmonary function in patients with CF, after two years of use they have been associated with significant side effects, including cataracts, lower bone mineral density, and diabetes; therefore, glucocorticoids are usually only used in acute or end-stage lung disease [101, 102]. Inhaled corticosteroids (ICS) have minimal side effects, but have not been found to decrease inflammation significantly, most likely due to difficulties in getting such medications to penetrate through the mucus and reach lower airways [103-108].

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, mediate their anti-inflammatory effects through inhibiting cellular cyclooxygenase activity and prostaglandin synthesis; however, they do not mediate any suppression of cytokine release, although high-dose ibuprofen (>50 μg/ml) may reduce neutrophil migration to the lung [109]. High-dose ibuprofen was found to slow the progression of lung disease in patients with CF [110], but its use has been limited by the need to obtain individual pharmacokinetics and concern about side effects, such as the risk of gastric and pulmonary bleeds and renal failure, a specific concern in patients with CF because they are almost continuously on aminoglycoside antibiotics, which like ibuprofen, are cleared by the kidneys. When ibuprofen is used in combination with aminoglycosides (e.g., tobramycin) the blood levels of the aminoglycoside may increase, presumably because the elimination of aminoglycosides from the body is reduced.

Azithromycin is a macrolide antibiotic which inhibits protein synthesis of the bacterial ribosome by blocking the exit of the growing peptide chain in sensitive microorganisms

[111]. It has recently been found to have additional anti-inflammatory characteristics that may involve inhibition of proinflammatory cytokine expression (IL-8 and TNF-α) through interaction with NFκB [111-113]. Azithromycin use three times a week has demonstrated a modest improvement or temporary halt in the decline in forced expiratory volume (FEV)-1 (a measure of pulmonary function), reduction in hospitalizations (47%), increase in weight gain (0.8 g), decreased need for antibiotics, decreased or no change in bacterial infection [97-99], and a generally improved quality of life [97-99] with far fewer side effects making it a more commonly used anti-inflammatory agent in CF.

A significant amount of inflammation persists in the CF airway despite current therapeutics, indicating more potent anti-inflammatory agents are needed, some of which are in clinical trials. A recent clinical trial with inhalation of 25 mg of α1-anti-trypsin (AAT) for 4 weeks decreased elastase activity, neutrophil migration, pro-inflammatory cytokine production, and colonization with *P. aeruginosa,* but had no effect on lung function [114]. Higher doses, 250 mg and 500 mg, of AAT for 4 weeks resulted in a trend toward reduction in NE and myloperoxidase (MPO) activity, markers of neutrophil activity and migration, respectively [115].

Another major inhibitor of NE, secretory leukocyte protease inhibitor (SLPI), may also increase levels of anti-oxidants, such as glutathione (GSH), which are decreased in the airways of patients with CF [116]. Reduction of GSH *in vitro* has been associated with enhanced susceptibility to oxidative stress, an increased inflammatory cytokine release, and impairment of T cell responses [117, 118]. In patients with CF, recombinant human SLPI given by aerosol at 100 mg for 7 days decreased NE levels by 67% [119]. Additionally, GSH prodrug oral N-

acetylcysteine found to decrease sputum elastase levels, neutrophil migration to the lung, and IL-8 in the sputum of patients with CF [120].

A preliminary study in 8 patients with CF showed that administration of colchicine over 6 months resulted in better respiratory function tests in 5 and clinical status improved in all [121]. Colchicine has anti-inflammatory properties which include inhibition of neutrophil migration, chemotaxis, and phagocytosis [122].

In addition to clinical trials, researchers are investigating a number of anti-inflammatory agents. IL-10 and interferon (INF)-γ are counter-regulatory cytokines that inhibit TNF-α, IL-1β, and IL-8, key proinflammatory cytokines responsible for the damaging inflammation present in the CF airway. Anti-microbial peptides against *P. aeruginosa* have been shown to decrease bacterial burden and proinflammatory cytokines in rats [123]. Mercaptoethylguanidine (MEG), a selective inhibitor of inducible nitric oxide synthase (iNOS) and a scavenger of the potent oxidant peroxynitrite, has been found reduce weight loss, lung injury, proinflammatory cytokine production (IL-1β, TNF-α, and macrophage inflammatory protein [MIP]-2), and MPO activity, while not increasing bacterial load in the lungs of rats chronically infected with *P. aeruginosa* [124]. Proteosome inhibitors, such as MB-132, inhibit proteasome-mediated IκB degradation and TNF-α induced IL-8 production in A549 cells (lung carcinoma cell line) by limiting NFκB-mediated gene transcription [125].

1.4. Interleukin 10

Patients with CF have low levels of IL-10 intracellularly (in alveolar macrophages obtained by bronchoscopy) and in airway secretions (sputum and BAL) when compared to their levels of proinflammatory cytokines (TNF-α, IL-6, IL-8) [28-30]. This elevation has

been observed after an infection has been resolved as well as in the absence of infection (measured in uninfected infants), suggesting that inflammation in CF is not dependent on infection [27]. Microarrays have been used to investigate the gene expression profile in a CF bronchial epithelial cell line (IB3-1) as it was altered by the addition of the wild-type CFTR gene, IL-10, and/or infection with *Pseudomonas aeruginosa* and demonstrated that IL-10 is able to reverse some of the phenotypic changes in CF cells that are not observed in non-CF cells [38]. These studies suggest lack of IL-10 may contribute to the initiation/progression of CF lung disease and that IL-10 may have therapeutic potential in CF.

a. Molecular Biology of IL-10

The basic parameters of IL-10 signaling have been under investigation for a while (around 14 years), indicating the complexities of the pathway; however, it is clear IL-10 signaling is a non-redundant pathway that serves to limit inflammatory responses [126].

The receptor for IL-10 is a complex composed of four transmembrane polypeptides; the two chains of IL-10Rα (or IL10R1) bind the ligand and the two chains of IL-10Rβ (or IL10R2) initiate signal transduction [127]. Janus kinase (JAK)-1 is constitutively bound to IL-10Rα while tyrosine kinase (TYK)-2 constitutively associates with IL-10Rβ. Binding of IL-10 to the IL10 receptor activates JAK-1 and TYK-2 (presumably through cross-phosphorylation), initiating phosphorylation of the IL-10Rα chain and recruiting signal transducers and activators of transcription (STAT)-3 to the IL-10 receptor complex. Subsequently, phosphorylation of STAT-3, STAT-2, and STAT-5 by JAK-1 and TYK-1 occur, producing homodimers and heterodimers of the STAT proteins that translocate to the nucleus to inhibit STAT responsive genes, initiating the anti-inflammatory cascade (**Figure**

1.7). Many receptors use the STAT3 pathway, but do not mediate anti-inflammatory responses due to the activities of the suppressor of cytokine signaling (SOCS) [128]. Specifically, SOCS3 regulates STAT3 signaling from receptors such as IL-6, preventing activation of these receptors from mediating an anti-inflammatory response. While SOCS3 expression is strongly induced in macrophages upon IL-10 stimulation, IL-10R is not inhibited by any member of the SOCS family and maintains a strong anti-inflammatory signal [126].

IL-10 regulates a subset of genes induced by TLR stimulation, not as general block on TLR-induced gene transcription [129, 130]. Although the target of STAT3 that executes the anti-inflammatory response has not been found, specific mechanisms are believed to include augmenting IκBα mRNA expression [131] to inhibit NFκB translocation to the nucleus and gene transcription and decreasing gene-specific mRNA, as has been shown for a number of cytokines including IL-6 and IL-8 [132].

Figure 1.7

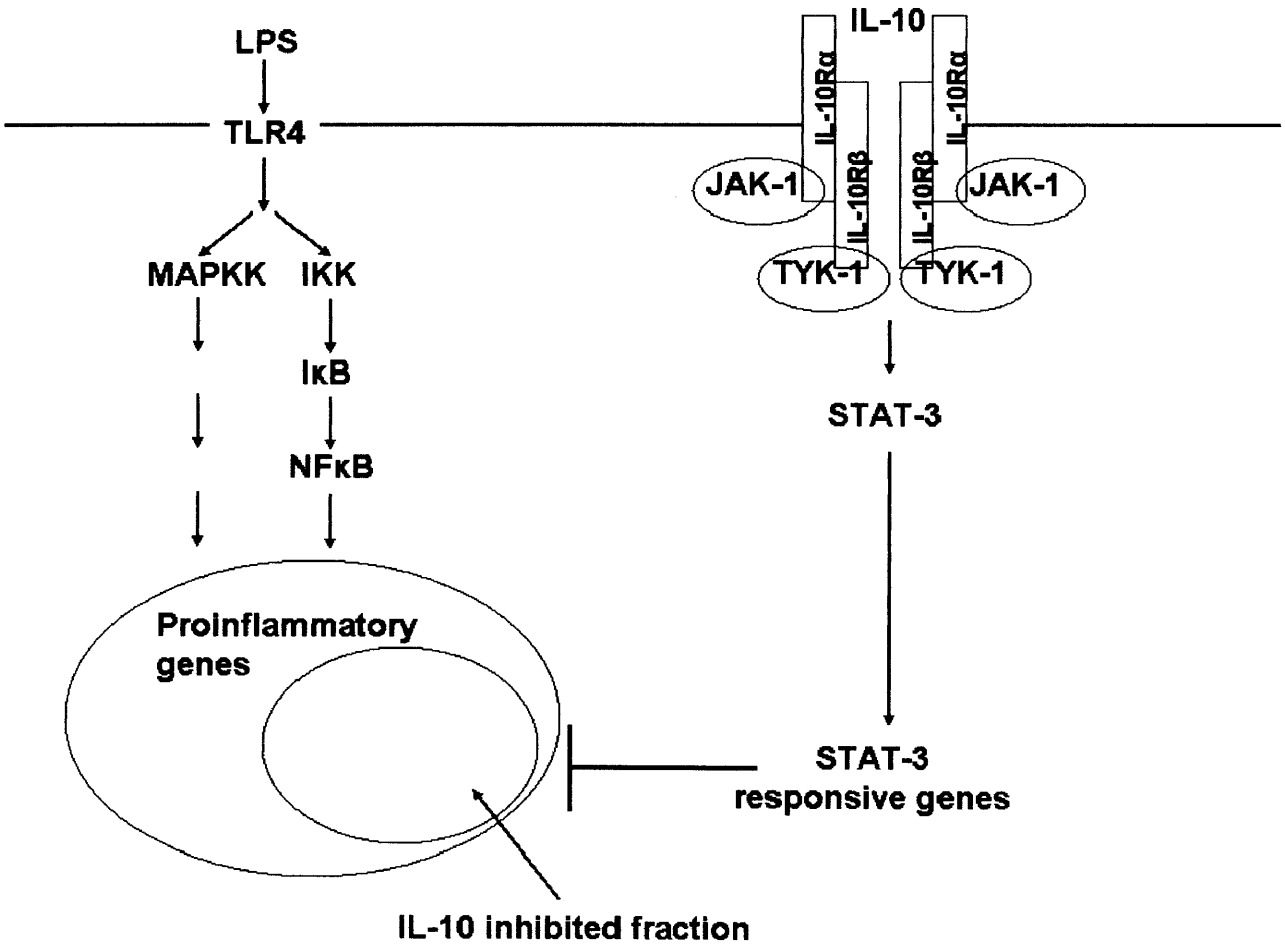

LPS
TLR4
MAPKK
IKK
IκB
NFκB
Proinflammatory genes
IL-10
IL-10Rα
IL-10Rβ
IL-10Rβ
IL-10Rα
JAK-1
JAK-1
TYK-1
TYK-1
STAT-3
STAT-3 responsive genes
IL-10 inhibited fraction

Figure 1.7 The overall mechanism for IL-10 signal transduction. IL-10 signals through JAK1 then STAT3, activating STAT3 responsive genes and inhibiting proinflammatory cytokine production. JAK1 is the only JAK family member required for IL-10 signaling and STAT3 is the only STAT protein required for the anti-inflammatory effects of IL-10 [126]. This example shows proinflammatory cytokine production through TLR activation with subsequent signaling through mitogen-activated protein kinase and NFκB pathways. The target of STAT3 and mechanistic link between the pathways has not been elucidated. IKK, IκB kinase; MKK, mitogen activated protein kinase kinase. Adapted from [126].

The mechanism of IL-10 preventing cytokine production was originally believed to be an inhibition of the antigen presentation by macrophages and dendritic cells, but there is also direct inhibition of cytokine synthesis for several genes in addition to preventing the inflammatory effects of these cytokines on their target cells [127]. To limit ongoing immune responses and inflammation, IL-10 plays important roles in blocking cytokine production, the expression of co-stimulatory molecules, and chemokine secretion as well as controlling the differentiation and proliferation of many immune cells, such as T, B, and antigen-presenting cells (APC) [133, 134]. In addition, IL-10 modifies chemokine receptor expression, increases cell adhesion molecule expression such as intercellular adhesion molecule (ICAM)-1, and reduces the production of inflammatory mediators such as nitric oxide and free radicals [127]. IL-10 inhibits major histocompatability complex (MHC)-II expression and presentation, macrophage activation, and may decrease myeloid cells and prevent cell to cell communication between macrophages and T cells, further resulting in decreased production of proinflammatory cytokines and cytotoxic factors [135]. IL-10 also regulates inflammation/immune responses by inhibiting neutrophil-derived cytokine, chemokine, and mediator production in response to different stimuli, particularly LPS [136].

The primary producers of IL-10 are T cell subsets; either TH-1 or TH-2 polarized subsets can produce IL-10, regulating inflammation in TH-1 and TH-2-driven immune responses [126]. An *in vivo* model of T-cell specific inactivation of IL-10 has demonstrated that IL-10 production from T cells is critical for regulation of chronic inflammation while IL-10 production from TLR-activated macrophages and dendritic cells is important for regulation of acute inflammation [126, 137]. In chronic infections with bacteria such as *P. aeruginosa*, IL-10 may control the intensity of inflammation through controlling T-cell

tolerance to the pathogens [138]; however, because IL-10 is produced from several different sources (antigen specific T and B cells as well as myloid-derived cells) in a chronic infection, the mechanistic basis of IL-10 production in T cells verses myeloid-derived cells (macrophages and dendritic cells) and in chronic versus acute inflammation is still not well-developed [126].

Lack of IL-10, as shown by studies in IL-10-deficient transgenic (IL-10T) mice, leads to greater neutrophil recruitment, more severe weight loss, higher pro-inflammatory cytokine production (IL-6, KC) in the BAL, and increased areas of lung inflammation than seen in wild-type mice when chronically infected with endobronchial *P. aeruginosa* [139], but does not have an effect on bacterial load. In addition, a functional IL-10 deficiency in the lungs of CFTR knockout and IL-10T mice was found to enhance T-cell co-stimulatory molecule, B7 and co-stimulatory activation of alveolar macrophages, suggesting reductions in pulmonary IL-10 in CF may enhance B7 expression and local immune responses [140].

b. Use as a Therapeutic in Inflammation

Many of the pathologies noted with *P. aeruginosa* infection were reversed by administration of IL-10 in wild-type mice [139, 141] and CFTR knockout mouse models [37, 140]. In a model of chronic *P. aeruginosa* infection of CD-1 mice, intraperitoneal administration of recombinant IL-10 (rIL-10) resulted in increased survival, fewer neutrophils in the BAL, and decreased areas of lung inflammation when compared to placebo-treated mice with no decrease in bacterial burden [139]. Recombinant IL-10 was intraperitoneally administered 1 hr before, 8 hrs after, or before and after acute *P. aeruginosa* infection in Balb/c mice [141]. The most successful dosing was the pre/post administration,

demonstrating decreased lung injury and mortality, and a decreased inflammatory response (increase in IFN-γ and slight decrease in TNF-α).

In CFTR knockout and IL-10-deficient transgenic mice, intraperitoneal injection of rIL-10 resulted in decreased expression of T-cell co-stimulatory molecule, B7, and reduced co-stimulatory activity of bronchoalveolar macrophages (BALMs) [140]. An acute model of intratracheal lipopolysaccharide (LPS) challenge in CFTR knockout mice resulted in a significant decrease of PMNs and TNF-α, which correlated with decreased NFκB activity and increased IκBα, upon subcutaneous administration of rIL-10 [37].

1.5. Adeno-associated Virus (AAV)

To date, the therapies available for inflammation in CF have had modest success and are limited in usage by their side-effect profiles. Gene transfer has become a promising alternative, as it has the potential for stable maintenance of therapeutic genes inside defective cells, ideally providing long-term treatment within the diseased tissue without serious side effects. Adeno-associated virus (AAV)-based vector gene transfer has much promise as a therapeutic for CF and has proven safe in multiple clinical trials.

a. Background

Adeno-associated virus (AAV) was first discovered as a contaminate of Adenovirus (Ad) stocks (**Figure 1.8**), hence the name "Adeno-Associated," but was later characterized as a distinct virus with no known cytopathic effect in mammalian cell lines or animal models [142].

Figure 1.8

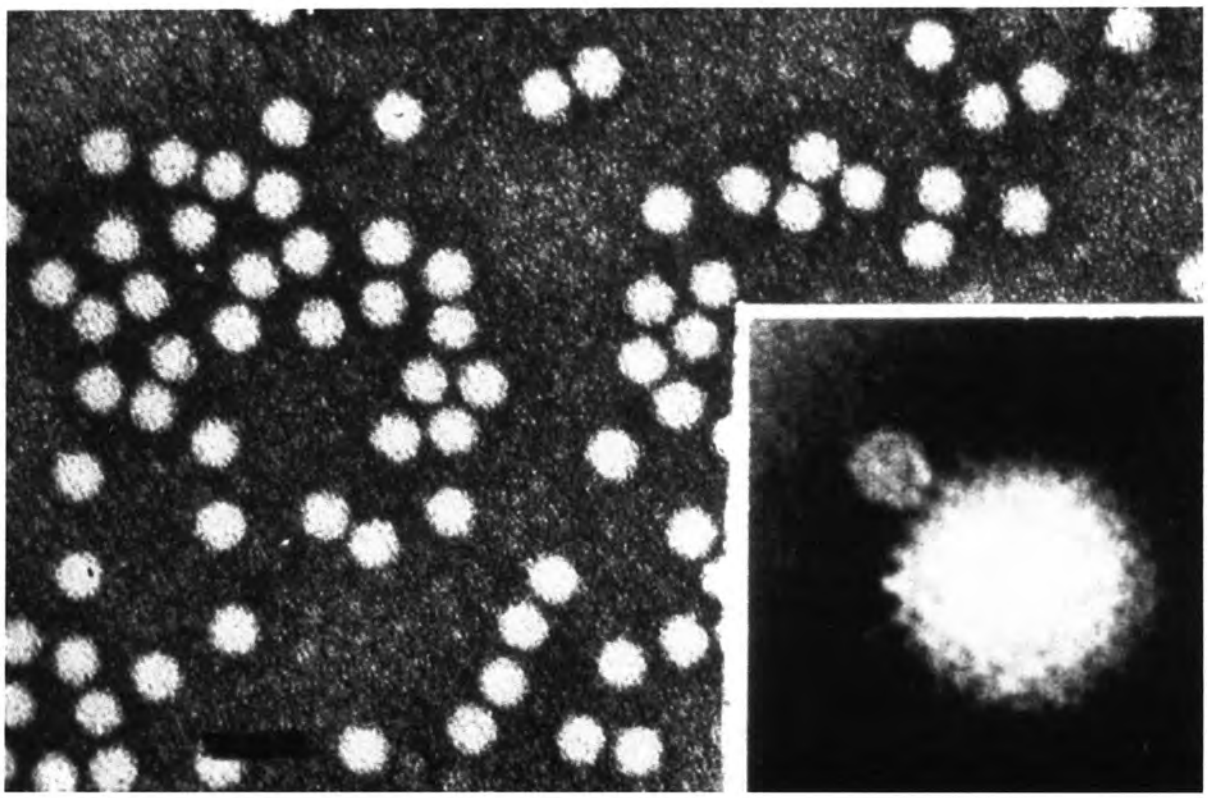


Figure 1.8 Electron micrograph of AAV and adenovirus. The broader field shows purified wild-type AAV2 particles, ~25 nm in diameter. The inset shows a side-by-side micrograph of an AAV2 particle and an Adenovirus type 5 particle [143].

AAV is a member of the *Parvoviridae* family and *Dependoviridae* genus, which means it relies the "helper" functions of a co-infecting virus (typically Ad or herpes simplex virus [HSV]) to complete its lytic life cycle [144, 145]. In the absence of helper virus, AAV integrates, site-specifically, to position 13-3-qter in chromosome 19 in the host genome, a region defined as AAVS1 [146]. The linear single-stranded DNA genome of AAV is approximately 4.7 kilobases (kb) and is flanked by two 145 nucleotide (nt) sequences, inverted terminal repeats (ITRs) that are responsible for integration, serve as the origin for genome replication, and also have putative promoter activity [147]. The genome is surrounded by a non-enveloped icosahedral protein capsid [145, 148].

The AAV genome includes two open reading frames (ORFs), ORF1 and ORF2, and one polyadenylation (pA) signal (**Figure 1.9**). The ORFs contain three promoters, designated p5, p19, and p40 based on their map unit positions in the viral genome. In ORF1, termed the *rep* gene, four mRNAs result from alternative splicing of the p5 and p19 transcripts and produce the non-structural proteins Rep78, Rep68 (a splice variant of Rep78), Rep53, and Rep 40 (a splice variant of Rep52). ORF2, the *cap* gene, contains the p40 promoter and transcribes the viral capsid proteins (VP)-1, VP-2 (a splice variant of VP-1), and VP-3 (transcribed from an alternative stop codon within VP-2). Transcription of the *rep* and *cap* genes is regulated by the presence or absence of helper virus, as well internal regulation between the transcripts [145].

When AAV infects a cell, the unspliced p5 transcript and resulting Rep78 protein is the first to be processed. Shortly after, the p19 and p40 transcripts and their encoded proteins appear (Rep52, VP-1). Later in infection, splicing of the p5 and p19 transcripts (Rep68 and Rep40) occurs through the action of Rep78 or by helper virus interaction [145].

Figure 1.9

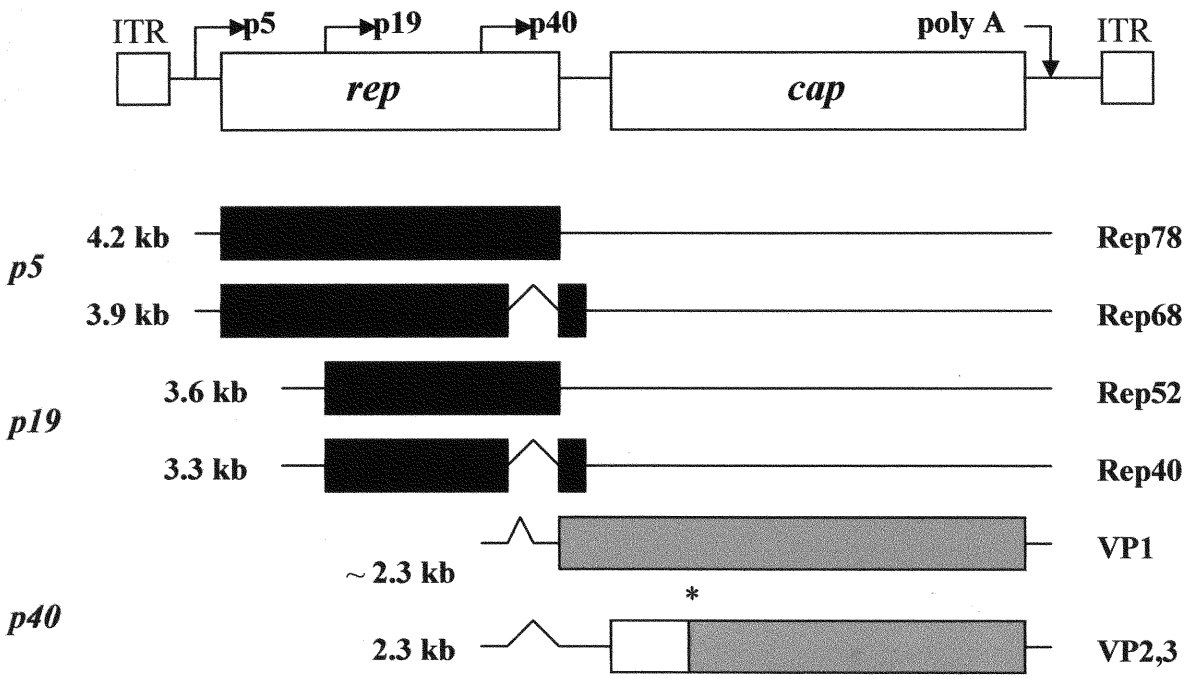

ITR
p5
p19
p40
poly A
ITR
rep
cap
p5
4.2 kb
Rep78
3.9 kb
Rep68
p19
3.6 kb
Rep52
3.3 kb
Rep40
~ 2.3 kb
VP1
*
p40
2.3 kb
VP2,3

Figure 1.9 Transcriptional map of AAV2. AAV has three promoters, defined by their map positions: p5, p19, and p40. AAV2 contains two ORFs, *rep* and *cap*, and two inverted terminal repeats (ITR), represented by boxes. Below each genome are the major mRNA synthesized by AAV2. The number to the left is the size of the transcript and the name to the right are the viral proteins translated from each mRNA. * indicates transcription from an alternate start codon. Adapted from [145].

If there is no helper virus present, AAV enters the lysogenic or latent phase of its life cycle (**Figure 1.10**). During this phase, only a small amount of Rep78 is produced, and it binds the p5 Rep binding element (RBE) site, located within p5, to inhibit further transcription. Rep52 and Rep40 also inhibit p5 promoter activity, but they do not contain RBE domains and their mechanism for inhibition is not clear, although it may include interaction with host transcription factors. Rep52 additionally inhibits p19 transcription. Repression of the p40 promoter has not been demonstrated, but in the absence of helper infection, there is little accumulation of p40 transcripts or Cap proteins [145]. Rep78 interacts with the AAV ITRs and the host genome at the AAVS1 site (19q) to mediate site-specific integration of the AAV genome [149]. Alternately, Rep68 may bring chromosome 19 and the AAV genome together through Rep-Rep contacts usually resulting in integration of multiple copies in head-to-tail tandem within the host genome, although AAV can also persist in episomal forms [150].

Figure 1.10

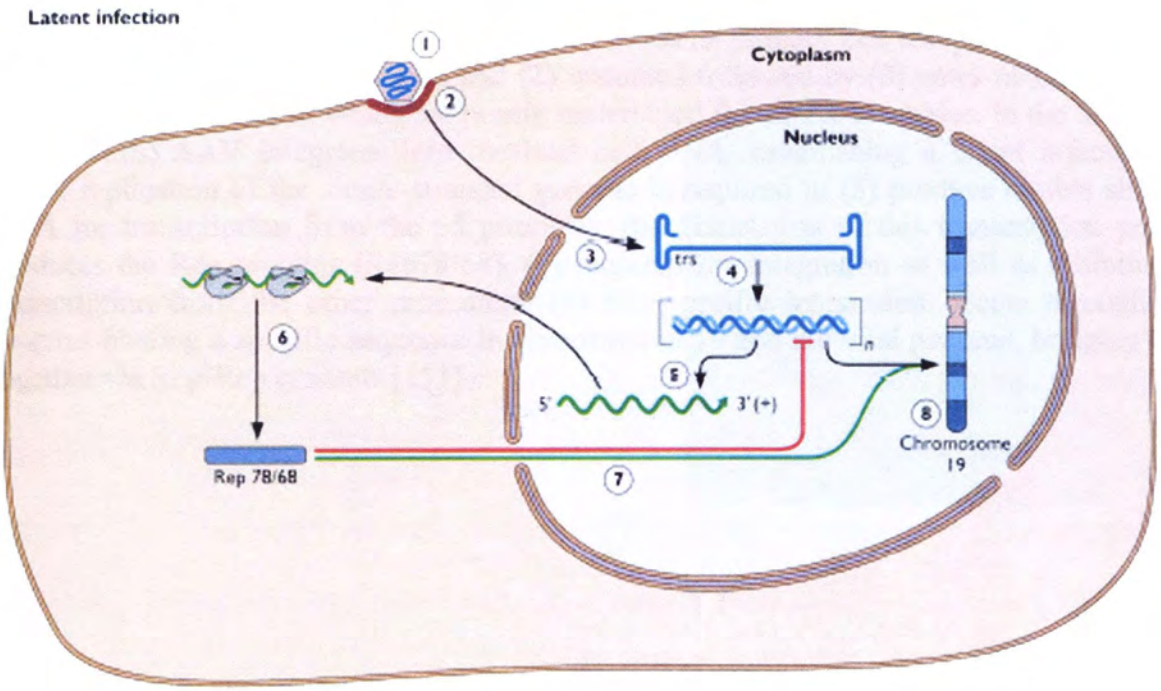

Latent infection
Cytoplasm
Nucleus
1
2
3
trs
4
n
5
5'
3'(+)
6
7
8
Rep 78/68
Chromosome
19

Figure 1.10 Latent life cycle of AAV. AAV2 binds its primary cell receptor, heparin sulfate proteoglycans, and is **(1)** adsorbed and **(2)** uncoated followed by **(3)** entry of DNA into the nucleus, the processes of which are poorly understood for all *Parvoviridae*. In the absence of helper virus, AAV integrates into the host cell DNA, establishing a latent infection. **(4)** Minor replication of the single-stranded genome is required to **(5)** produce double stranded DNA for transcription from the p5 promoter. **(6)** Translation of this transcription product produces the Rep proteins (Rep78/68), **(7)** required for integration as well as inhibition of transcription from the other promoters. **(8)** Site-specific integration occurs through Rep proteins binding a specific sequence in chromosome 19 and the viral genome, bringing them together via Rep-Rep contacts [151].

In the presence of Ad, HSV, or other helper co-infection, AAV goes through its lytic phase (**Figure 1.11**), beginning with rapid induction of AAV gene transcription. In the case of Ad superinfection, Ad early (E)1A transactivator (TA) protein induces transcription from the p5 promoter to generate high levels of Rep78 and Rep68. Rep78 and Rep68 induce p5 promoter expression by interacting with the TATA binding protein (TBP) or repress p5 by binding p5RBE, also self-inhibiting. The mechanism is unclear, but may be through interaction with YY1, a cellular protein, which can function as a transactivator (TA) or repressor [145, 152] and may displace Rep78 and Rep68 from the p5RBE, thereby 'de-repressing' the p5 promoter. Rep78 and Rep68 also induce transcription from the p19 promoter. Rep52 and Rep 40 also inhibit p5 in the absence of Ad or de-repress p5 when Ad is present. Additionally, the overexpression of Rep78 and Rep68 inhibits Cap protein at the post-transcriptional level [145].

The net effect is tight regulation of these elements for a constant ratio of the Rep and Cap proteins, which is important because overexpression of *rep* increases the replicative pool of DNA at the expense of packaging and overexpression of *cap* promotes premature deletion of the pool of replicative AAV intermediates required to sustain a productive infection [151].

Figure 1.11

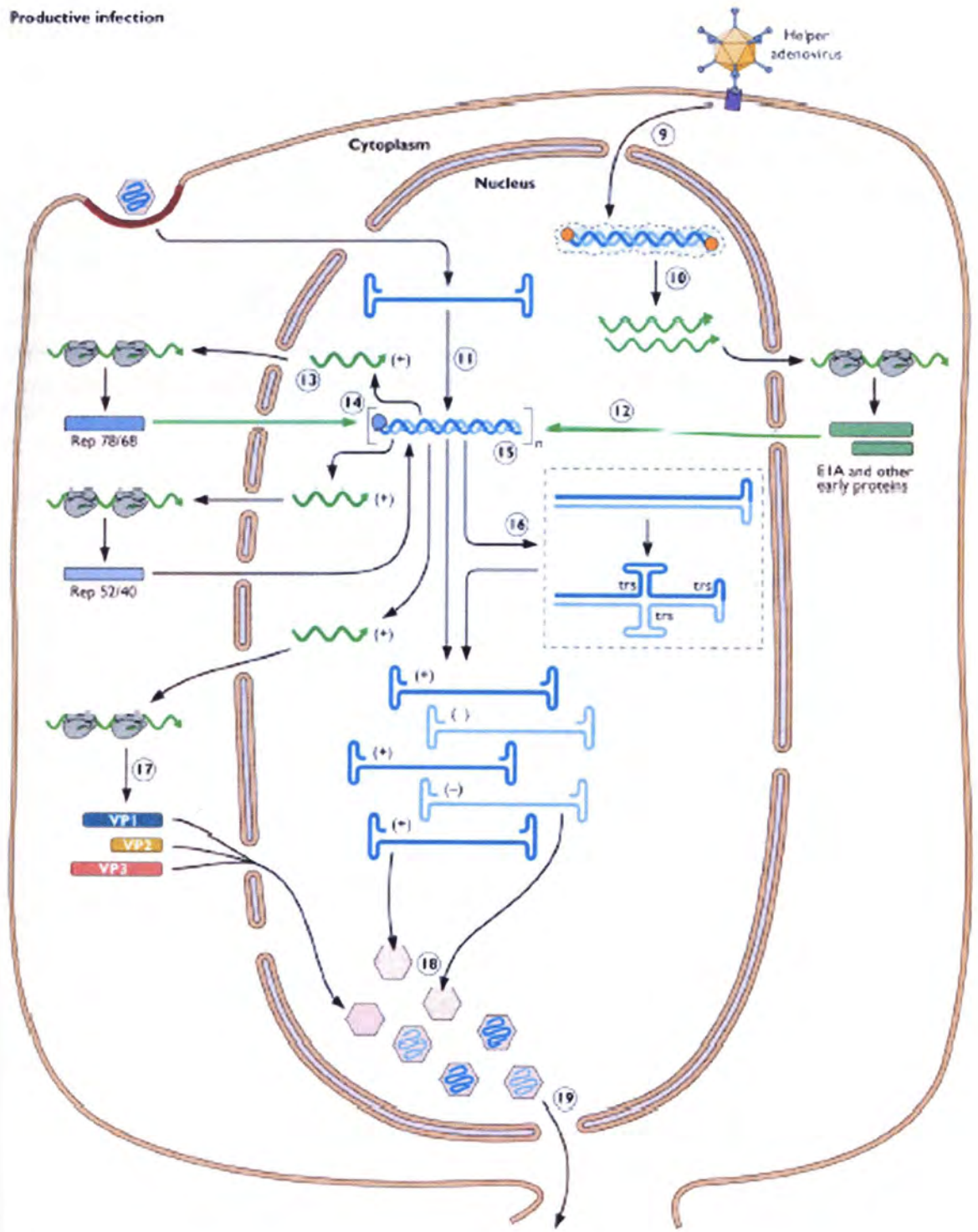

Productive infection
Helper adenovirus
Cytoplasm
Nucleus
9
10
11
12
13
14
15
16
17
18
19
(+)
(-)
Rep 78/68
Rep 52/40
E1A and other early proteins
trs
VP1
VP2
VP3

Figure 1.11 Lytic life cycle of AAV. In the presence of **(9)** co-infection with a helper virus, or rescue by superinfection, AAV enters a productive infection. In the case of adenovirus, **(10)** expression of the early genes E1A, E1B, E4, and E2A is required for entry of the host cell into S phase and expression of host cellular proteins needed for **(11)** viral DNA replication. Expression of the adenoviral E1A and other early proteins **(12)** induces transcription from the p5 promoter, **(13)** producing large amounts of Rep78/68 mRNA and proteins. While in latency these proteins were powerful repressors, in the lytic life cycle they become potent transcriptional activators, **(14)** inducing transcription from p5 and p19 promoters. Viral DNA replication is initiated by recognition of the terminal resolution site (trs) by the Rep78/68 proteins, **(15)** producing a large number of replicating forms (10^6 ds genomes/cells) **(16)** which can be in dimeric head-to-head and tail-to-tail orientations. The **(17)** capsid proteins are produced in the cytoplasm, **(18)** self-associate in the nucleus during viral assembly, and **(19)** progeny virions are released usually upon destruction of the cell [151].

b. AAV-based Vectors

AAV-based vectors have been studied extensively and predominate as one of the most promising vectors for gene transfer in CF (**Table 1.1**). Vectors derived from AAV can transduce dividing and non-dividing target cells [153] and provide prolonged gene expression in the host through episomal persistence or integration [154-157] in such models as the lungs of rhesus macaques and New Zealand white rabbits following a single dose administration [143, 158]. In addition, AAV vectors deliver therapeutic genes without co-transfer of any viral genes and, therefore, induce minor inflammatory responses. Indeed, experiments of AAV-mediated gene transfer into skeletal muscle cells have shown persistent transgene expression (up to 1.5 years) with no inflammatory responses in animal models [159-162]. AAV-based vectors have also been found safe in a number of animal models and human clinical trials [143, 155].

Table 1.1 Properties of AAV supporting its potential role in gene therapy.

Non-pathogenic
Capable of persistent infection
Present in over 100 variants with diverse cell tropisms
Generally elicits mild innate cytokine response
Genome readily modified in proviral plasmids
Recombinant production and purification methods in place

Adapted from [163].

i. Design and Production

Due to the small packaging capacity of AAV, which has been found to be 4.0 to 4.8 kb, AAV-based vectors traditionally do not contain either ORF from the wild-type genome as genomes <4.1 kb are packaged in duplicate and genomes >4.9 kb are inefficiently packaged [164]. Instead, this region is replaced with the transcription cassette of interest and only the ITRs of the wild-type virus are retained [165]. The ITRs are needed for integration of the vector and are also recognized by Rep for replication and Cap for packaging. Therefore, to produce AAV-based vectors, Rep and Cap must be supplemented in *trans,* along with the helper virus genes needed, to promote replication and packaging of the vector.

Currently, there are several methods of AAV vector production: multiple transfection, wild-type or recombinant helper super-infection, and AAV packaging cell lines. The multiple transfection method consists of the transfection of two or three plasmids: the AAV vector (containing the expression cassette of interest), a plasmid carrying the helper genes (from Ad or HSV), and a plasmid carrying the AAV *rep* and *cap* genes; a double transfection can be performed if the helper genes and AAV *rep* and *cap* genes are supplied from one plasmid. The plasmids are re-transfected with each expansion of the culture to produce high titer of the vector [166]. Although laborious, this method is currently the technique of choice when generating good-manufacturing-practice (GMP)-grade vector for clinical trials because there is no helper contamination, which can cause an adverse immune response [167].

The second technique for AAV vector production uses AAV packaging cell lines which contain the AAV *rep* and *cap* genes stably integrated into their genome [168] and are transfected with the AAV vector and then infected/transfected with helper virus components, which would promote *rep* and *cap* expression. The predominant limitation of these cell lines

is that they only contain one copy of Rep per cell, when it has been determined that the ratio of Rep to AAV vector is optimal when 5:1 [164]. Additionally, HeLa (human cervical cancer cell line) and A549 (human lung carcinoma cell line) are the current packaging cell lines [169-171] which cannot be used for production of GMP-grade vector

Another method of AAV vector production is wild-type helper super-infection (typically with Ad), which occurs after co-transfection of the AAV vector and the *rep/cap* construct. This technique is the most successful at generating high titer AAV vector due to the multiple copies of helper genes expressed in each cell and the reduction of the number of plasmids for transfection. The limitation of this technique is the high amount of helper virus contamination, which requires additional steps to obtain a pure stock of AAV vector. The most common technique for Ad and AAV separation is Cesium chloride (CsCl) gradient purification, based on AAV being denser than Ad. Separation can also be done by chromatography-based systems using the coxackie associated receptor (CAR) for Ad and heparin sulfate proteoglycan (HSP) for AAV-2 [167, 172-175] or by heat treatment (55°C/1 hr) which inactivates Ad, but not AAV; however, this is less commonly used because the components of the helper virus are still present to elicit an immune response.

Additionally, recombinant adenoviruses have been constructed to carry the AAV *rep* and *cap* genes for the purpose of higher multiplicity of infection (MOI) and multiple copies of Rep and Cap (R/C) per cell [176]. These strategies require a single transfection of the AAV vector followed by recombinant (r) Ad.R/C super-infection. Unfortunately, it is difficult to generate these recombinant helper vectors due to the extreme cytotoxicity and inhibition of helper virus replication mediated by the Rep proteins, predominantly Rep78 and

Rep 68 [176]. This method currently lacks an efficient mechanism to ensure recombinant helper virus production is highly reduced or eliminated to prevent contamination.

ii. Immune Response

AAV-based vectors do not contain any viral open reading frames and, therefore, do not produce viral proteins, making these vectors particularly useful due to their lack of host cell mediated immunity or acute inflammation. There are few studies examining the innate immune response to AAV vectors [177]. The lack of inflammatory responses documented in numerous *in vivo* gene transfer models employing AAV vectors suggest that these agents do not interact significantly with the innate immune system [143]. In contrast to Adenovirus vectors, AAV vectors do not induce chemokine/cytokine expression in epithelium-derived cells [15]. Additionally, intravenous administration of AAV-LacZ resulted in rapid induction of TNF-α mRNA in the liver, but returned to normal after 6 hours compared to the prolonged induction (> 24 hours) of TNF-α mRNA levels and TNF-α expression exhibited by Ad-LacZ [15]. The increased cytokine expression was accompanied by neutrophil migration to the liver, transient with AAV-LacZ and greater in magnitude and prolonged with Ad-LacZ. AAV vectors may interact significantly with macrophages as the absence of liver macrophages (Kupffer cells) eliminated the innate immune response elicited by the vector [15]. These studies suggest that the reduced inflammatory properties of AAV vectors are due to the vector not efficiently activating the innate immune system; however, transient activation might contribute to the adaptive response generating neutralizing antibodies to the AAV capsid.

Thirty-five to eighty percent (depending upon age and geographical location) of the population have neutralizing activity to AAV2 [177]; therefore, making the humeral immune response to AAV capsid proteins an important barrier against re-infection for clinical gene transfer. Capsid neutralizing antibody production elicited by AAV is both T-cell-independent and -dependent, possibly varying by route of vector administration. Intramuscular administration of AAV vector in mice and rhesus monkeys demonstrated T-cell-dependent antibody production [178] as did tail vein administration, completely inhibiting antibody production with depleting CD4-antibody [179]. Inhibiting T-cell responses when AAV vector was administered into the portal circulation had no effect on antibody production, although the T-cell independent B cell response was short-lived compared to the T-cell-dependent response [179].

Route and time between administrations also contribute to overall antibody production as demonstrated by lung-specific gene transfer. Intranasal administration of an AAV2/5 vector to the mouse lung was found to be successful 5 months after the original administration [180]. Aerosol delivery of an AAV2 vector by three serial doses (three administrations) was found to be successful despite increasing titers of neutralizing antibodies in the lungs of rhesus macaques [181]. Aerosol and maxillary sinus deliver of tg-AAV.CF resulted in serum neutralizing antibodies in treated patients with CF [19, 20, 182], but gene transfer was detected by bronchoscopy. Importantly, repeat administration is safe and well-tolerated in clinical studies.

Current strategies to overcome humoral immunity to the AAV capsid include AAV capsid modifications which may have altered immune function and overcome pre-existing immunity [177]. Additionally, alternative serotypes of AAV have decreased presence of

neutralizing antibodies and may be used to pseudotype the AAV2 vector genome into capsids of other AAV serotypes. A study comparing neutralizing antibodies in the normal adult and CF population found adults in both populations were seropositive for AAV2 (~30%), but at a lower rate for AAV6 (20-30%) and AAV5 (10-20%) [183]. Children with CF (<18 yrs) were seropositive to AAV2, AAV5, or AAV6, but at much lower rates (4-15%). This study indicates pre-existing immunity will not limit AAV-based gene transfer for the majority of individuals with CF and vectors made with AAV5 and AAV6 capsids will enhance evasion of the adaptive immune response.

iii. Targets for Optimization of Gene Transfer and Expression

Although DNA transfer efficiency and safety have been consistently favorable, thus far gene expression from AAV-based vectors has been variable and insufficient for a successful therapeutic effect. There are several modifications to optimize AAV vectors for gene transfer being developed which include capsid mutagenesis, the use of alternate AAV serotypes, and the development of stronger, more compact promoters.

The efficiency of AAV-2 entry of the airway epithelial cells through the apical surface is less efficient than that from the basolateral side of the epithelia, presumably due to the low density of heparin sulfate proteoglycan (HSP) receptors. Altering the targeting of AAV by modifying or inserting binding ligands into the structural proteins of the AAV capsid has led to promising results with respect to enhancing and/or directing vector infectivity to preferred cell types the virus would not normally infect [184, 185].

The tropism of AAV is very important for efficient transduction of airway cells, especially considering all the barriers to CF gene therapy in the lung. Recent experiments

comparing the differential transduction efficiency of AAV serotypes have determined AAV-1 [186, 187], AAV-5 [187, 188], and AAV-6 [189, 190] demonstrate higher levels of transduction in the airway epithelia when compared to the traditionally used AAV-2. AAV-5 has markedly different capsid proteins from AAV-2, which alter its tropism [191, 192] and allow it to bind the apical surface of airway epithelial cells more efficiently via receptors other than HSP and result in a 50-fold increase in gene transfer to the airway [188].

Studies in the translocation and processing of AAV upon viral infection demonstrated a delay in viral entry to the nucleus, leading to the accumulation of vector particles persisting up to 48 hrs in a paranuclear location [193]. Engelhardt and colleagues have determined that AAV-2 and AAV-5-based vectors are susceptible to ubiquitin/proteosome interactions that interfere with the ability of the virions to complete their life cycles and enter the nucleus; the use of proteasome inhibitors significantly enhances transduction of both serotypes from the apical membrane [193, 194].

The limited packaging capacity of AAV has made it difficult to use this vector for the transfer of large genes (such as the CFTR) with efficient promoters. Early studies showed the optimal size range for AAV packaging to be between 4.0 and 4.8 kb [164]. Although AAV can package a vector up to 5.0 kb, the packaging efficiency decreases sharply with increasing vector size. As the complementary (c)DNA of the CFTR gene is 4.6 kb and the AAV vector itself is 300 base pairs (bp), including only the two ITR regions, it is difficult to incorporate a strong promoter to express the CFTR cDNA using the AAV-based system. This size limitation is the reason the current AAV-based vector in clinical trials (tg-AAV.CF) relies on the promoter activity of the already-needed ITRs to transcribe the CFTR [147]. Circumventing the packaging capacity of AAV relies on an inherent feature of the virus

where independent genomes can form large circular concatemers through intermolecular recombination [195-197]. Manipulation of this feature has been successful in expanding the AAV vector packaging capacity through a trans-splicing event mediated by AAV heteroconcatemerization [198-201]. This approach divides either the transcription regulation components or the transgene itself between two distinct AAV viruses. Co-expression of the two viruses leads to reconstruction of the intact expression cassette of the transgene with its regulatory components through ITR-mediated intermolecular concatemerization. The efficiency level of transgene expression ranges from very low to reportedly as high as a single vector system carrying the complete gene [199, 200, 202, 203] although it was recently reported that AAV5 pseudotyped vectors are more efficient than AAV2, suggesting that efficiency may be serotype specific [201]. This natural mechanism of AAV can allow full-length or enhanced transgene expression through heterodimerization.

Several promoters have been optimized for AAV gene expression. One which is compact (500 bp) and produces high levels of gene expression in the lungs was developed by combining essential features (immediate-early, ie) of the commonly-used and robust cytomegalovirus (CMV) promoter to produce the CMVie/chicken beta-actin hybrid (Cβ) promoter and resulted in significantly increased gene expression of human AAT both *in vitro* and *in vivo* as measured in the serum and lung [204]. Human AAT expression peaked at 8 weeks, achieving therapeutic levels which persisted for 1 year at which time the mice were sacrificed. Another promoter, CC10, was derived from 350 bp of the Clara cell promoter and is epithelial cell-specific [205], exhibiting comparable protein levels to CMV in the murine lung and also achieving therapeutic levels of transgene expression, persisting to 5 months after vector delivery [180]. A third, the *FoxJ1* promoter (900 bp), was analyzed with the

development of transgenic mice and found the promoter to be lung-specific, required for ciliated cell differentiation, and activated in the presence of cilia, in the tracheal, bronchial, and nasal epithelium [206].

While an AAV vector essentially "gutted" of all wild-type proteins is viewed beneficial from a host immune response standpoint, it also results in the deletion of features need for Rep-dependent site-specific integration. There are three RBEs in AAV, two identical ones in the ITR regions (RBE[itr]) and one in p5 (RBE[p5]) [207, 208]. A 16 bp section of RBE(itr) was found to be sufficient for mediating Rep-dependent site-specific integration, which was more effective and specific than the RBE(p5) in Rep-dependent integration at the AAVS1 site [207].

1.6. Animal Models of CF Lung Disease

Analysis of potential therapeutics *in vivo* for CF has been hampered by the lack of a representative animal model. While the current animal models of CFTR gene targeting do not spontaneously develop chronic obstructive airway disease and die of respiratory failure, the most common cause of death in CF, great progress has been made in developing an *in vivo* model of CF lung disease, characterized by neutrophil migration, rise in inflammatory cytokines, and histopathological changes similar to that seen in patients with CF.

a. CFTR Knockout Mice

Several CF transgenic mice have been made through deletions, insertions and mutations of the CFTR gene, but none of them have the lung complications that are the most

common symptom in humans [209]. This may be due to an alternate chloride channel in the mouse lung [1, 55], the physiology of the mouse airway [209], or the lack of sodium hyperabsorption in the mouse airway epithelia [210]. The majority of CF mice have a lethal intestinal phenotype from bowel obstruction which results in a limited lifespan and difficulty of breeding; therefore, a transgenic CFTR knockout (homozygous for the S489X mutation of the CFTR gene) mouse [211] was gut-corrected by having the CFTR reintroduced into the mouse under the control of a Fatty acid-binding promoter (FABP) [212]. Gut-corrected CFTR knockout mice do not have gut complications, but are still smaller in size and body weight than their wild-type littermates [212]. CFTR targeting of other animals such as ferrets [213] and pigs [214] are being conducted to find an *in vivo* model with spontaneous lung disease.

b. Murine Models of Chronic *P. aeruginosa* Lung Infection

Because CFTR knockout mice do not have lung disease, it must be induced to create animal models of CF lung disease. In CF mice, infection with *Pseudomonas aeruginosa* produces an *in vivo* model with histology and cytokine elevation representative of chronic CF lung disease. *In vivo* chronic infection with *P. aeruginosa* has been accomplished by colonizing the mouse lung through feeding mice drinking water containing *P. aeruginosa* [215] and repeatedly challenging mice in an enclosed chamber with aerosolized *P. aeruginosa* [216].

Intratracheally injecting beads that have been embedded with *P. aeruginosa* [217] is a short-term infection (3-5 days); however, this model is the most appropriate *in vivo* representation of CF lung disease, producing histologic and inflammatory changes

characteristic of a chronic infection, and is often used as a "chronic infection" model. In fact, the *P. aeruginosa*-laden bead approach is the most widely-used and least variable model for chronic infection, originally developed in rats [218], mice [219], and optimized for reproducible results [217] (**Figure 1.12**). In non-CF mice, infection with *P. aeruginosa*-laden beads can persist for up to 28 days [220-222], while CFTR knockout mice appear to have more severe mortality and inflammation, characterized by high levels of pro-inflammatory mediators (TNFα, KC, and MIP-2) 3 days after exposure to the beads, despite having the same bacterial load as their non-CF counterparts [220, 223].

Three days post-inoculation with the beads, *P. aeruginosa*-infected mice have leukocyte infiltration involving the airways, bronchus-associated lymphoid tissue (BALT), and adjacent parenchyma with early fibrosis occurring in the more severely affected regions of the lung [220]. These histopathology features closely mimic those of individuals with CF. Impaired bacteria killing occurs between days 3 and 7 after infection of CF mice with 80% of death on day 7, compared to wild-type mice with 50% mortality rate [220, 224]. A sacrifice date of 3-4 days is documented to be optimal for analysis of cytokines and lung pathology [217].

Figure 1.12

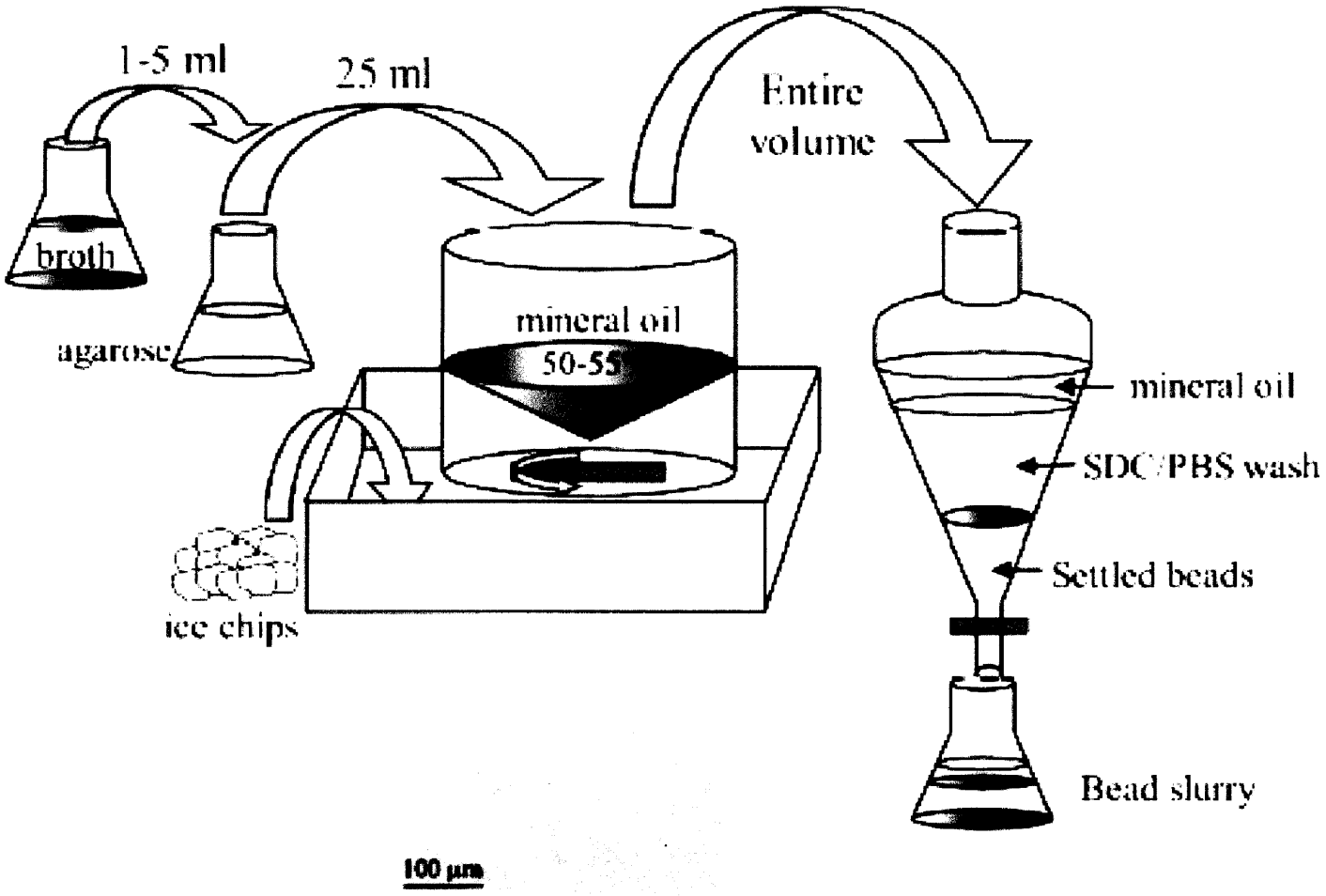

1-5 ml
25 ml
Entire volume
broth
agarose
mineral oil
50-55
mineral oil
SDC/PBS wash
Settled beads
ice chips
Bead slurry
100 µm

Figure 1.12 Schematic for making *P. aeruginosa*-embedded agarose beads. An aliquot of late-log phase bacteria is added to warm (50-55°C) 2% low melting agarose in PBS, then an aliquot of bacteria-agar mixture is added to warm (50-55°C) heavy mineral oil containing a stir bar. The mixture is stirred rapidly at room temperature, followed by cooling with slow additions of ice. The volume of agarose beads and mineral oil are transferred to a separatory funnel; the mineral oil removed and agarose beads washed with sodium deoxycholate (SDC) and PBS. The settled *P. aeruginosa*-laden bead slurry is collected, titered, and instilled in the animal airway to create a model of chronic infection [217].

c. IL-10 Deficient Mice

IL-10-deficient transgenic (IL-10T) mice have been genetically altered to be deficient in IL-10 production, resulting in more severe infection as measured by greater neutrophil recruitment, more severe weight loss, and increased areas of lung inflammation than seen in wild-type mice when chronically infected with endobronchial *P. aeruginosa* [37, 139, 225]. These findings are similar to those of *P. aeruginosa*-infected CF mice [37, 215, 220, 226]. IL-10T mice repeatedly exposed to mucoid *P. aeruginosa* have higher mortality rates and more severe lung pathology when compared to C57BL/6 controls that were similarly infected [216] and have a prolonged inflammatory response to acute *P. aeruginosa* challenge [35]. These characteristics of IL-10T mice further suggest lack of IL-10 may contribute to the initiation/progression of CF lung disease.

Chapter 2.

IL-10 delivery by adeno-associated vector attenuates inflammation in mice with *Pseudomonas* pneumonia.

2.1 Introduction

Cystic fibrosis (CF) is the most common monogenic disorder in Caucasians. In CF, the dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) results in a complex phenotype. Individuals with CFTR mutations have abnormalities in chloride and sodium ion transport across epithelial membranes, contributing to the development of progressive obstructive airway disease, malabsorption, sinusitis, liver dysfunction, and CF-related diabetes. As the disease progresses, the airways of patients with CF become infected and colonized with a variety of opportunistic pathogens, predominantly *Pseudomonas aeruginosa* [227]. Presently, the foremost cause of morbidity and mortality in CF is respiratory failure due to persistent pulmonary infections with *P. aeruginosa*, intense neutrophil-dominated airway inflammation, and progressive bronchiectasis and lung damage.

A number of studies support the premise that CF lungs have an abnormal, upregulated proinflammatory cytokine profile [26-30, 64, 65, 68, 228, 229]. Whether the mechanism of the altered immune response in CF is directly or indirectly due to CFTR mutations and/or other polymorphisms present in patients with CF is not known. However, there is a body of evidence supporting the presence of a relative IL-10 deficiency in patients with CF [29, 230]. IL-10 inhibits cytokine production by T lymphocytes and potently suppresses macrophage production of activating and/or chemotactic cytokines, including the PMN chemoattractant IL-8 and other NF-κB-dependent cytokines, including TNF-α, IL-1β, and IL-6 [231-234]. IL-10 may also facilitate resolution of lung inflammation by promoting apoptosis of PMNs

[235]. Deficiency of IL-10 production, as shown by studies in IL-10-deficient transgenic (IL-10T) mice [236], leads to greater neutrophil recruitment, more severe weight loss, and increased areas of lung inflammation compared to wild-type mice with analogous chronic endobronchial *P. aeruginosa* infection [37, 139, 225]. These findings are similar to those of *P. aeruginosa*-infected CF mice [37, 215, 220, 226]. IL-10T mice repeatedly exposed to mucoid *P. aeruginosa* have higher mortality rates and more severe lung pathology when compared to C57BL/6 controls similarly infected [216]; additionally, IL-10T mice have a prolonged inflammatory response to acute *P. aeruginosa* challenge [237]. Treatment with IL-10 has been documented to reduce leukocyte recruitment, proinflammatory cytokine production, weight loss, neutrophil recruitment, and tissue injury in the airways of sensitized animals following antigen exposure or *P. aeruginosa* challenge [37, 139-141, 238, 239], supporting the use of IL-10 in ameliorating inflammation.

Gene transfer of IL-10 would convey the advantage of long-term gene expression, an inherent feature of adeno-associated virus (AAV)-based vectors [143, 155, 158]. These vectors have a proven safety profile and the ability to elicit a minimal inflammatory response in comparison to other gene transfer agents [17, 19, 182, 240]. To increase lung specific delivery and promote high levels of gene expression, we used an AAV5 pseudotyped vector for efficient airway transduction [188, 241, 242] and the CMV/chicken-beta-actin hybrid (Cβ) promoter, a promoter with robust activity in the murine lung [204]. Using these elements, we hypothesized that AAV-based gene transfer of IL-10 would result in high levels of lung-specific IL-10 expression, ameliorating the excessive response in the lung without systemic immunosuppression. In this study, we characterize AAV5.Cβ-mIL10 in an IL-10T mouse

model of chronic *P. aeruginosa* lung infection to determine whether IL-10 gene delivery and expression specifically reduce the proinflammatory response in the lung.

2.2 Materials and Methods

Mice

Eight-week old IL-10-deficient transgenic (IL-10T) mice (C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME), shipped in protective, filtered containers, transported in climate-controlled trucks, and allowed to acclimate for at least 3 days prior to use. Mice were fed autoclaved or irradiated Harlan Teklad Sterilizable Rodent Blox 8656 (Harland Teklad, Indianapolis, IN) and bedded on autoclaved 1/8" corn cob bedding and corn husk nesting material (Harland Teklad, Indianapolis, IN). Mice were housed, 2-4, in Micro-Isolator Top Flow Ventilated cages (Tecniplast, Exton, PA) in ventilated racks. All caging supplies were cleaned with an automated cage wash and autoclaved prior to use in animal rooms. MUSC is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC, International), and the mice were maintained in specific pathogen-free (SPF) conditions. Each rodent rack in the facility was assigned 1-4 cages of sentinels which are housed in microisolator cages without filter tops. Sentinel animals are exposed to dirty bedding from different shelves of animals at each cage change, but serology was negative for Sendai virus (SEND), Pneumonia virus of mice (PVM), Mouse hepatitis virus (MHV), Mouse polio virus (TMEV), Reovirus (REO), *Mycoplasma pulmonis* (MPUL), Mouse arvovirus (MPV), and

Mouse rotavirus (EDIM). Mouse feces were negative for Helicobacter. The procedures were approved by the Institutional Animal Use and Care Committee (IACUC) at MUSC.

Recombinant AAV-interleukin-10 vector

Murine cDNA for the cytokine IL-10 were cloned into the pAAV2.Cβ plasmid containing AAV-2 inverted terminal repeats (ITRs), a CMV/chicken-beta-actin hybrid (Cβ) promoter including an intron, and the SV40 polyadenylation region [243]. To facilitate pseudotyping, pAAV.Cβ-mIL10 was co-transfected with pXYZ5, an adenoviral helper plasmid with AAV2 rep/AAV5 cap [167]. Anion-exchange chromatography was used to purify the AAV5 pseudotyped vector and the dot-blot assay was used to determine the titer of rAAV virions harboring vector genomes. The pseudotyped packaged vector was then designated as AAV5.Cβ-mIL10 (**Figure 2.1**).

Figure 2.1

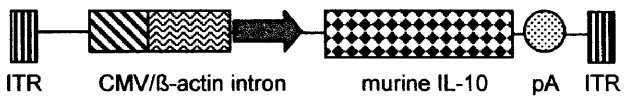


Figure 2.1 Schematic of the AAV5.Cβ-mIL10 vector. AAV5.Cβ-mIL10 contains the CMV/chicken-beta-actin hybrid promoter including an intron, the murine IL-10 coding sequence, and the SV40 polyadenylation region. Adapted from [243].

***Pseudomonas aeruginosa*-laden bead preparation**

The mucoid *Pseudomonas aeruginosa* isolate, PA 15719, was obtained from the sputum of a patient with CF using a protocol approved by the Institutional Review Board (IRB) at MUSC. Bacteria were maintained as glycerol stocks and stored at -80°C. Bacteria-impregnated agar beads were prepared as described by van Heeckeren et al [217, 220] with minor modifications. Briefly, an inoculation loop was used to collect a sample of frozen mucoid *P. aeruginosa* stock, streaked for isolation on a tryptic soy agar (TSA) plate, and incubated at 37°C overnight. One colony was picked from the plate and used to inoculate 50 ml tryptic soy broth (TSB) in a 125 Erlenmeyer flask, which was placed, shaking, overnight at 37°C. A 0.1 ml aliquot of the culture was used to inoculate a second flask of 50 ml TSB in a 125 Erlenmeyer flask. Bacteria were grown to late log phase, shaking, in a 37°C incubator. The bacterial culture was concentrated by centrifugation, resuspended in 10 ml TSB, and 5 ml added to warm (50°C) 2% low melting agarose (Type 1 low EEO; Sigma) in PBS. The bacteria-agar mixture was added to warm (50°C) heavy mineral oil (0122-4, Fisher) containing a stir bar, and stirred rapidly at room temperature for 6 min, followed by cooling with slow additions of ice over a 10-min period. The agar beads were transferred to a separatory funnel; the mineral oil removed, and washed one time with 0.5% sodium deoxycholate (SDC), one time with 0.25% SDC, and four times with PBS. The *P. aeruginosa*-laden agarose beads were collected and allowed to settle. Additional liquid was removed to obtain a final volume of 75% beads and homogenized using a Kinematica Polytron PT10/35 homogenizer for 4 min at high speed to obtain beads predominantly 100–120 μm in diameter. The size of the beads was measured microscopically before and after homogenization. Using serial dilutions, quantitative bacteriology was performed on an

aliquot of the homogenized bead slurry to determine bacterial concentration in colony forming units (cfu) per milliliter. Sterile agar beads were prepared by the same method, without the addition of *P. aeruginosa* culture, and were verified for sterility by serial dilutions plated on TSA plates. The homogenized bead slurries for both preps were prepared the day before inoculation of the mice, stored at 4°C, and concentration of bacteria calculated on the day of inoculation.

Assessment of weight loss

Body weight was measured in all mice before PBS or AAV5.Cβ-mIL10 administration, before infection with sterile beads or *P. aeruginosa*-laden beads (6 weeks later), and at time of sacrifice (3 days after infection). A record was made of any hunching, poor grooming, fatigue, or increased respiratory effort following inoculation with agarose beads.

Intratracheal injection

In preparation for intratracheal injection, mice were sedated through inhalation of isoflurane. A transverse cervical incision was made, and the trachea was exposed and intubated with a sterile, flexible 21-gauge SURFLO winged infusion set (Terumo Corporation, Tokyo, Japan) attached to a 1.0-ml syringe. Thirty microliters containing $1x10^{12}$ vg AAV5.Cβ-mIL10 or PBS was injected into the trachea. All incisions were closed by suture. Six weeks after treatment with AAV5.Cβ-mIL10 or PBS, the mice underwent

intratracheal injection with sterile agarose beads or *P. aeruginosa*-laden beads. Anesthesia and intratracheal injection were performed as before, with 50 µl of sterile beads or $5x10^6$ cfu *P. aeruginosa*-laden beads being injected.

Collection and processing of samples

At 3 days following *P. aeruginosa*-laden bead infection, the mice were anesthetized with isoflurane and sacrificed by exsanguination. Blood and bronchoalveolar lavage fluid (BALF) were collected. For the BAL collection, the trachea was isolated by blunt dissection. An incision was made in the upper portion of the trachea, which was cannulated with a 21-gauge shielded I.V. catheter (BD Bioscience, San Hose, CA) attached to a 5 ml syringe. The alveoli were washed 3 times, each with 1 ml PBS. The volume of bronchoalveolar lavage fluid (BALF) recovered was approximately 1-2 ml. Lungs were collected and weighed for histology and cytokine analysis of lung homogenate. For histology, the lungs were inflated and fixed with 4% paraformaldehyde, while for lung homogenization, the lungs were immediately submerged in liquid Nitrogen and stored at -80°C.

To obtain homogenate cytokine values, the lungs were thawed and washed in 0.5 ml cell wash buffer (Bio-Rad, Hercules, CA). One milliliter of cell lysis buffer (Bio-Rad) was added to each lung, which was then homogenized for 60 seconds at high speed using Kinematica Polytron PT10/35 homogenizer (Brinkmann Instruments Co., Westbury, NY). The homogenized lung samples were frozen at -80°C, thawed on ice, and then sonicated in ice-cold water. They were centrifuged at 1500xg at 4°C for 10 min; the supernatant was

aspirated and the homogenate stored at -80°C. Cytokine analysis was performed within 24 hrs of homogenization.

Measurement of cytokine levels

Cytokine levels in the serum, BAL, and lung homogenate were measured using a customized Bio-Plex cytokine kit (Bio-Rad) containing detection for IL-1β, TNF-α, IFN-γ, IL-10, KC, and MIP-1α. Analysis was conducted using the Bio-Plex multiplex suspension array system (Bio-Rad) per the company's protocol. Each sample was performed in duplicate. Epithelial lining fluid (ELF) values were obtained through correction of the BAL dilution using a ratio of urea nitrogen concentrations in the airway fluid and in the serum by the Enzymatic Urea Nitrogen (blood urea nitrogen [BUN]) procedure (Stanbio, Boerne, TX). Homogenate levels were standardized by lung weight for a final value of picogram (pg) per gram of lung.

Histological analysis

After the lungs were fixed in 4% paraformaldehyde for 48 hrs, they were embedded in paraffin and cut in 150-200 μm step-sections. The step-sections were left unstained (for immunohistochemistry) or stained with hematoxylin and eosin (H&E) using standard techniques.

Immunohistological staining protocol

Five μm lung paraffin sections were soaked in Hemo-de for 10 min followed by rehydration in graded alcohol (100%, 80%, 40%, 20%) and water, each for 5 min. The sections were blocked with universal blocking solution (KPL, Gaithersburg, MD) for 30 min at 25°C and rinsed with water and Tris buffer. Then, the sections were blocked with goat serum at 25°C for 15 min and incubated with rat anti-mouse IL-10 monoclonal antibody (1:1000 dilution; BD PharMingen, San Diego, CA) overnight at 4°C in a humidity chamber. The next day, the sections were washed in Tris buffer and incubated with biotinylated goat anti-rat IgG-horseradish peroxidase (HRP) (1:100 dilution; BD PharMingen) at 25°C for 1 hr. The sections were further incubated with HRP-streptavidin (KPL) for 30 min at 25°C and then incubated with true blue substrate solution (KPL) for 10 min at 25°C. After being washed in water and counter-stained in orcein (KPL) for 3 min, the sections were dehydrated in graded alcohol and soaked in Hemo-de for 10 min.

Statistical analysis

Data are presented as average ± SD. Change in body weight is expressed as the percentage of weight lost from the original weight three days before inoculation. Statistical analysis of percentage weight loss, and cytokine levels were determined by unpaired, two-tailed Student's t test using the Graph Pad InStat statistics software program (GraphPad Software, San Diego, CA). Welch correction was applied when standard deviations were

significantly different. For all data sets, p values less than 0.05 were determined to be statistically significant.

2.3 Results

AAV5.Cβ-mIL10 and PBS-treatment result in similar percentage weight loss in IL-10T mice infected with *P. aeruginosa*-laden beads.

IL-10T mice were inoculated, intratracheally, with $1x10^{12}$ vector genomes (vg) of AAV5.Cβ-mIL10 or PBS. Six weeks later, the mice that received AAV5.Cβ-mIL10 were infected with $5x10^6$ cfu of *P. aeruginosa*-laden beads (n=5). Mice that received PBS were either given sterile beads (n=4) or infected with $5x10^6$ cfu of *P. aeruginosa*-laden beads (n=6). Mice were sacrifice after 3 days.

To ensure that AAV5.Cβ-mIL10 did not impact mouse weight, the mice were weighed before administration with AAV5.Cβ-mIL10 or PBS and 6 wks later before *P. aeruginosa*-laden bead infection. There was no significant difference in the percentage weight gain between the groups ($p>0.05$).

The mice were weighed a second time before administration with *P. aeruginosa*-laden beads and 3 days later before sacrifice. The average pre-infection weight of the mice receiving AAV5.Cβ-mIL10 (n=5) was 23.3 g with mice losing an average body weight of 16.4% $\pm$ 9.9 over the 3 days after *P. aeruginosa* infection and before sacrifice. This profile was similar to the mice in the PBS group infected with *P. aeruginosa* (n=6): initial weight was 22.2 g with mice losing an average body weight of 22.4% $\pm$ 1.3. Mice that were not infected and, instead, received sterile beads as a control for surgery (n=4) had an average

initial weight of 22.6 g, losing an average body weight of 5.3% $\pm$ 1.6. There was no significant difference in the percentage weight loss between AAV5.Cβ-mIL10 and PBS mice infected with *P. aeruginosa*-laden beads ($p>0.05$); statistical difference could not be obtained from mice receiving sterile beads due to the small sample size.

Intratracheal administration of AAV5.Cβ-mIL10 produces significant levels of IL-10 protein in the lung.

IL-10 expression following AAV5.Cβ-mIL10 administration in the lungs of IL-10T mice infected with *P. aeruginosa* was measured to verify gene transfer and expression efficiency. In the AAV5.Cβ-mIL10-treatment group, mice were infected with $1x10^5$ cfu *P. aeruginosa*-laden agarose beads by intratracheal injection. Mice receiving PBS were either given sterile beads or infected with *P. aeruginosa*-laden agarose beads. Three days later, the mice were sacrificed and BALF, lung tissue, and blood were collected from each animal.

The administration of AAV5.Cβ-mIL10 successfully resulted in the production of IL-10 as measured in the ELF (average, 25,000 pg/ml) and lung homogenate (average, 12,000 pg/gram-lung) (**Figure 2.2**). IL-10 production was also measured in the serum to determine whether production was systemic. IL-10 was undetectable in the serum of mice in all treatment groups (data not shown), thus confirming localized secretion of IL-10 from the AAV5.Cβ-mIL10 vector.

Figure 2.2

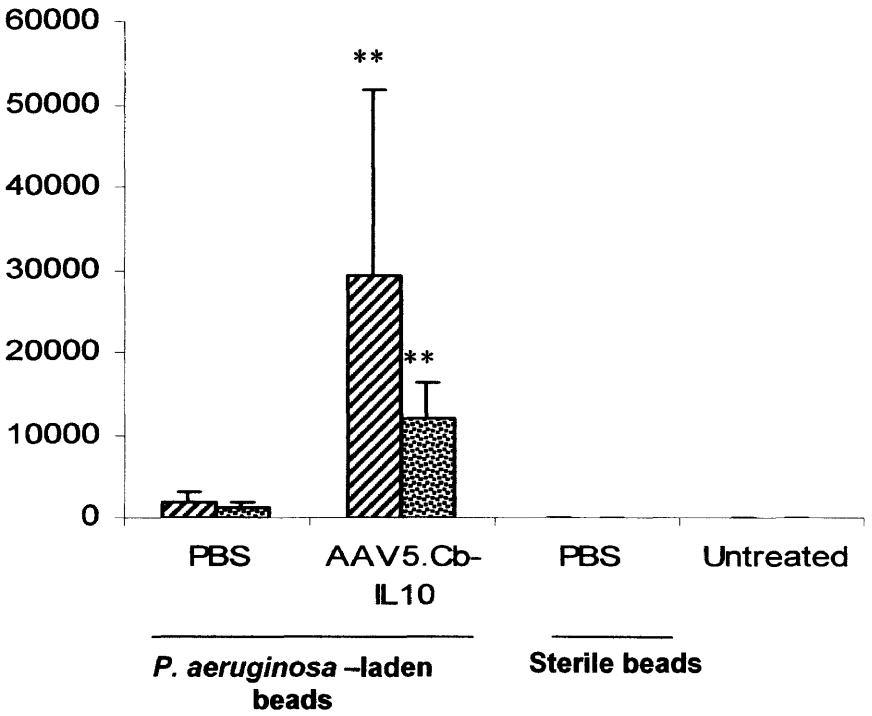

60000
50000
40000
30000
20000
10000
0
**
**
PBS
AAV5.Cb-IL10
PBS
Untreated
P. aeruginosa –laden beads
Sterile beads

Figure 2.2 Successful intratracheal administration of AAV5.Cβ-mIL10 to the murine lung. Anesthetized mice were intratracheally injected with PBS or $1x10^{12}$ vg of AAV5.Cβ-mIL10. After 6 weeks, AAV5.Cβ-mIL10-treated mice were intratracheally infected with $5x10^{6}$ cfu *P. aeruginosa*-laden beads while PBS-treated mice were infected with *P. aeruginosa*-laden beads or sterile beads. Untreated mice did not receive the vector, PBS, or agarose beads. Mice were sacrificed 3 days later. IL-10 protein levels were measured in the ELF (▨) and lung homogenate (▩). **$p<0.005$ compares PBS and AAV5.Cβ-mIL10 treatments in IL-10 levels in the ELF and lung homogenate, respectively. Data shown are average ± standard deviation for 3 or more animals in each group from 2 experiments.

AAV5.Cβ-mIL10 mediates IL-10 protein expression in the alveoli.

In addition to determining gene expression levels, we sought to identify the specific cells responsible for protein expression. An immunostaining protocol for IL-10 was developed using paraffin-embedded lung sections. Positive IL-10 staining was evident in lung sections from *P. aeruginosa*-infected IL-10T mice that received AAV5.Cβ-mIL10 (**Figure 2.3A**) with no IL-10 staining in the lung tissue harvested from IL-10T mice that received PBS (**Figure 2.3B**). These data confirm that resident alveolar cells have been successfully infected with the IL-10 vector and are responsible for protein production.

Figure 2.3

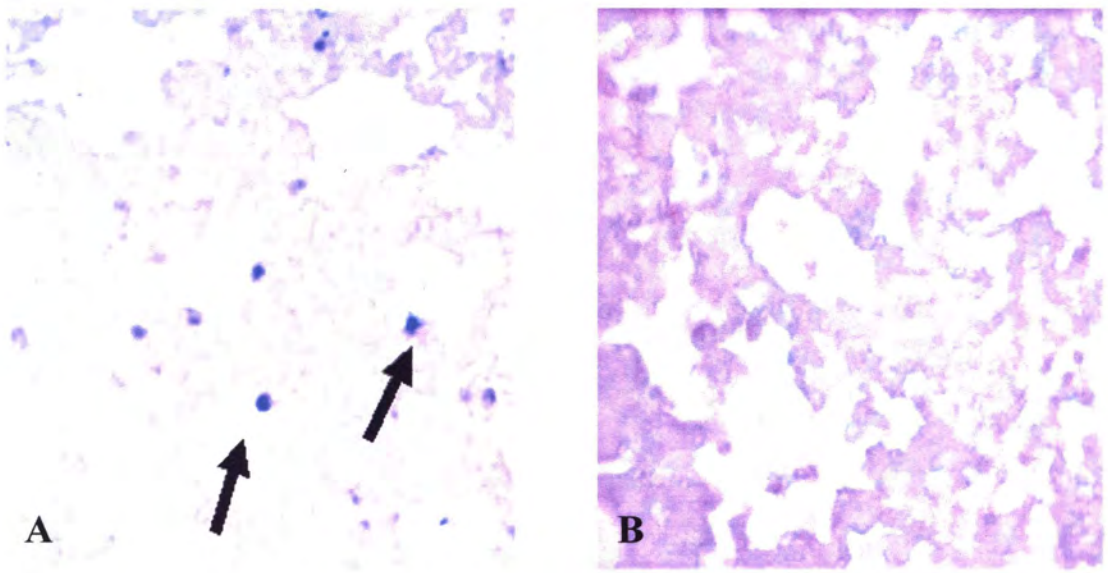


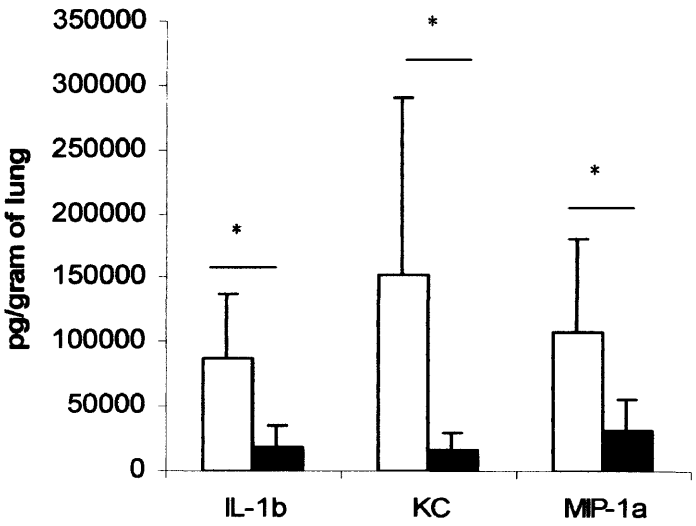
Figure 2.3 Lung sections from IL-10T mice receiving AAV5.Cβ-mIL10 stain positive for IL-10 expression in the alveoli. Anesthetized mice were intratracheally injected with PBS or $1x10^{12}$ vg of AAV5.Cβ-mIL10. After 6 weeks, anesthetized mice were intratracheally infected with $5x10^{6}$ cfu *P. aeruginosa*-laden beads. Mice were sacrificed 3 days later. Lungs were collected and fixed in paraformaldehyde, then sectioned and immunostained for IL-10 protein expression. Arrows point to blue staining to indicate IL-10 protein expression. **(A)** AAV5.Cβ-mIL10 **(B)** PBS. Magnification 40x. Observations represent the findings from 4 or more animals from 2 experiments.

AAV5.Cβ-mIL10 produces a significant decrease in proinflammatory cytokine levels in the lung.

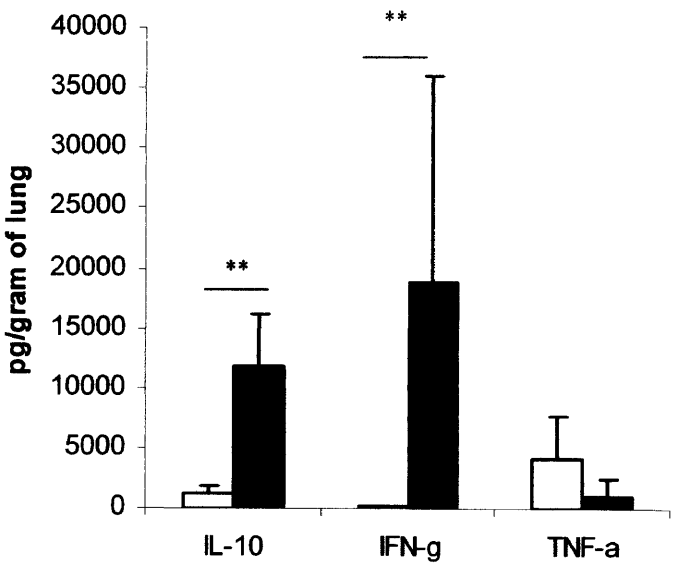
The lung homogenates from IL-10T mice receiving AAV5.Cβ-mIL10 or PBS and infected with *P. aeruginosa* were analyzed for cytokine levels. AAV5.Cβ-mIL10 reduced chemokines levels: a 9-fold reduction in KC and a 4-fold reduction in MIP-1α compared to PBS treatment (**Figure 2.4A**). There was also a decrease in proinflammatory cytokine levels 5-fold reduction in IL-1β (**Figure 2.4A**) and a 0.5 fold reduction in TNF-α (**Figure 2.4B**) with an increase (10-fold) of IFN-γ. Chemokine and cytokine levels in the ELF showed a trend towards decreasing IL-1β, KC, and MIP-1α levels, and an increase IFN-γ (data not shown). To determine if the dramatic reduction in lung proinflammatory cytokines had a systemic effect, we measured circulating proinflammatory cytokine concentrations in the serum and did not observe a decrease (data not shown).

Figure 2.4

A.


350000
300000
250000
200000
150000
100000
50000
0
pg/gram of lung
*
*
*
IL-1b
KC
MIP-1a

B.


40000
35000
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pg/gram of lung
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IL-10
IFN-g
TNF-a

Figure 2.4 AAV5.Cβ-mIL10 decreases proinflammatory cytokine levels in the lungs of IL-10T mice. Anesthetized mice were intratracheally injected with PBS or $1x10^{12}$ vg of AAV5.Cβ-mIL10. After 6 weeks, anesthetized mice were intratracheally infected with $5x10^{6}$ cfu *P. aeruginosa*-laden beads. Mice were sacrificed 3 days later. Lungs were collected, homogenized, and cytokine levels measured. **(A)** IL-1β, KC, and MIP-1α were measured with PBS (□) or AAV5.Cβ-mIL10 treatment (■). **(B)** IL-10, IFN-γ, and TNF-α. were measured with PBS (□) or AAV5.Cβ-mIL10 treatment (■). *$p < 0.05$, **$p < 0.005$ Data shown are average ± standard deviation for 5 or more animals in each group from 2 experiments.

IL-10 expression from AAV5.Cβ-mIL10 decreases inflammation-associated lung pathology.

Despite fairly high IL-10 levels in the lung, there was no indication of systemic infection or immunosuppression modulated by IL-10. This was confirmed by histological examination of lung sections obtained from mice receiving AAV5.Cβ-mIL10 and infected with *P. aeruginosa*, which contained fewer neutrophils and lymphocytes, primarily found in discrete nodules (**Figure 2.5A, B**). The lung sections of PBS-treated mice infected with *P. aeruginosa* had extensive inflammatory cell infiltration and lung consolidation (**Figure 2.5C, D**) while PBS-treated mice given sterile beads did not exhibit any inflammation-associated histopathology (**Figure 2.5E, F**). This pathology corroborates our cytokine findings and supports our hypothesis that AAV5.Cβ-mIL10 can effectively reduce inflammation in *P. aeruginosa*-infected lungs.

Figure 2.5

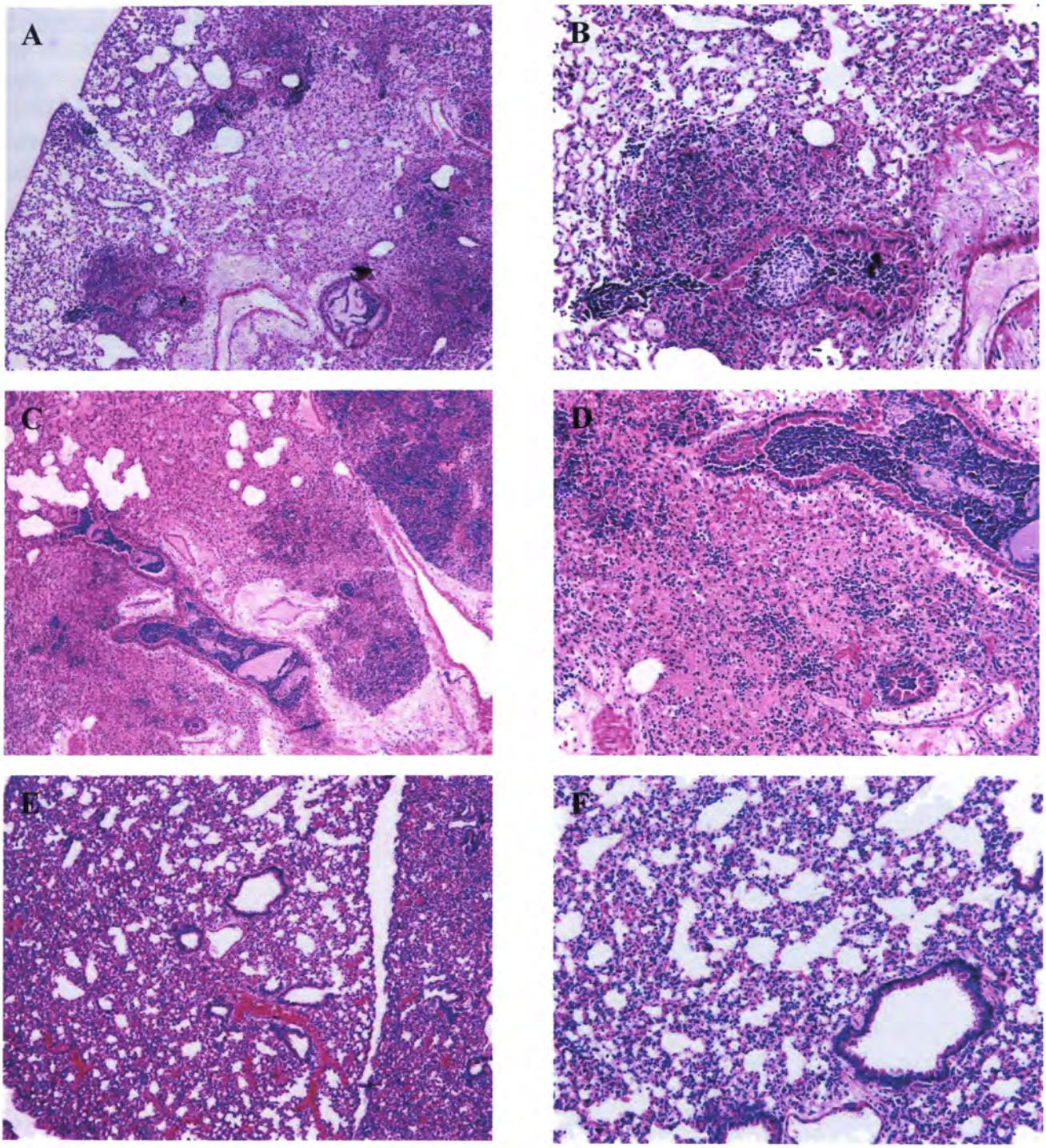


Figure 2.5 Inflammation-associated pathology is reduced in AAV5.Cβ-mIL10-treated mice. Anesthetized mice were intratracheally injected with PBS or $1x10^{12}$ vg of AAV5.Cβ-mIL10. After 6 weeks, AAV5.Cβ-mIL10-treated mice were intratracheally infected with $5x10^{6}$ cfu *P. aeruginosa*-laden beads while PBS-treated mice were infected with *P. aeruginosa*-laden beads or sterile beads. Mice were sacrificed 3 days later. Lungs were collected and fixed in paraformaldehyde, then sectioned and H&E stained. **(A, B)** AAV5.Cβ-mIL10 with *P. aeruginosa*-laden beads, **(C, D)** PBS with *P. aeruginosa*-laden beads, and **(E,** F) PBS with sterile beads. Magnifications are 4x (A, C, E) and 10x (B, D, F). Observations represent the findings from 3 or more animals from 2 experiments.

2.4 Discussion

IL-10 is necessary to balance the proinflammatory response and serves to limit and terminate the cascade of inflammatory cytokines triggered by NFκB activation. By regulating cytokine secretion and the differentiation and proliferation of many immune cells, such as T, B, natural killer (NK), antigen-presenting cells (APC), mast cells, and granulocytes, IL-10 helps maintain immune homeostasis. Stimulation of the IL-10 receptor regulates signaling pathways, including JAK/STAT3, PI 3-kinase, MAPK, and suppressor of cytokine signaling (SOCS) [128] by selective inhibition of transcription through STAT3, not as a general block on NFκB activation [130]. IL-10 may also decrease myeloid cells and prevent cell to cell communication between macrophages and T cells, resulting in decreased production of proinflammatory cytokines and cytotoxic factors. Via these mechanisms, IL-10 reduces the production and release of TH-1 cytokines (including IL-2, IL-6, IL-8, and TNF-α).

Decreased IL-10 production has been documented in CF and is thought to contribute to the excessive inflammation present in the airway during active and chronic infection [26, 30]. Supporting this theory are studies of chronic *P. aeruginosa* infection in IL-10T and CFTR knockout mice, demonstrating dramatic weight loss, greater PMN infiltration, and higher concentrations of proinflammatory cytokines following infection compared to wild-type mice [37, 139, 215, 220, 225, 226]

In this study, we performed intratracheal injection of AAV5.Cβ-mIL10 6 weeks prior to chronic *P. aeruginosa* infection in IL-10T mice. IL-10 was expressed in the alveoli and produced at significantly higher levels in the ELF and lung homogenate in vector-treated mice compared to mice that received PBS. IL-10 production correlated with a significant decrease in proinflammatory cytokines IL-1β, KC, and MIP-1α, as well as a decrease in

TNF-α, and inflammation-associated lung pathology (neutrophil influx and lung consolidation). This corroborated the results found by others when IL-10 was administered as recombinant protein [37, 139, 141]. In a chronic *P. aeruginosa* infection in CD-1 mice, intraperitoneal administration of recombinant IL-10 (rIL-10) resulted in increased survival, fewer neutrophils in the BAL, and decreased area of lung inflammation when compared to placebo-treated mice with no decrease in bacterial burden [139]. A study was also done with intraperitoneal injection of rIL-10 to CFTR knockout mice resulting in decreased T-cell co-stimulatory molecule, B7, and poor co-stimulatory activity of bronchoalveolar macrophages (BALMs) [140]. Because BALMs from patients with CF have been shown to actively produce the proinflammatory cytokines which are elevated in CF BAL [26, 29], treatment with IL-10 may suppress synthesis of these proinflammatory cytokines and alter T-cell responses. Sawa et al administered recombinant IL-10 (rIL-10) intraperitoneally before or after acute *P. aeruginosa* infection in Balb/c mice [141], demonstrating decreased lung injury and mortality, and a decreased inflammatory response (increase in IFN-γ and slight decrease in TNF-α) as shown in our studies with AAV5.Cβ-mIL10. Recombinant IL-10 was also administered, subcutaneously, in an acute model using intratracheal LPS challenge in CF mice and resulted in a significant decrease of PMNs and TNF-α, which correlated with decreased NFκB activity and increased IκBα [37].

Importantly, blood levels of proinflammatory cytokines and IL-10 from mice receiving AAV5.Cβ-mIL10 were not significantly different from those measured in PBS-treated mice. This finding supports a localized IL-10 effect, an important safety feature because IL-10 leakage into the circulation could mediate systemic immunosuppression or even become

immunostimulatory at high systemic doses [244]; rIL-10 administered systemically was previously found to attenuate both local and systemic effects [139].

There were several limitations in this study. Instead of using CFTR knockout mice, we used the IL-10T mouse model because of its strong similarities to CFTR knockout mice in response to both chronic and acute *P. aeruginosa* lung infections. Greater neutrophil recruitment, more severe weight loss, and increased areas of lung inflammation result compared to wild-type mice [35, 37, 139, 215, 220, 225, 226] suggesting an important role for IL-10 in regulating the inflammatory response to *P. aeruginosa.* IL-10T mice still produce background levels of IL-10, demonstrated in our PBS-treated mice infected with *P. aeruginosa* (**Figure 2.2**); however this level was significantly less than the levels of IL-10 produced by AAV5.Cβ-mIL10 ($p<0.005$).

We did not use a sham AAV vector to ensure the AAV did not impact *P. aeruginosa* infection or IL-10 production. While AAV-mediated gene transfer leads to the development of antibodies against the vector capsid, possibly preventing vector re-administration, the lack of inflammatory responses documented in numerous *in vivo* gene transfer models employing AAV vectors suggest that these agents do not interact significantly with the innate immune system [143]. Studies in non-human primates with AAV-CFTR showed the vector did not result in an increase in proinflammatory cytokines and neutrophil influx as measured in the BALF [143]. Additionally, studies in murine models have shown that AAV-GFP has the same response as PBS when measuring percentage weight loss and neutrophil migration (Dr. Tom Ferkol, unpublished studies), supporting our use of PBS as a negative control.

In the current study, we did not measure bacterial burden. We observed that mice receiving IL-10 prior to *P. aeruginosa* infection appeared to be healthier (more active, less

respiratory distress, better groomed) than mice given PBS. That high levels of IL-10 do not promote bacteremia is supported by studies showing chronic *P. aeruginosa* infection in IL-10T and CF mice models have no difference in bacterial burden when compared to wild-type mice [220]. Additionally, the bacterial burden following systemic IL-10 administration is unchanged in IL-10T and CF mice [139]. Furthermore, treatment of CF patients with anti-inflammatory therapeutics slows the progression of lung disease, but does not exacerbate infection [96, 99, 100]. At present, we are testing AAV5.Cβ-mIL10 in a chronic *P. aeruginosa* infection model in CFTR knockout mice to substantiate these findings and will examine bacterial load in all infected mice to verify that a decrease in the inflammatory response does not promote the spread of infection in the lung or systemically.

The model of chronic infection using *P. aeruginosa*-laden agarose beads was over a short period of time (3 days) and seems counterintuitive as a chronic model; however, this most commonly-used approach of chronic lung infection and most appropriate *in vivo* representation in experimental models of CF. In non-CF mice, infection with *P. aeruginosa*-laden beads can persist for up to 28 days [220-222], while CFTR knockout mice appear to have more severe mortality and inflammation, characterized by high levels of pro-inflammatory mediators (TNF-α, KC, and MIP-2) 3 days after exposure to the beads. Impaired bacteria killing occurs between days 3 and 7 after infection of CF mice with 80% of death on day 7, compared to wild-type mice with 50% mortality rate [220, 224]. A sacrifice date of 3-4 days is documented to be optimal for analysis of cytokines and lung pathology [217].

Historically, AAV gene transfer has not led to sufficient gene expression in the CF lung due to a number of factors including the deficiency of the AAV2 receptor (heparin sulfate)

on the apical surface of epithelial cells, the lack of a strong promoter in AAV-based vector cassettes, and the presence of significant physical barriers to viral entry in epithelial cells. We addressed two of these barriers in our vector construct by employing an AAV5 pseudotyped vector, which binds to apically-expressed receptors [2,3-linked sialic acid and the platelet-derived growth factor (PDGF)] [188, 241, 242] and a potent promoter (Cβ), previously demonstrated to be highly effective in the murine lungs [204]. Furthermore, because our therapeutic gene, IL-10, is produced by a variety of cells and is a secreted protein, it has more available targets and may demonstrate its anti-inflammatory effects in sites removed from the successfully transduced cells.

In recent decades, many advances have been made in the treatment of cystic fibrosis, contributing to a longer life span and less morbidity for patients with CF. However, airway inflammation and infection continue to be problematic for the majority of patients, leading to progressive airway obstruction, lung damage, and, eventually death. The role of inflammation in the pathogenesis of CF has been controversial, but the majority of current evidence supports the hypothesis that airway inflammation in CF is exaggerated and pathologic [26-30, 64, 65, 68, 228, 229]. Although anti-inflammatory treatments for CF airway disease have been associated with modest improvements in lung health, none have achieved a high rate of success, and several carry the risk of significant side effects. Therefore, the development of safer and more effective anti-inflammatory therapies continues to be essential. In this study, we prove our hypothesis that in a chronic *P. aeruginosa* infection model, AAV5.Cβ-mIL10 mediates high levels of lung-specific IL-10 expression and reduction in proinflammatory cytokines with no systemic leakage. The anti-inflammatory activity of AAV5.Cβ-mIL10 is an important step towards finding new, effective therapies for inflammation in CF and validating the use of

AAV gene transfer to alter the local proinflammatory immune response in the lung. Future studies should analyze the duration of AAV-mediated gene expression, shown for some transgenes to persist for 1.5 years in animal models [159-162], a feature that is clinically relevant for a population of patients with CF that receive multiple therapies a day [245].

Chapter 3.

Non-invasive delivery of *P. aeruginosa*-laden beads produces a murine model of chronic lung disease.

3.1 Introduction

The leading cause of morbidity and mortality in patients with CF is chronic endobronchial infections with mucoid *Pseudomonas aeruginosa* [83, 84]. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR); however, similar mutations of the CFTR gene to create an animal model of CF have not resulted in a spontaneous *in vivo* model of CF lung disease [209]. CFTR knockout mice are generally difficult to breed, yielding small litters, and having early mortality, succumbing to a lethal intestinal phenotype, rather than the obstructive airway disease commonly seen in patients with CF. The high mortality of CFTR knockout mice led to the creation of gut-corrected CFTR knockout mice, CFTR knockout mice that have overexpression of human CFTR from the fatty acid-binding promoter (FABP) to improve the intestinal phenotype and increase viability [212]. While the mortality rates were dramatically reduced, gut-corrected CFTR knockout mice are still smaller in size and body weight than their respective litter-mates, making them more sensitive to manipulation [217, 246].

Because CFTR knockout mice do not have lung disease, it must be induced in animal models of CF lung disease. The most commonly-used model of chronic lung infection *in vivo* uses intratracheal injection of *P. aeruginosa*-laden agarose beads, a technique developed by Cash et al in rats [218], modified by Stark et al for mice [219], and further optimized for reproducible results by van Heeckeren et al [217]. In non-CF mice, the infection can persist for up to 28 days [220-222], while CFTR knockout mice appear to have more severe mortality and inflammation, characterized by high levels of pro-inflammatory mediators

(TNF-α, KC, and MIP-2) 3 days after exposure to the beads. Impaired bacteria killing occurs between days 3 and 7 after infection of CF mice with 80% of death on day 7, compared to wild-type mice with 50% mortality rate [220, 224]. This finding demonstrates that mice lacking CFTR function are relevant *in vivo* models for studying CF lung disease because they are predisposed to bacterial lung infection, but the mechanism of increased susceptibility is unclear, although it is suspected that the underlying CF-specific host factors in animal models are the same as in humans.

Although intratracheal injection of *P. aeruginosa*-laden beads produces chronic lung infection characteristic of CF, the complexity of reproducing *P. aeruginosa* lung infection *in vivo* coupled with the difficulties of using CFTR knockout mice has led researchers to seek alternative, non-invasive methods of administration. Guilbalt et al recently published a method of non-invasive *P. aeruginosa*-laden bead administration using microscopic guidance for delivery to the trachea [246] and the model resulted in increased weight loss, higher bacterial burden, and more elevated polymorphonuclear alveolar cell recruitment into the lungs in CFTR knockout mice compared to wild-type mice. Aspiration challenge has also been used successfully for *P. aeruginosa*-laden bead delivery [247], but neither approach has been directly compared in successful lung delivery to the widely-used intratracheal injection method. While the microscopic guidance method was better characterized for reproducibility of *P. aeruginosa* infection, the technical difficulty of performing this procedure led us to focus on aspiration challenge for *P. aeruginosa*-laden bead delivery.

Aspiration challenge was originally described by Wills-Karp et al [248] who later made minor modifications to improve lung delivery and reproducibility between animals [249]. This non-invasive method results in even distribution to the lung with reportedly no

delivery to the esophagus or stomach [248, 250] and is seldom used, but could be a beneficial procedure as it is less technically difficult and could result in less mortality associated with surgical instillation of the bacteria.

The purpose of these experiments is to refine the method of aspiration challenge using surfactant phospholipids as a carrier for delivery of *P. aeruginosa*-laden beads as a viable alternative to intratracheal injection. Surfactant, a mixture composed of phospholipids and surfactant associated (SA) proteins, lowers surface tension and, when used as a vehicle, aids in uniform distribution to the peripheral and distal regions of the lungs [251, 252]. We hypothesize that aspiration challenge with surfactant phospholipids results in similar or enhanced lung delivery of *P. aeruginosa*-laden beads when compared to intratracheal injection. If the non-invasive technique produces the same characteristics of chronic lung infection (weight loss, cytokine release, histopathology, and bacterial burden) with reproducibility and less mortality, then it can serve as a model of CF lung disease.

3.2 Materials and Methods

Mice

Eight-week-old CD-1 mice were purchased from the Charles River Laboratories, Inc (Wilmington, MA), shipped in protective, filtered containers, transported in climate-controlled trucks, and allowed to acclimate for at least 3 days prior to use. Mice were fed autoclaved or irradiated Harlan Teklad Sterilizable Rodent Blox 8656 (Harland Teklad, Indianapolis, IN) and bedded on autoclaved 1/8" corn cob bedding and corn husk nesting material (Harland Teklad, Indianapolis, IN). Mice were housed, 2-4, in Micro-Isolator Top Flow Ventilated cages (Tecniplast, Exton, PA) in ventilated racks. All caging supplies were cleaned with an automated cage wash and autoclaved prior to use in animal rooms. MUSC is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC, International), and the mice were maintained in specific pathogen-free (SPF) conditions. Each rodent rack in the facility was assigned 1-4 cages of sentinels which are housed in microisolator cages without filter tops. Sentinel animals are exposed to dirty bedding from different shelves of animals at each cage change, but serology was negative for Sendai virus (SEND), Pneumonia virus of mice (PVM), Mouse hepatitis virus (MHV), Mouse polio virus (TMEV), Reovirus (REO), *Mycoplasma pulmonis* (MPUL), Mouse arvovirus (MPV), and Mouse rotavirus (EDIM). Mouse feces were negative for Helicobacter. The procedures were approved by the Institutional Animal Use and Care Committee (IACUC) at MUSC.

Amido black dye

Anesthetized mice received 0.04% amido black dye (Bio-Rad, Hercules, CA) in phosphate buffered saline (PBS) through aspiration challenge with surfactant phospholipids or intratracheal injection. After 1 hr, anesthetized mice were sacrificed by cervical dislocation and lungs, stomach, and spleen collected.

***Pseudomonas aeruginosa*-laden bead preparation**

The mucoid *Pseudomonas aeruginosa* isolate, PA 15719, was obtained from the sputum of a patient with CF using a protocol approved by the Institutional Review Board (IRB) at MUSC. Bacteria were maintained as glycerol stocks and stored at -80°C. Bacteria-impregnated agar beads were prepared as described by van Heeckeren et al [217, 220] with minor modifications. Briefly, an inoculation loop was used to collect a sample of frozen mucoid *P. aeruginosa* stock, streaked for isolation on a tryptic soy agar (TSA) plate, and incubated at 37°C overnight. One colony was picked from the plate and used to inoculate 50 ml tryptic soy broth (TSB) in a 125 Erlenmeyer flask, which was placed, shaking, overnight at 37°C. A 0.1 ml aliquot of the culture was used to inoculate a second flask of 50 ml TSB in a 125 Erlenmeyer flask. Bacteria were grown to late log phase, shaking, in a 37°C incubator. The bacterial culture was concentrated by centrifugation, resuspended in 10 ml TSB, and 5 ml added to warm (50°C) 2% low melting agarose (Type 1 low EEO; Sigma) in PBS. The bacteria-agar mixture was added to warm (50°C) heavy mineral oil (0122-4, Fisher) containing a stir bar, and stirred rapidly at room temperature for 6 min, followed by cooling with slow additions of ice over a 10-min period. The agar beads were transferred to a separatory funnel; the mineral oil removed, and washed one time with 0.5% sodium

deoxycholate (SDC), one time with 0.25% SDC, and four times with PBS. The *P. aeruginosa*-laden agarose beads were collected and allowed to settle. Additional liquid was removed to obtain a final volume of 75% beads and homogenized using a Kinematica Polytron PT10/35 homogenizer for 4 min at high speed to obtain beads predominantly 100–120 μm in diameter. The size of the beads was measured microscopically before and after homogenization. Using serial dilutions, quantitative bacteriology was performed on an aliquot of the homogenized bead slurry to determine bacterial concentration in colony forming units (cfu) per milliliter. Sterile agar beads were prepared by the same method, without the addition of *P. aeruginosa* culture, and were verified for sterility by serial dilutions plated on TSA plates. The homogenized bead slurries for both preps were prepared the day before inoculation of the mice, stored at 4°C, and concentration of bacteria calculated on the day of inoculation.

Surfactant phospholipid liposomes preparation

DPPC and POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine) phospholipids were purchased (Avanti Polar Lipids, Alabaster, AL) with stock concentrations of 20 mg/ml (DPPC in chloroform) and 10 mg/ml (POPC in chloroform). For every ml of final liposome suspension, 17.5 mg DPPC (0.875 ml stock) and 7.5 mg POPC (0.75 ml POPC stock) were added in a clean, sterile 13x100 mm glass tube. The tube was capped and mixed well via vortex. The solvent was removed via nitrogen stream, with the tube submerged in 40°C water bath, and mixed during the drying process. After the samples were dry, the tubes were transferred to a lyophylization flask and vacuum-dried overnight without additional heating. PBS (1.0 ml) was added to each tube, and then the tubes were

heated at 45°C for 15 min, vortexed for 30 sec, and sonicated for 10 sec. This step was repeated 3 times to ensure the liposome preparation had consistent viscosity and did not contain large liposome aggregates. The liposomes were transferred to sterile screw-cap plastic cryovials using sterile pipette tips and stored at 4°C. The stock phospholipid liposome concentration was 7:3 (mol/mol) DPPC/POPC at 25 mg/ml total phospholipid. For administration, a concentration of 4 mg/ml phospholipid liposomes was used.

Intratracheal injection

Mice were anesthetized through inhalation of a mixture of oxygen and isoflurane in a closed chamber, then transferred and immobilized in a supine position on a surgical table (**Figure 3.1A**). During the procedure, mice were under continuous anesthesia with isoflurane through a conical nosepiece. A small transverse incision was made (about 0.5 mm) just cranial to the thoracic inlet followed by blunt dissection of the salivary glands and musculature to expose the trachea. A sterile, flexible 21-gauge SURFLO winged infusion set (Terumo Corporation, Tokyo, Japan) was inserted into the trachea between the tracheal rings for delivery to both lobes of the lung. A one milliliter syringe pre-loaded with 0.050 ml containing sterile beads or 4mg/ml surfactant phospholipid liposomes with $5x10^6$ cfu *P. aeruginosa*-laden beads was attached to the winged infusion set and the plunger depressed for injection into the trachea (the same procedure was followed for the amido dye injection). The attached set was removed and incision closed by suture. Mice were monitored for recovery which occurred within 10 min of procedure completion.

Aspiration challenge with surfactant phospholipids

Aspiration challenge was conducted using previously published methods [248, 249] with minor modifications. Mice were anesthetized through inhalation of a mixture of oxygen and isoflurane for 5 min in a closed chamber. Mice were removed from anesthetization chamber and suspended by their front incisors in a supine position on an acrylic platform at a 60^{o} angle (**Figure 3.1B**). Coated forceps were used to extend the tongue from blocking the trachea (**Figure 3.1C**). Using a p200 pipette, 0.040 ml containing sterile beads or 4mg/ml surfactant phospholipids with $5x10^{6}$ cfu *P. aeruginosa*-laden beads was instilled at the back of the oral cavity above the tracheal opening (the same procedure was followed for the amido dye injection). Immediately after, the mice aspirated the liquid, evidenced by the mouse gulping. Mice were monitored for recovery which occurred within 5 min of the administration.

Figure 3.1

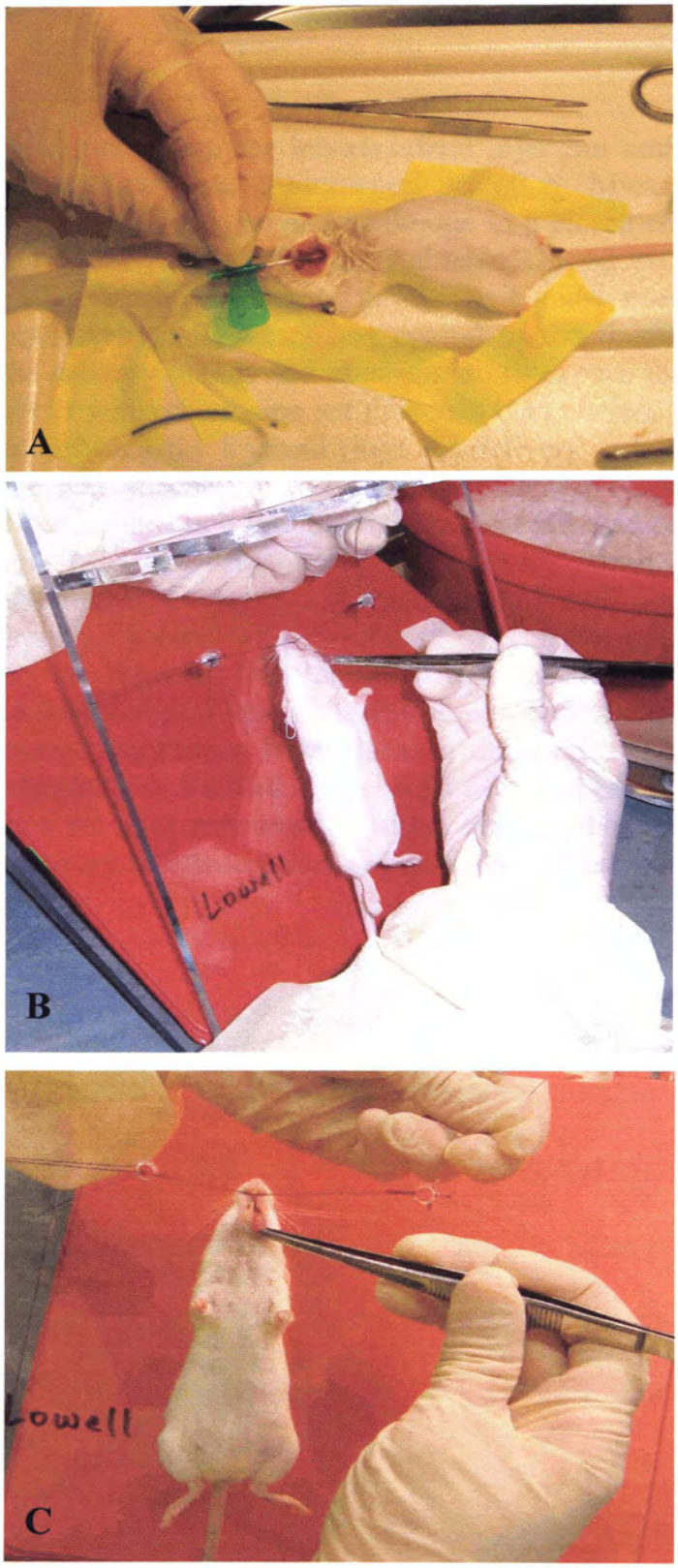


Figure 3.1 Apparatus set-up for intratracheal injection and aspiration challenge with surfactant phospholipids. A. Intratracheal injection. Mice were anesthetized through inhalation of a mixture of oxygen and isoflurane in a closed chamber, then transferred and immobilized in a supine position on a surgical table. During the procedure, mice were under continuous anesthesia with isoflurane through a conical nosepiece. A small transverse incision was made (about 0.5 mm) just cranial to the thoracic inlet followed by blunt dissection of the salivary glands and musculature to expose the trachea. A sterile, flexible 21-gauge SURFLO winged infusion set (Terumo Corporation, Tokyo, Japan) was inserted into the trachea between the tracheal rings for delivery to both lobes of the lung. A one milliliter syringe was attached to the winged infusion set and the plunger depressed for sample (dye or beads) injection into the trachea. The attached set was removed and incision closed by suture. Mice were monitored for recovery which occurred within 10 min of procedure completion. **B, C.** Aspiration challenge with surfactant phospholipids. Mice were anesthetized through inhalation of a mixture of oxygen and isoflurane for 4 min in a closed chamber. Mice were removed from anesthetization chamber and mice suspended by their front incisors in a supine position on an acrylic platform at a 60^{o} angle (**B**). Coated forceps were used to extend the tongue from blocking the trachea (**C**). Using a p200 pipette, the sample (dye or beads) was instilled at the back of the oral cavity above the tracheal opening. Immediately after, the mice aspirated the liquid, evidenced by the mouse gulping. Mice were monitored for recovery which occurred within 5 min of administration.

Assessment of weight loss

Body weight was measured in all mice before *P. aeruginosa* or sterile bead inoculation and at time of sacrifice (3 days later). A record was made of any hunching, poor grooming, fatigue, or increased respiratory effort following inoculation with agarose beads. At 3 days following *P. aeruginosa*-laden bead or sterile bead inoculation, mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Blood was obtained from all mice.

Bronchoalveolar lavage collection

Following sacrifice, bronchoalveolar lavage fluid (BALF) was collected. The trachea was isolated by blunt dissection and an incision made in the upper portion of the trachea, which was cannulated with a 21-gauge shielded I.V. catheter (BD Bioscience, San Hose, CA) attached to a 5 ml syringe. The alveoli were washed 3 times, each with 1 ml sterile PBS. Approximately 1-2ml of bronchoalveolar lavage fluid (BALF) was recovered. Cytokine levels were measured using a customized Bio-Plex cytokine kit (Bio-Rad) for detection of IL-1β, IL-6, TNF-α, KC, and MIP-1α. Analysis was conducted using the Bio-Plex multiplex suspension array system (Bio-Rad) following the company's protocol. Each sample was performed in duplicate. Cytokine concentrations were normalized for urea dilution using a ratio of urea nitrogen concentrations in the airway fluid and in the serum by the Enzymatic Urea Nitrogen (BUN) procedure (Stanbio, Boerne, TX). Final values were expressed as pg/ml epithelial lining fluid (ELF).

Lung histopathology

Lungs were inflated and fixed with 4% paraformaldehyde. After 48 hr, the lungs were embedded in paraffin, cut in 150-200 μm step-sections, and stained with hematoxylin and eosin (H&E) using standard techniques. Histopathologic scoring parameters were based on published criteria [217] with the following designations: 0=within normal limits; 1=darker red than normal; 2=0-2 lymphocytic infiltrated nodules present; 3=3-6 lymphocytic infiltrated nodules and <¼ lung consolidated; 4=¼ to ½ lung consolidated; and 5=½ to ¾ lung consolidated.

Lung homogenate collection and quantitative bacteriology

Lungs were collected and placed on ice. The same day of sacrifice, the lungs were weighed (OHAUS Adventurer balance, 1.000 g sensitivity) and washed in 0.5 ml cell wash buffer (Bio-Rad, Hercules, CA). Using surgical scissors, lungs were cut into small pieces, 2 ml of PBS was added, and the lungs were homogenized for 60 seconds at high speed using Kinematica Polytron PT10/35 homogenizer (Brinkmann Instruments Co., Westbury, NY).

For quantitative bacteriology, the lung homogenate was diluted with PBS in ten-fold serial dilutions, spread on TSA plates, and incubated at 37°C overnight.

For cytokine analysis, the homogenized lungs were further processed by freezing at -80°C, thawing on ice, and sonicating in ice-cold water for 45 sec. The processed lung homogenates were centrifuged at 1500xg at 4°C for 10 min, supernatants collected, and stored at -80°C. Cytokine levels were measured using a customized Bio-Plex cytokine kit (Bio-Rad) for detection of IL-1β, IL-6, TNF-α, KC, and MIP-1α. Analysis was conducted using the Bio-Plex multiplex suspension array system (Bio-Rad) following the company's

protocol. Each sample was performed in duplicate. Cytokine concentrations were standardized for lung weight by multiplying the cytokine value by the volume of sample (2ml) and dividing by the lung weight to obtain final values in picogram (pg) per gram of lung tissue.

Statistical analysis

Percentage body weight, cytokine levels and quantitative bacteriology are presented as average $\pm$ SD. Change in body weight is expressed as the percentage of weight lost from the original weight three days before inoculation. Statistical analysis of cytokine levels and quantitative bacteriology was determined by unpaired, two-tailed Student's t test with Welch correction using the Graph Pad InStat statistics software program (GraphPad Software, San Diego, CA). Histological scoring is presented as individual grades per lung section; significance was determined by Mann-Whitney statistical analysis. For all data sets, p values less than 0.05 were determined to be statistically significant.

3.3 Results

Administration by aspiration challenge with surfactant phospholipids and intratracheal injection results in similar dye distribution.

To compare the location of inoculation between the two procedures, preliminary experiments were performed using amido black dye to ascertain the administration route. Anesthetized mice received amido black dye through aspiration challenge with surfactant phospholipids or intratracheal injection. After 1 hr, mice were sacrificed and the lungs, stomach, and spleen collected. Both methods resulted in distribution throughout the lungs with some delivery to the stomach, but no delivery to the spleen (**Figure 3.2**). The experiment was repeated and the data shown representative of 4 or more animals.

Figure 3.2

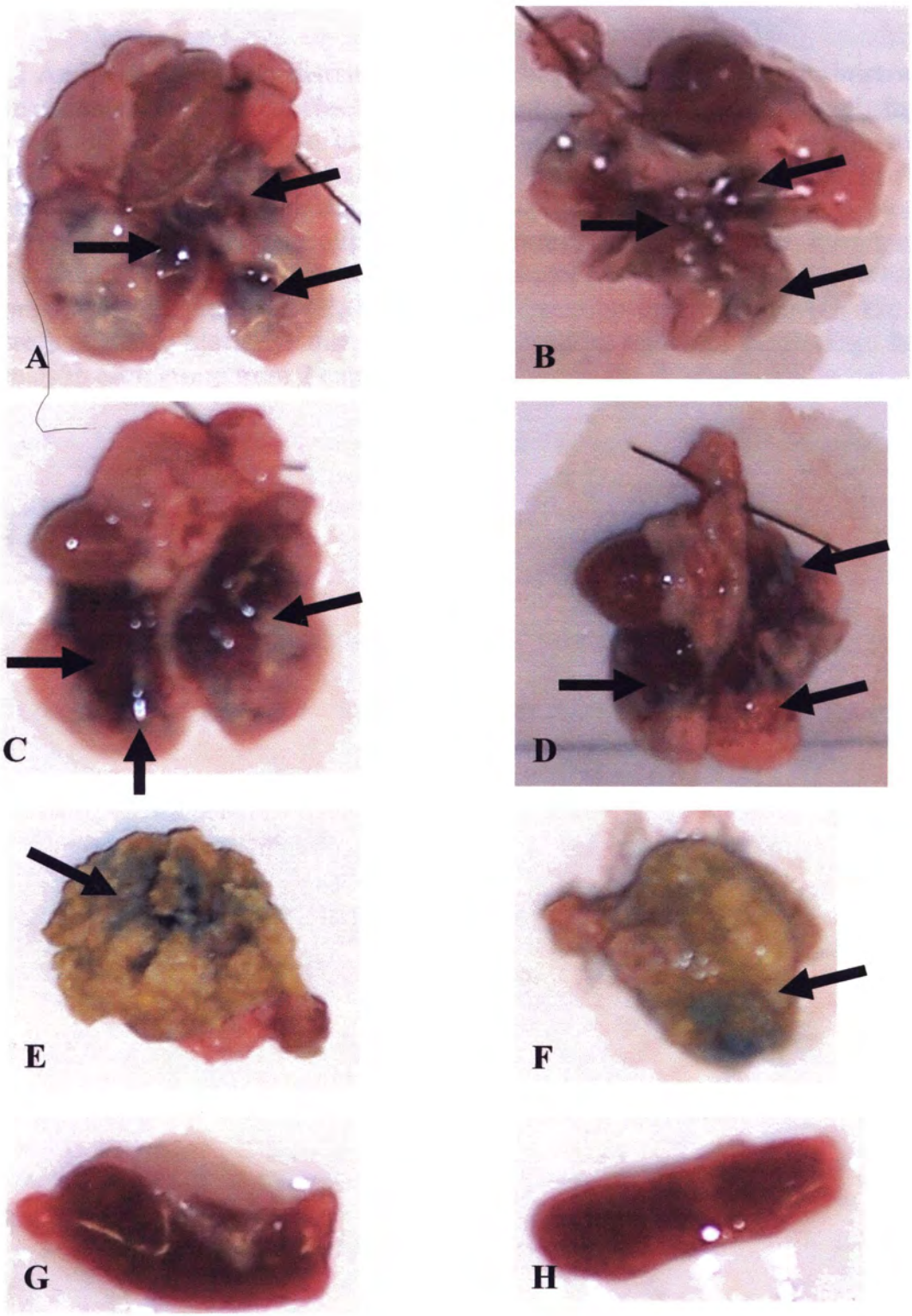


Figure 3.2 Amido black dye is distributed throughout the lungs after administration by aspiration challenge with surfactant phospholipids and intratracheal injection. Anesthetized mice received amido black dye through aspiration challenge with surfactant phospholipids or intratracheal injection. After 1 hr, mice were sacrificed by cervical dislocation and the lungs, stomach, and spleen collected. Administration by aspiration challenge with surfactant phospholipids **(left column)** or intratracheal injection **(right column)** resulted in delivery predominantly to the frontal (**A, B**) and dorsal (**C, D**) lung, with some expression in the stomach (**E, F**), and no expression in the spleen (**G, H**). Arrows indicate areas of amido black dye distribution. Observations represent the findings from 4 or more animals in each group from 2 experiments.

Delivery of *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids results in the same weight loss as intratracheal delivery.

Weight loss following *P. aeruginosa* infection by aspiration challenge with surfactant phospholipids or intratracheal injection to CD-1 mice was measured to determine the severity of infection. The mice were weighed before administration with $5x10^6$ cfu *P. aeruginosa*-laden beads or sterile beads and 3 days later before sacrifice. The average pre-infection weight of the mice in the aspiration challenge with surfactant phospholipids group (n=29) was 28.8 g; mice lost an average body weight of 17.4% $\pm$ 5.1 after administration of the *P. aeruginosa*-laden beads. The average pre-infection weight of the mice (n=22) in the intratracheal administration group was 27.2 g with mice losing an average body weight of 18.9% $\pm$ 5.4 following infection. There was no mortality in the mice from the infection; however, 3 mice in the intratracheal injection group died as a result of complications in the injection operation (i.e. excessive bleeding). While there was no significant difference between the groups in percent weight loss following infection ($p>0.05$), the aspiration challenge with surfactant phospholipids and intratracheal injection of *P. aeruginosa*-laden beads had significantly greater percentage weight loss when compared to intratracheal injection of sterile beads ($p<0.05$ for both groups).

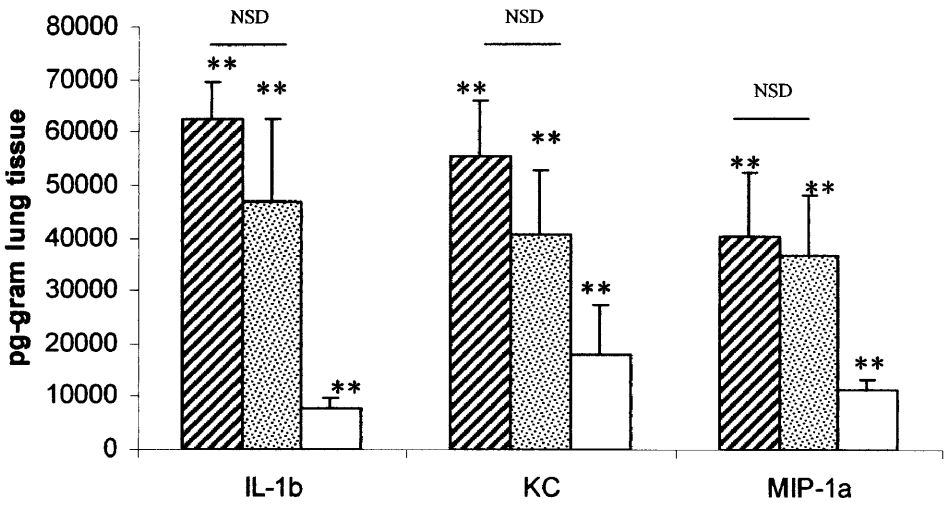
***P. aeruginosa* infection results in lung homogenate inflammatory cytokine levels that are not significantly different by the method of delivery.**

Pro-inflammatory cytokine expression following administration with *P. aeruginosa* was analyzed to compare the immune response to infection between the administration groups. CD-1 mice were inoculated with $5x10^6$ cfu *P. aeruginosa*-laden beads by aspiration

challenge with surfactant phospholipids, intratracheal injection, or with sterile beads by intratracheal injection. Three days later, the mice were sacrificed, lungs collected, and cytokines levels measured in the lung homogenate. Cytokine levels in the aspiration challenge with surfactant phospholipids group: IL-1β (63 ± 7 ng/gram-lung), IL-6 (9 ± 4 ng/gram-lung), TNF-α (13 ± 4 ng/gram-lung), KC (56 ± 11 ng/gram-lung), and MIP-1α (40 ± 12 ng/gram-lung) were not significantly different from those in the intratracheal injection group: IL-1β (47 ± 16 ng/gram-lung), IL-6 (17 ± 7 ng/gram-lung), TNF-α (10 ± 5 ng/gram-lung), KC (40 ± 12 ng/gram-lung), and MIP-1α (37 ± 11 ng/gram-lung) (**Figure 3.3A, B**). Both groups had significantly higher cytokine levels than mice intratracheally injected with sterile beads (**Figure 3.3A, B**).

Figure 3.3

A.


80000
70000
60000
50000
40000
30000
20000
10000
0
pg-gram lung tissue
NSD
NSD
NSD
**
IL-1b
KC
MIP-1a

B.

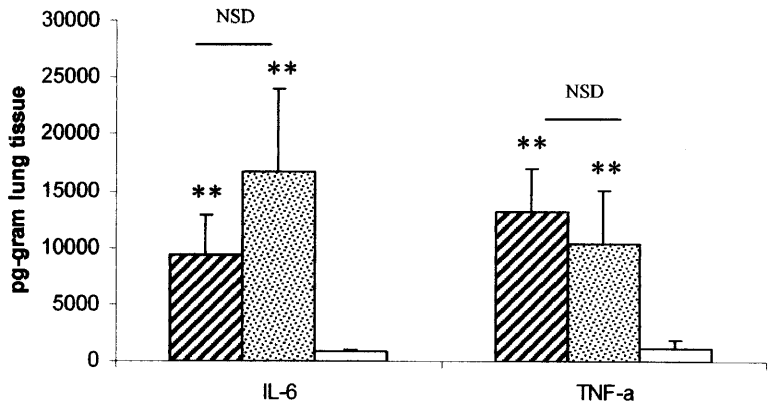

30000
25000
20000
15000
10000
5000
0
pg-gram lung tissue
NSD
NSD
**
IL-6
TNF-a

Figure 3.3 ***P. aeruginosa*** **infection causes rise in lung homogenate inflammatory cytokine levels that are not significantly different in the method of administration.** Anesthetized mice received $5x10^6$ cfu *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids or intratracheal injection or sterile beads by intratracheal injection. Mice were sacrificed 3 days later. Lungs were collected and homogenized. Cytokines **(A)** IL-1β, KC, and MIP-1α and **B)** IL-6 and TNF-α. were measured in the groups of aspiration challenge with surfactant phospholipids (□) or intratracheal injection for delivery of *P. aeruginosa*-laden beads (▨) or administration of sterile beads by intratracheal injection. NSD is not significantly different ($p>0.05$) and $^{**}p<0.005$, comparing the *P. aeruginosa* infection methods to delivery of sterile beads. Data shown are the average ± standard deviation for 5 mice in each group from 2 experiments.

Inflammatory cytokine levels in the epithelial lining fluid are not significantly different by the method of *P. aeruginosa*-laden bead delivery.

The bronchoalveolar lavage fluid (BALF) was collected and analyzed for inflammatory cytokine levels. Cytokine concentrations were normalized for urea dilution using a ratio of urea nitrogen concentrations in the airway fluid and in the serum. Levels of cytokines in the BALF were not significantly different upon infection of mice with *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids compared to intratracheal injection (**Figure 3.4**). The serum was also analyzed to determine whether proinflammatory cytokine production was systemic. There was no significant difference ($p>0.05$) in any of these cytokine levels in the serum when comparing the two *P. aeruginosa*-laden bead administration groups, aspiration challenge with surfactant phospholipids (n=15) and intratracheal injection (n=12) (data not shown), thus confirming a lung-specific infection.

Figure 3.4

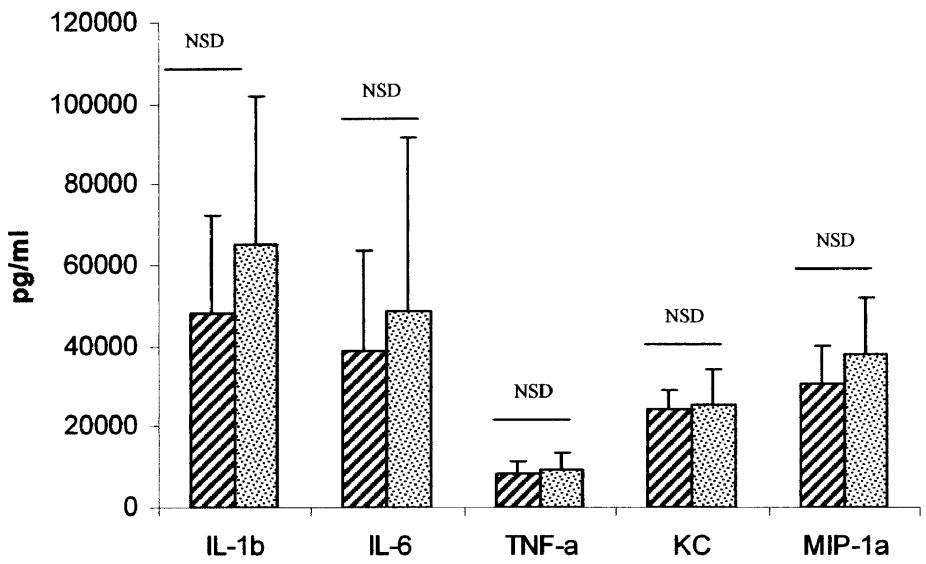

120000
100000
80000
60000
40000
20000
0
pg/ml
NSD
NSD
NSD
NSD
NSD
IL-1b
IL-6
TNF-a
KC
MIP-1a

Figure 3.4 Inflammatory cytokine levels in the epithelial lining fluid are not significantly different in the administration method used for *P. aeruginosa* delivery. Anesthetized mice received $5x10^6$ cfu *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids or intratracheal injection. Mice were sacrificed 3 days later. BALF was collected, cytokine levels analyzed, and the measurements corrected for urea nitrogen dilution to obtain ELF values. Levels of IL-1β, IL-6, TNF-α, KC, and MIP-1α were measured with administration by aspiration challenge with surfactant phospholipids (▨) or intratracheal injection for delivery of *P. aeruginosa*-laden beads (▩). NSD is not significantly different ($p>0.05$). Data shown are the average ± standard deviation for 12 mice in the aspiration challenge group and 8 mice in the intratracheal injection group from 2 experiments.

Administration methods produce similar levels of inflammation-associated lung pathology.

To determine if increased inflammatory cytokines correlated with histopathology, lung sections were obtained from mice receiving *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids or intratracheal injection. Histological examination revealed similar levels of inflammatory cell infiltration and lung consolidation (**Figure 3.5A, B**). Histological scoring was used to show that the inflammatory changes were not significantly different when comparing the two administration groups ($p>0.05$, **Table 1**).

Figure 3.5

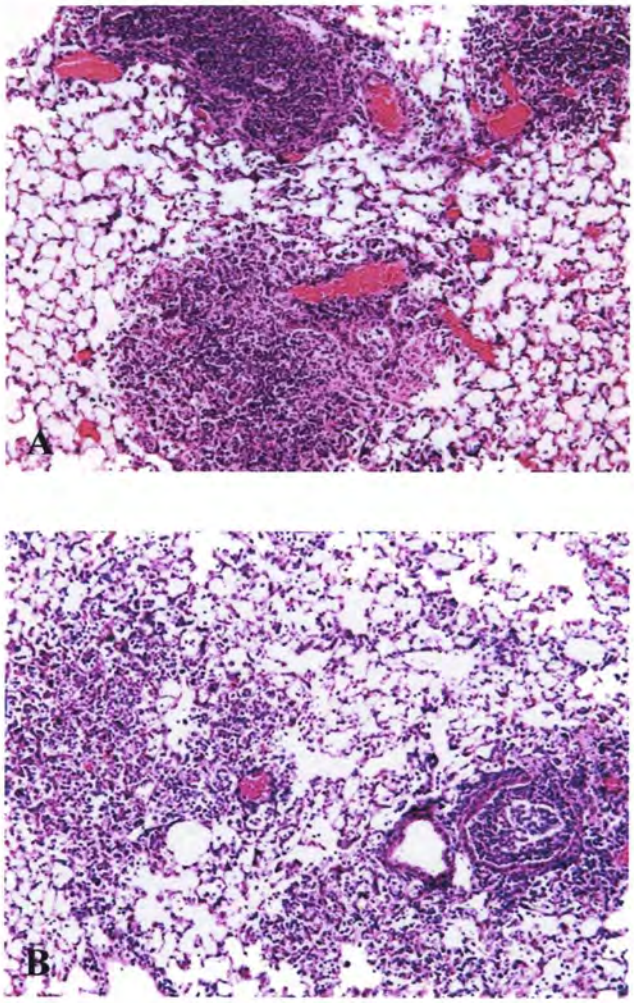


Figure 3.5 Administration methods produce similar levels of inflammation-associated lung pathology. Anesthetized mice received $5x10^6$ cfu *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids or intratracheal injection. Mice were sacrificed 3 days later. Lungs were collected and fixed in paraformaldehyde, then sectioned and H&E stained. **(A)** Aspiration challenge with surfactant phospholipids administration of *P. aeruginosa*-laden beads and **(B)** intratracheal injection administration of *P. aeruginosa*-laden beads. Magnification is 10x. Observations represent the findings from 9 animals in each group from 2 experiments.

Table 3.1 Histopathologic scores of lung sections from CD-1 mice infected with $5x10^6$ cfu *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids or intratracheal injection.

P. aeruginosa *administration method*	*Number of mice*	*Score*	*Average± SD*
Aspiration challenge with surfactant phospholipids	9	4 4 5 4 4 4 4 4 3	4.0 ± 0.5
Intratracheal injection	9	4 3 4 4 3 4 4 4 4	3.8 ± 0.4
p value			**p>0.05**

Anesthetized mice were infected with $5x10^6$ cfu *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids or intratracheal injection. Mice were sacrificed 3 days later. Lungs were collected and fixed in paraformaldehyde, then sectioned and H&E stained. Sections were graded by a blinded pathologist.

Histopathologic scoring parameters were based on previously published criteria [217].
0=within normal limits
1=darker red than normal
2=0-2 lymphocytic infiltrated nodules present
3=3-6 lymphocytic infiltrated nodules, <¼ lung consolidated
4=¼ to ½ lung consolidated
5=½ to ¾ lung consolidated
6=>¾ lung consolidated

Aspiration challenge with surfactant phospholipids and intratracheal injection of *P. aeruginosa*-laden beads results in comparable levels of bacterial burden in the lung.

In addition to determining the cytokine response and pathology, we wanted a direct measurement of bacteria delivered to the lung. Homogenized lungs obtained from mice in both administration groups were spread in 10-fold serial dilutions, in duplicate, on TSA plates. Colonies were counted on each plate and bacterial load was not significantly different between the administration groups (**Figure 3.6**). These data confirm that aspiration challenge with surfactant phospholipids for delivery of *P. aeruginosa*-laden beads produces the same infection in the lung as intratracheal injection. These data corroborate our cytokine and histopathologic findings, supporting our hypothesis that delivery of *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids produces the same chronic lung infection as intratracheal injection.

Figure 3.6

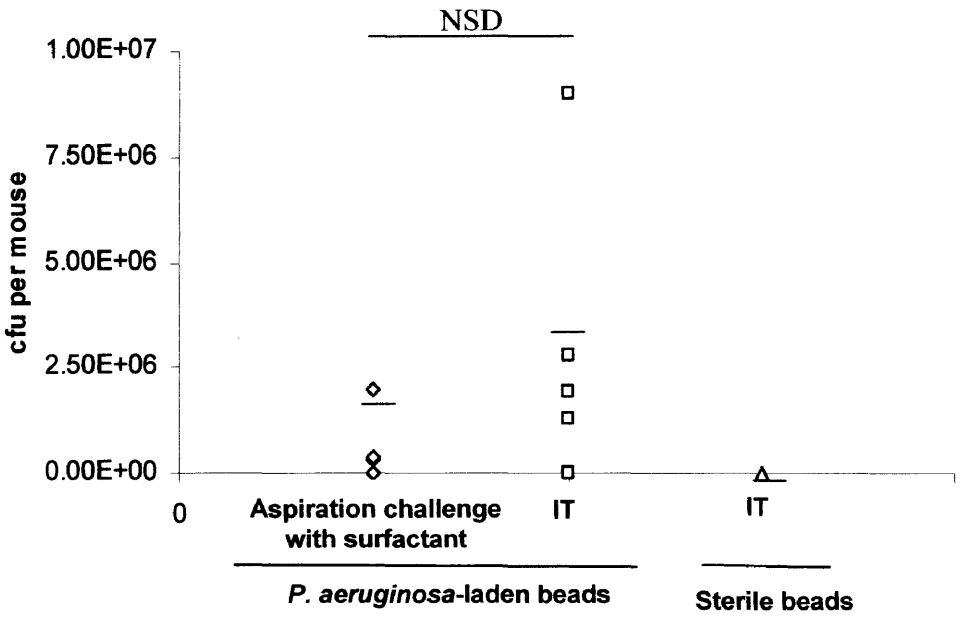

NSD
1.00E+07
7.50E+06
5.00E+06
2.50E+06
0.00E+00
cfu per mouse
0
Aspiration challenge with surfactant
IT
IT
P. aeruginosa-laden beads
Sterile beads

Figure 3.6 Similar level of bacterial burden results from *P. aeruginosa* administration by aspiration challenge with surfactant phospholipids and intratracheal administration. Anesthetized mice received $5x10^6$ cfu *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids or intratracheal injection or sterile beads by intratracheal injection. Mice were sacrificed 3 days later. Mice were sacrificed 3 days later. Lungs were collected, homogenized, and spread in serial dilutions on TSA plates. After 24 hr colonies were counted and bacterial titer calculated. Data is shown for administration of*P. aeruginosa*-laden beads by aspiration challenge with phospholipids (◇) or intratracheal injection (□) and sterile beads administered by intratracheal injection (△). NSD is not significantly different ($p>0.05$). Data points are bacterial burdens for 5 mice in each group from 2 experiments; the black line is the average of each group. IT is intratracheal injection.

3.4 Discussion

A representative *in vivo* model of CF lung disease is essential for characterizing potential therapeutics. Because CFTR knockout mice do not have spontaneous lung disease, it must be induced, commonly through infection with *Pseudomonas aeruginosa*, the pathogen that most highly correlates with clinical outcome in patients with CF [83, 84]. Chronic lung infection has been achieved by colonization of the lungs of mice through *P. aeruginosa* in drinking water [215] and repeated *P. aeruginosa* aerosol aspiration challenge [216]. The most commonly-used model of chronic lung infection *in vivo* uses intratracheal injection of *P. aeruginosa*-laden beads. Administration of *P. aeruginosa* trapped in agar beads slows the growth of the bacteria, resulting in a chronic infection that most accurately resembles that seen in patients with CF [220]. The beads localize the bacteria in the distal airways and mimic the morphology of bacterial infection observed in the CF lung [220, 224, 253]. Unfortunately, intratracheal instillation is particularly challenging to perform in CFTR knockout mice because they are smaller in size and more sensitive to manipulation than wild-type mice [217, 246].

Because of the extensive use of intratracheal injection of *P. aeruginosa*-laden beads, we were interested in making a direct comparison to characterize fully the resulting infection and inflammation in this method when compared to a non-invasive method of infection. Additionally, due to the surface tension properties of surfactant, we used the phospholipid fraction of surfactant to enhance lung-targeted delivery. Surfactant forms a thin layer over the alveolar surface and is comprised of lipids and proteins [254]. The lipid fraction (90%) includes Dipalmitoylphosphatidylcholine (DPPC) and Phosphatidylglycerol (PG); the protein fraction (5-10%) includes hydrophilic surfactant-associated proteins (SP-A and SP-B) and

hydrophobic proteins (SP-B and SP-C). Surfactant lowers surface tension, aids in rapid adsorption and spreading and contributes to stability of the airway film [254]. It is non-immunogenic and plays an important role in host defense of the lung, specifically surfactant-associated proteins (SP-A and SP-D) bind bacteria and have antimicrobial properties *in vitro* [255]. To eliminate any antimicrobial activity against *P. aeruginosa*, as shown against other bacteria, we removed the surfactant-associated protein fraction and used the phospholipid portion as a carrier to aid in spreading and delivery of *P. aeruginosa*-laden beads to the murine airway.

A preliminary experiment with administration of dye showed that 1 hr post-administration by aspiration challenge with surfactant phospholipids and intratracheal injection both resulted in delivery primarily to the lung. Our main goal was to compare methods for delivery of *P. aeruginosa*-laden beads. Mice received $5x10^6$ cfu *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids or intratracheal injection and were sacrificed 3 days later. No significant difference in percentage weight loss was found when the groups were compared nor was there a difference in inflammatory cytokine levels (IL-1β, IL-6, TNF-α, KC, and MIP-1α) in the lung homogenate or BALF. Tissue sections revealed that the groups were pathologically similar in inflammation, infiltration, and lung consolidation, using a grading scale. Bacterial burden, as measured by serial dilutions of the lung homogenate, was not significantly different between administering *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids or intratracheal injection, although bacterial load was higher in these groups than in mice receiving sterile beads by intratracheal injection. None of the mice in the aspiration challenge with surfactant phospholipids group died as a result of the chronic *P. aeruginosa* administration; however, 3

mice in the intratracheal injection group died from surgical complications. Importantly, measurements of infection and inflammation from chronic *P. aeruginosa* administration by aspiration challenge with surfactant phospholipids were as reproducible as intratracheal injection when comparing the standard deviation between the groups.

There were several limitations to this study. We did not compare surfactant phospholipids to total surfactant for enhanced lung delivery or measure specific antimicrobial activity against *P. aeruginosa* due to the interaction of surfactant proteins and lipopolysaccharide (LPS) [255]. In an earlier experiment, we found no difference in *P. aeruginosa* growth in the presence or absence of surfactant phospholipids when plating in serial dilutions on TSA plates. Because intratracheal injection and aspiration challenge with surfactant phospholipids both resulted in delivery to the stomach, we did not quantify the expression level. Delivery to the stomach with aspiration challenge was a surprise and contradictory to other literature [248, 250]. A better marker to visualize lung distribution such as a viral vector with a reporter gene would have allowed for quantification and more clear definition of lung delivery. Our use of surfactant phospholipids did not result in enhanced lung delivery over intratracheal injection and may be due to removing the surfactant-associated proteins.

The non-invasive model of *P. aeruginosa*-laden bead administration described here results in the same infection and inflammation characterized by intratracheal administration of the bacteria, currently the most-employed method for creating CF lung disease *in vivo*. The technique could be especially beneficial for investigators working with CFTR knockout mice because they are generally more sensitive to surgical manipulations [246] strongly

impacting experimental outcomes associated with adverse reaction to the surgical instillation of *P. aeruginosa*-laden beads in the trachea.

Future directions include using this method of administration in CFTR knockout mice to determine if mortality is reduced and to compare dosing of *P. aeruginosa*-laden beads. It would also be interesting to administer free *P. aeruginosa* to determine if an acute model of infection could be developed with this administration method. Delivery of other agents such as drugs and gene transfer agents have been shown to be enhanced using surfactant as a carrier [256, 257] and could be used in our aspiration challenge model for non-invasive delivery and characterization of therapeutics for lung disease.

Chapter 4.

Non-invasive method of AAV vector administration with surfactant phospholipids results in successful vector distribution and transgene production in the murine lung.

4.1 Introduction

Gene transfer with recombinant adeno-associated virus (AAV)-based vectors has been studied extensively for diseases which impact the lung such as cystic fibrosis (CF) and alpha-1 antitrypsin (AAT) deficiency. Vectors derived from AAV can transduce dividing and non-dividing target cells [153] and provide prolonged gene expression in the host through episomal persistence or integration [154-157] following a single dose administration [143, 158]. In addition, AAV vectors deliver therapeutic genes without co-transfer of any viral genes and, therefore, are not likely to induce inflammatory responses [159-162]. AAV-based vectors have also been found safe in a number of animal models and human clinical trials [143, 155].

One of the current limitations for therapeutic gene delivery to the epithelia by AAV is the low level of transgene expression [22]. This can be influenced by a number of obstacles that impede or limit access of vectors to receptors or binding sites on epithelial cells following vector administration. Physical barriers such as mucins, surface fluid lining layers, glycoproteins, as well as the availability of cell entry receptors are present in the airway of normal lungs and often increased in diseased lungs [258].

The method of vector administration to the lungs can affect efficiency of delivery and lung-specific transgene expression. While therapeutic efficiency of AAV vectors *in vivo* has been characterized by administration through intranasal [180, 190], aerosol [181], intrapleural [259], and intratracheal [204, 260, 261] routes for delivery to the lungs, the

optimal method of AAV vector delivery to the lungs has not been determined. The most commonly-used approach is catheter or bronchioscopic-directed vector delivery, but this method requires surgical instillation. However during surgical instillation, the addition of perfluorochemical (PFC) liquids, chemically inert fluorinate carbon chains with high solubility for O_2 and CO_2, has been shown to enhance lung transgene expression in the distal airway and alveolar epithelium of experimental animals when co-instilled with adenoviral [252, 262-264] and AAV vectors [262, 265], requiring less vector for efficient transduction. Properties of PFC liquids, such as high density and low surface tension, aid in widespread distribution throughout the lungs following intratracheal administration [251]. Surfactant, a mixture of phospholipids and surfactant-associated (SA) proteins, also has the capacity to lower surface tension and has been shown to aid in a uniform distribution pattern in the lungs when used as a carrier following intratracheal delivery in hamsters [251]. When compared, PFC liquids and surfactant have been found to aid in similar distribution of intratracheally injected adenoviral vectors throughout the rat lung [252].

We were interested in developing a murine model of non-invasive lung delivery for AAV vectors. While intratracheal delivery has been superior to nebulized delivery of surfactant [266], administration by aspiration challenge has not been evaluated for surfactant. In our laboratory we observed that delivery of *P. aeruginosa*-laden beads with surfactant phospholipids through aspiration challenge results in the same chronic lung disease as administration of *P. aeruginosa*-laden beads by intratracheal injection.

The present study provides a strategy for non-invasive AAV delivery to the murine lung using surfactant phospholipids as a vehicle. We hypothesized that aspiration challenge with surfactant phospholipids will result in AAV distribution and gene expression in the

lungs similar or enhanced to intratracheal injection. We used an AAV5 pseudotyped vector because this serotype results in higher levels of transduction and gene expression when compared to other serotypes due to the prevalence in its primary receptor, N-linked sialic acid (N-SA), in apical airway epithelia [188, 204]. We compared aspiration challenge with surfactant phospholipids in lung delivery and protein expression to intratracheal injection to determine the effectiveness of this administration method for *in vivo* characterization of AAV-based therapeutics.

4.2 Materials and Methods

Mice

Eight-week old C57BL/6 mice were purchased from the Charles River Laboratories, Inc (Wilmington, MA). Mice were housed in a specific pathogen-free facility at the Medical University of South Carolina (MUSC). The experimental protocols were approved by the Institutional Animal Use and Care Committee (IACUC) at MUSC.

Recombinant AAV-α1 anti-trypsin vector

A recombinant AAV vector carrying alpha-1 anti-trypsin (AAT) was used for these studies. AAV.Cβ-AAT (see **Figure 4.1**) contains AAV2 inverted terminal repeats (ITRs), a CMV/chicken-beta-actin hybrid (Cβ) promoter including an intron, a full length human alpha-1 anti-trypsin (AAT) sequence, and the SV40 polyadenylation region. AAV.Cβ-AAT was cloned, pseudotyped with the AAV5 capsid, and titered using previously published methods [167, 243]. The pseudotyped packaged vector was then designated as AAV5.Cβ-AAT.

Figure 4.1

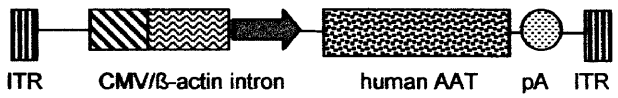


Figure 4.1 Schematic of the AAV5.Cβ-AAT vector. AAV5.Cβ-AAT contains the CMV/chicken-beta-actin hybrid promoter including an intron, the human AAT coding sequence, and the SV40 polyadenylation region. Adapted from [204].

Surfactant phospholipid liposomes preparation

DPPC and POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine) phospholipids were purchased (Avanti Polar Lipids, Alabaster, AL) with stock concentrations of 20 mg/ml (DPPC in chloroform) and 10 mg/ml (POPC in chloroform). For every ml of final liposome suspension, 17.5 mg DPPC (0.875 ml stock) and 7.5 mg POPC (0.75 ml POPC stock) were added in a clean, sterile 13x100 mm glass tube. The tube was capped and mixed well via vortex. The solvent was removed via nitrogen stream, with the tube submerged in 40°C water bath, and mixed during the drying process. After the samples were dry, the tubes were transferred to a lyophylization flask and vacuum-dried overnight without additional heating. PBS (1.0 ml) was added to each tube, and then the tubes were heated at 45°C for 15 min, vortexed for 30 sec, and sonicated for 10 sec. This step was repeated 3 times to ensure the liposome preparation had consistent viscosity and did not contain large liposome aggregates. The liposomes were transferred to sterile screw-cap plastic cryovials using sterile pipette tips and stored at 4°C. The stock phospholipid liposome concentration was 7:3 (mol/mol) DPPC/POPC at 25 mg/ml total phospholipid. On the day C57BL/6 mice were infected, the phospholipid liposomes were diluted to 4 mg/ml with AAV5.Cβ-AAT.

Labeling of AAV vector with fluorophore dye

AAV5.Cβ-AAT was incubated with reactive dye solution (10mg/ml Alexa 610 succinimidyl ester fluorophore dissolved in DMSO, Invitrogen) with continuous stirring at room temperature for 1 hr. A Sephadex G column was used to separate the AAV5.Cβ-AAT

labeled with Alexa610 from the unreacted labeling reagent. The labeled AAV vector denoted AAV5.Cβ-AAT*Alexa610 was stored at 4°C.

Intratracheal injection

Mice were anesthetized through inhalation of a mixture of oxygen and isoflurane in a closed chamber, then transferred and immobilized in a supine position on a surgical table. During the procedure, mice were under continuous anesthesia with isoflurane through a conical nosepiece. A small transverse incision was made (about 0.5 mm) just cranial to the thoracic inlet followed by blunt dissection of the salivary glands and musculature to expose the trachea. A sterile, flexible 21-gauge SURFLO winged infusion set (Terumo Corporation, Tokyo, Japan) was inserted just into the trachea between the tracheal rings for delivery to both lobes of the lung. A 1-ml syringe pre-loaded with 0.050 ml containing PBS or 4mg/ml surfactant phospholipid liposomes with $1x10^{12}$ or $2x10^{12}$ vector genomes (vg) of AAV vector was attached to the winged infusion set, and the plunger depressed for injection into the trachea. The attached set was removed and incision closed by suture. Mice were monitored for recovery which occurred within 10 min of procedure completion. All incisions were closed by suture.

Aspiration challenge with surfactant phospholipids

Aspiration challenge was conducted using previously published methods [248, 249] with minor modifications. Mice were anesthetized through inhalation of a mixture of oxygen and isoflurane for 4 min in a closed chamber. Mice were removed from anesthetization chamber and mice suspended by their front incisors in a supine position on an acrylic

platform at a 60° angle. Using coated forceps, the tongue was extended and moved aside from blocking the trachea. Using a p200 pipette, 0.040 ml containing $1x10^{12}$ or $2x10^{12}$ vg of AAV vector was instilled at the back of the oral cavity above the tracheal opening. Immediately after, the mice aspirated the liquid, evidenced by the mouse gulping. Mice were monitored for recovery which occurred within 5 min after the administration.

Analysis of AAV vector distribution

Two hr after $1x10^{12}$ vg of AAV5.Cβ-AAT*Alexa610 was administered; mice were anesthetized with isoflurane and sacrificed by cervical dislocation. To determine whole organ distribution, the lungs, heart, and spleen were removed and visualized under UV light. For cellular distribution, the lungs were inflated and fixed with 4% paraformaldehyde. After 48 hr, the lungs were embedded in paraffin, cut in 150-200 μm step-sections, and viewed microscope with a Rhodamine filter.

Analysis of AAV-based protein expression

Sacrifice and sample collection

Six weeks following $2x10^{12}$ vg of AAV5.Cβ-AAT administration, mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Blood was obtained from all mice. Lungs were collected and placed on ice. The same day of collection, the lungs were weighed (OHAUS Adventurer balance, 1.000 g sensitivity) and washed in 0.5 ml cell wash buffer (Bio-Rad, Hercules, CA). Using surgical scissors, lungs were cut into small pieces, 2 ml of PBS was added, and the lungs were homogenized for 60 seconds at high speed using Kinematica Polytron PT10/35 homogenizer (Brinkmann Instruments Co., Westbury, NY).

The homogenized lungs were further processed by freezing at -80°C, thawing on ice, and sonicating in ice-cold water for 45 sec. The processed lung homogenates were centrifuged at 1500xg at 4°C for 10 min, supernatants collected, and stored at -80°C.

AAT ELISA

Human AAT expression in sera and lung homogenate was measured by enzyme-linked immunosorbent assay (ELISA). The plate was coated with goat anti-hAAT (Sigma) in Voller's buffer (2.76g Na_2CO_3, 1.916g $NaHCO_3$, 0.2g NaN_3 in 1 liter, pH 9.6). After washing the plate in PBS-Tween (PBS-T) 3 times, the serial dilution of standard AAT and samples (mice sera or lung homogenate) were added to the wells and incubated at 37°C for 60 min. The plate was washed and blocked by Bovine Serum Albumin (3% BSA in PBS-T) solution for 60 min at 37°C. After washing, the plate was incubated with 1:1000 dilution of rabbit anti-hAAT (Sigma) for 60 min at 37°C. Then, the plate was washed and incubated with 1:2500 dilution of goat anti-rabbit IgG conjugated HRP (Sigma) for 60 min at 37°C. The plate was washed and incubated with 100 µl of TMB substrate (Research Diagnostics Inc., Flanders, NJ) for 5 min. Then, 100 µl of 0.5N HCl was added to stop the reaction. The plate was read at 450 nm using a VERSAmax tunable microplate reader (Molecular Devices).

Statistical analysis

Data are presented as average ± SD. Statistical analysis of AAT levels in the lung homogenate was determined by unpaired, two-tailed Student's t test, and statistical analysis of AAT levels in the serum was determined by unpaired, two-tailed Student's t test with Welch correction using the Graph Pad InStat statistics software program (GraphPad

Software, San Diego, CA). For all data sets, p values less than 0.05 were determined to be statistically significant.

4.3 Results

Administration by aspiration challenge with surfactant phospholipids and intratracheal injection results in similar vector distribution.

AAV5.Cβ-AAT was labeled with an Alexa 610 probe to compare the delivery location between the two methods of administration. Anesthetized mice received $1x10^{12}$ vg of AAV5.Cβ-AAT*Alexa610 through aspiration challenge with surfactant phospholipids or intratracheal injection. After 2 hr, mice were sacrificed and lungs, stomach, and spleen collected. Aspiration challenge with surfactant phospholipids and intratracheal injection resulted in similar distribution throughout the lungs with some delivery to the stomach and no delivery to the spleen (**Figure 4.2**). AAV5.Cβ-AAT*Alexa610 was distributed to the bronchial epithelial cells (**Figure 4.3A**) and alveoli (**Figure 4.3B**) as defined by sectioning of lung tissue. Similar distribution of the vector in both aspiration challenge with surfactant phospholipids and the intratracheal injection occurred. The experiment was repeated and the data shown representative of 4 or more animals.

Figure 4.2

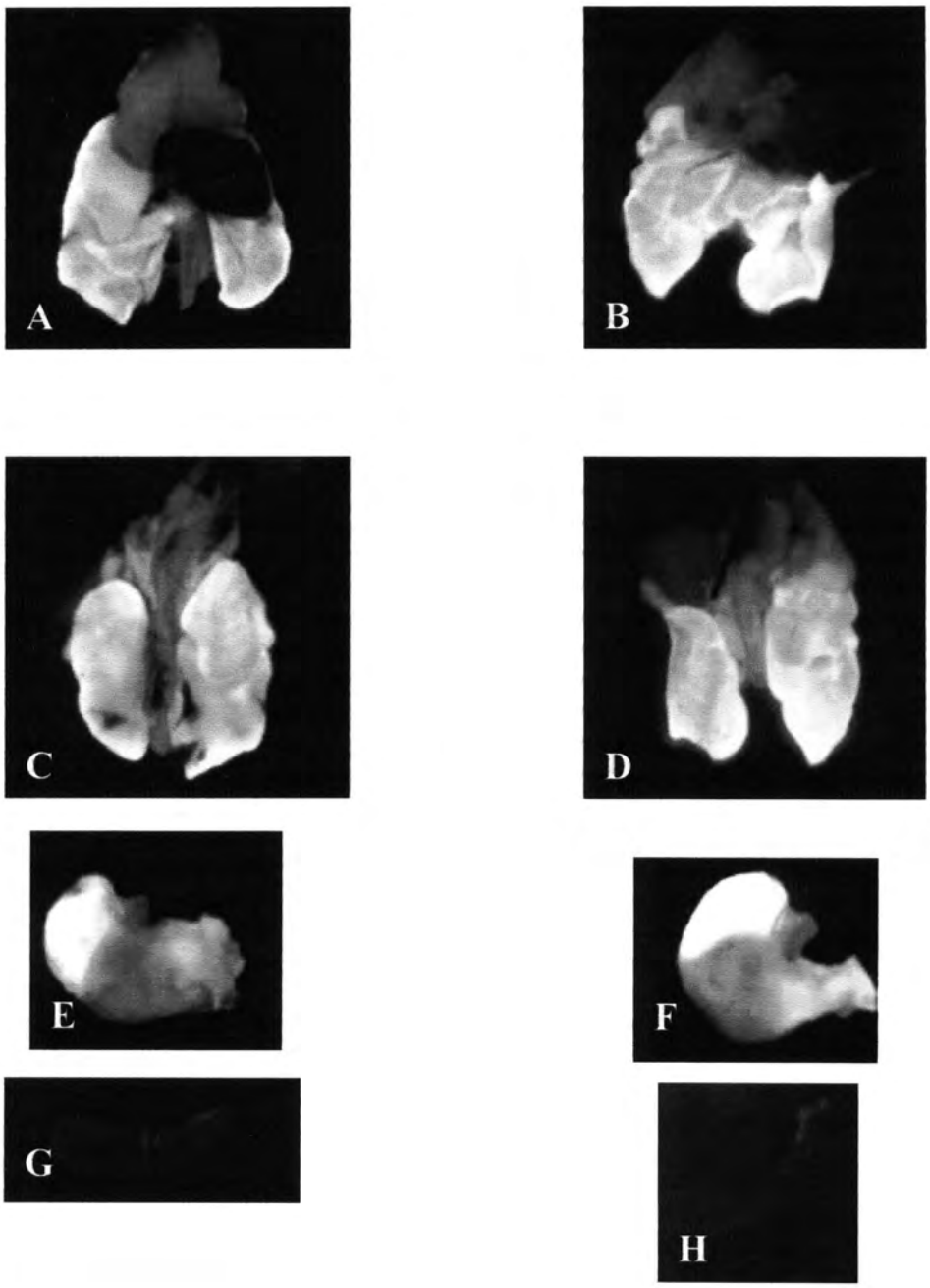


Figure 4.2 **AAV5.Cβ-AAT*Alexa610 is distributed throughout the lungs after administration by aspiration challenge with surfactant phospholipids and intratracheal injection.** Anesthetized mice received $1x10^{12}$ vg of AAV5.Cβ-AAT*Alexa610 through aspiration challenge with surfactant phospholipids or intratracheal injection. After 2 hr, mice were sacrificed and lungs, stomach, and spleen collected. Administration of AAV5.Cβ-AAT*Alexa610 by aspiration challenge with surfactant phospholipids **(left column)** or intratracheal injection **(right column)** predominantly distributed to the frontal **(A, B)** and dorsal **(C, D)** lung, with some expression in the stomach **(E, F)**, and no expression in the spleen **(G, H)** as viewed under ultraviolet light. All observations represent 4 or more animals from 2 experiments.

Figure 4.3A

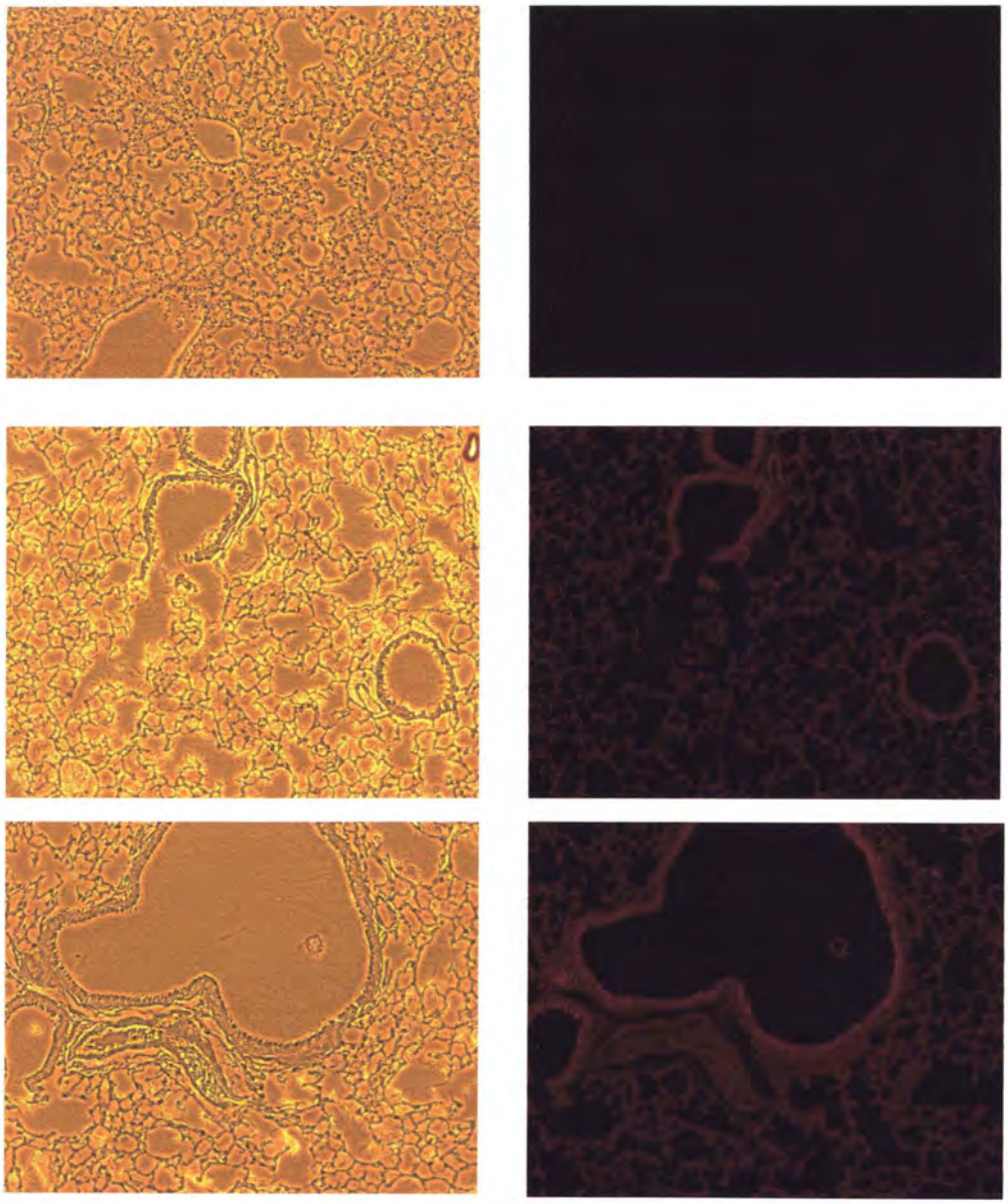

No AAV
Aspiration challenge with surfactant phospholipids
Intratracheal injection

Figure 4.3B

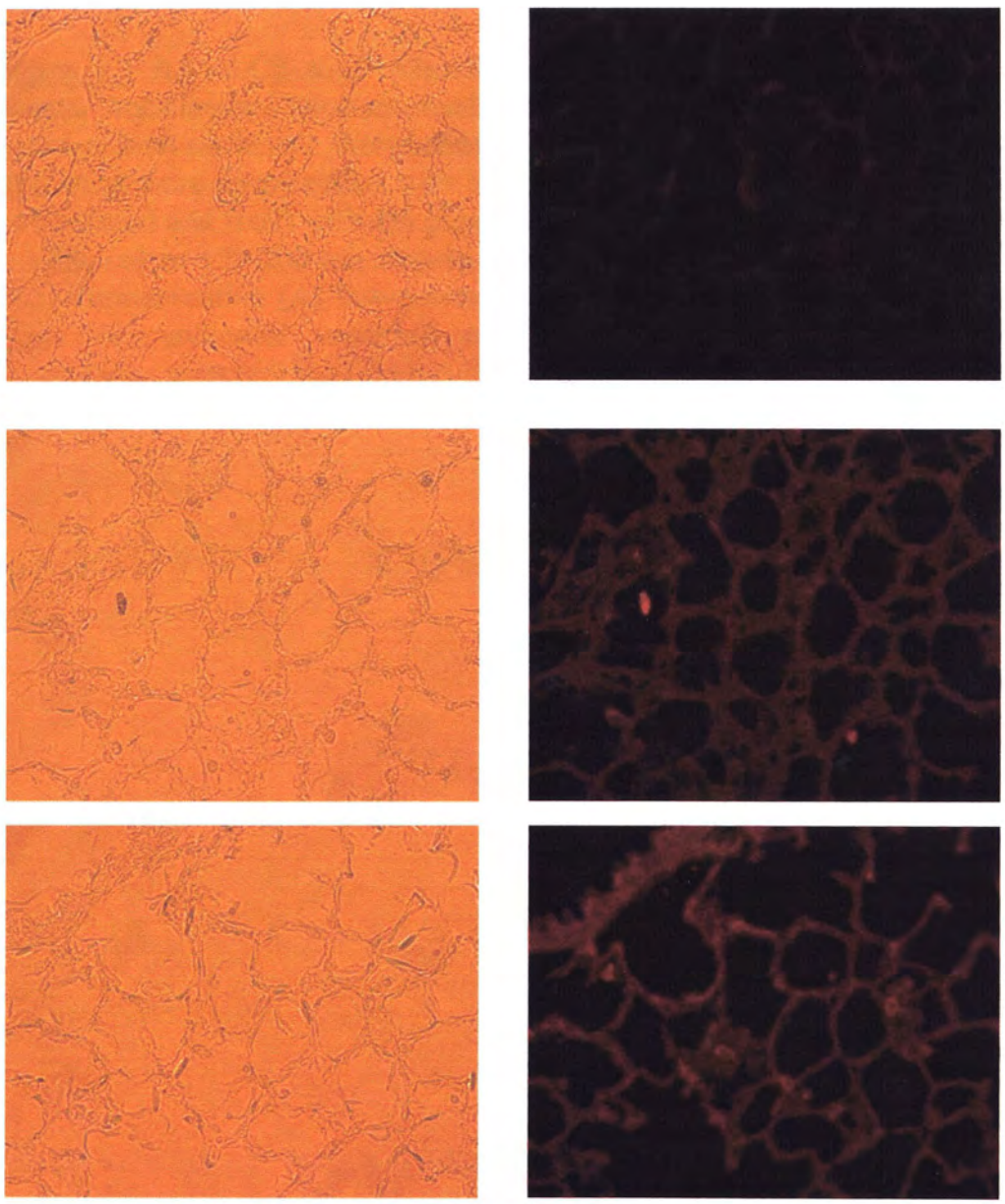

No AAV
Aspiration challenge with surfactant phospholipids
Intratracheal injection

Figure 4.3 AAV5.Cβ-AAT*Alexa610 has similar localization in both administration groups. Anesthetized mice received $1x10^{12}$ vg of AAV5.Cβ-AAT*Alexa610 by aspiration challenge with surfactant phospholipids or intratracheal injection, or were not treated. Two hr later, mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Lungs were inflated and fixed with 4% paraformaldehyde. After 48 hr, the lungs were embedded in paraffin, cut in 150-200 um step-sections, and microscopically viewed under a Rhodamine filter. Representative **(A)** low-power views (x10) and **(B)** higher-power views (x40) of the lungs after indicated treatment are shown. Untreated mice received no treatment. Observations represent the findings from 3 or more animals from 2 experiments.

Delivery of AAV5.Cβ-AAT by aspiration challenge with surfactant phospholipids results in the same protein expression levels as intratracheal delivery.

A second study was conducted to ascertain the similarity of AAV-mediated transgene expression by measuring gene transfer and protein production of AAT. AAT, a secreted protein, is easily detected in the serum and lung. Mice received $2x10^{12}$ vg of AAV5.Cβ-AAT by aspiration challenge with surfactant phospholipids (n=7) or intratracheal injection (n=7), and were sacrificed six weeks later. In the lung homogenate, AAT levels in the aspiration challenge with surfactant phospholipids group (47 $\pm$ 6 ng/ml) were not significantly different from those in the intratracheal injection group (43 $\pm$ 4 ng/ml) (**Figure 4.4**). Similar expression of AAT between the groups was also detected in the serum (**Figure 4.4**).

Figure 4.4

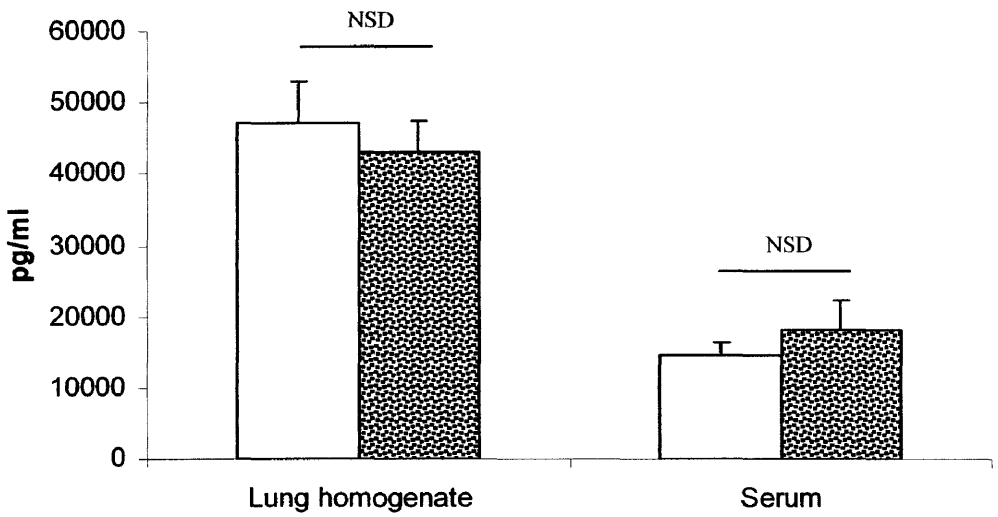

60000
50000
40000
30000
20000
10000
0
pg/ml
NSD
NSD
Lung homogenate
Serum

Figure 4.4 AAV5.Cβ-AAT produces AAT levels that are not significantly different in the lungs and serum in the administration groups. Anesthetized mice received $2x10^{12}$ vg of AAV5.Cβ-AAT by aspiration challenge with surfactant phospholipids or intratracheal injection. Mice were sacrificed 6 weeks later and blood collected. Lungs also were collected and homogenized. AAT protein levels were measured by administration of the vector aspiration challenge with surfactant phospholipids (□) or intratracheal injection (▩) in the serum and lung homogenate. NSD is not significantly different ($p>0.05$). Data shown are the average ± standard deviation for 7 mice in each group.

4.4 Discussion

Therapeutic gene transfer has been tested successfully in experimental models of chronic lung disease. These studies suggest the promise of gene therapy for lung diseases such as CF and AAT deficiency; however, gene transfer efficiency is limited by factors including host defenses and distribution of cell entry receptors. Circumventing these barriers and enhancing gene transfer will positively impact the use of these vectors as therapeutics. The small diameters of the airways in the periphery of the lung inhibit the movement of fluids with high surface tension, requiring high pressure for passage of these liquids in the airway. Surfactant, a mixture of phospholipids and SA proteins, lowers the surface tension resulting in rapid dispersion, beneficial for lung gene transfer. Use of surfactant as a vehicle has been shown to improve dispersion of adenoviral and AAV vectors in the lungs of rats [262, 265]. In these studies, minimal transgene expression was observed in the trachea and main bronchi, suggesting surfactant aids in distribution to the bronchial epithelium and alveoli.

For the first time, the current study compared the effectiveness of a non-invasive method (aspiration challenge) of AAV vector administration with surfactant phospholipids to intratracheal injection of AAV vector, the most-employed method of lung delivery in experimental models. We demonstrated that the use of surfactant phospholipids during aspiration challenge of AAV vector results in similar lung distribution and total transgene activity in the serum and lung homogenate when compared to intratracheal injection. Delivery was predominantly to the lung specifically to the columnar epithelium of the bronchioles and alveolar cells as was seen in a previous study [252]. Some expression was

observed in the stomach, but no expression in the spleen. AAT protein expression revealed high levels of transgene expression in the serum and lungs when delivered by both methods.

Our hypothesis that aspiration challenge with surfactant phospholipids would result in similar lung delivery to intratracheal injection was proven, but we did not observe the enhanced gene expression that was seen in a previous study [252]. This could be due to the removal of surfactant-associated (SA) proteins from our vehicle, which may further aid in peripheral spreading and clearance of the virus to the distal airway; however, we did not compare aspiration challenge with surfactant phospholipids to intratracheal injection with surfactant, the method of enhanced gene delivery used in this study.

There were several limitations to our experiments. There was no quantification of percentage AAV vector delivery to the stomach, although the expression level was similar to intratracheal injection. We did not employ a control of unlabeled AAV vector to determine the background of AAV vector dissemination, but AAV vector auto-fluorescence has not been reported. There was no comparison of total surfactant to surfactant phospholipids for AAV delivery. Removal of the SA fraction may not be necessary for antimicrobial reasons as it was not shown to impact adenoviral titer, but may aid in proximal lung delivery (a more appropriate target for CF) as previous studies demonstrated delivery was exclusively to the alveoli [252]. Vector delivery with surfactant phospholipids was localized to the peripheral (bronchioles) and distal lung (alveoli), but we did not determine which cells were transduced. This will be important to determine because surfactant is metabolized by type 2 alveolar epithelial cells, which raises the possibility that co-transport of AAV and surfactant into these cells may be a mechanism for uptake [252].

This study supports the use of surfactant as a carrier for non-invasive lung delivery of gene transfer agents in animal models. This administration method will be especially useful for researchers working with chronic infection models for CF lung disease. Current *in vivo* assessment of gene transfer therapeutics for CF relies on multiple surgeries for instillation of *P. aeruginosa*-laden beads and the therapeutic. Mortality in CFTR-targeted mice increases with each surgery and having a non-invasive method for both administrations is beneficial for mouse numbers as well as obtaining relevant inflammation data from the therapeutic agent, not influenced by surgical manipulation. In addition, the use of surfactant allowed for lower doses of adenovirus and AAV to be used in a previous study [252]. Obtaining high titers of AAV vectors is difficult and conducting a dosing study with AAV is a logical future study to increase the application of this vector as a gene transfer agent.

Chapter 5.

Adeno-associated vector delivery of IL-10 to the lungs of cystic fibrosis mice reduces inflammation in a model of chronic *P. aeruginosa* infection.

5.1 Introduction

Cystic fibrosis is the most common lethal autosomal recessive disease in the Caucasian population. In CF, dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) in airway epithelial cells and submucosal glands results in chronic disease of the respiratory tract, characterized by airway obstruction and persistent infections of the lung and sinuses, beginning early in life [85, 86]. The CF lung is particularly susceptible to infection with *Pseudomonas aeruginosa*, which plays an important role in development and progression of pulmonary disease in patients with CF [83, 84]. Almost all patients succumb to progressive lung disease associated with chronic infection of this organism due to an excessive, predominantly neutrophilic, inflammatory response that is more destructive than protective and causes the majority of tissue injury.

Patients with CF have elevated proinflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) and a negligible amount of anti-inflammatory cytokine, IL-10, in their bronchoalveolar lavage fluid (BALF) as compared to healthy subjects [26, 28]. In addition, airway epithelia cells and T lymphocyte clones from patients with CF are deficient in IL-10 production when compared to healthy controls. IL-10 inhibits production of IL-1β, IL-6, IL-8, and TNF-α and its constitutive expression in the lungs of healthy individuals may be responsible for maintaining a balance that inhibits inflammation. Likewise, a pathologic imbalance of inflammation may result from decreased or absent levels of IL-10. Deficiency in IL-10 production, as shown by studies

to greater neutrophil recruitment, more severe weight loss, and increased areas of lung inflammation inflammation than seen in wild-type mice when chronically infected with endobronchial *P. aeruginosa* [37, 139, 225]. These findings are similar to those of *P. aeruginosa*-infected CF mice [37, 215, 220, 226]. When repeatedly exposed to mucoid *P. aeruginosa*, IL-10T mice exhibit a prolonged inflammatory response to acute *P. aeruginosa* challenge [237] and have higher mortality rates and more severe lung pathology when compared to C57BL/6 controls similarly infected [216]. In acute and chronic infection models with *P. aeruginosa*, treatment with IL-10 has been shown to reduce weight loss and proinflammatory cytokine production, leukocyte and neutrophil recruitment, and tissue injury in the lungs [139, 141]. While the administration of exogenous IL-10 has been shown to reduce inflammation, over-expression of IL-10 could suppress normal host defenses and be detrimental, mediating systemic immunosuppression or even becoming immunostimulatory at high systemic doses [244]. Our laboratory demonstrated that gene transfer with IL-10 using an adeno-associated vector (AAV5.Cβ-mIL10) reduced pro-inflammatory cytokine production and lung pathology in IL-10T mice chronically infected with *P. aeruginosa*, further suggesting the importance of IL-10 in preventing excessive activation of the inflammatory cascade. While systemic administration of recombinant IL-10 was found to attenuate both local and systemic cytokine levels [139], our studies with AAV5.Cβ-mIL10 resulted in lung-specific IL-10 expression with no effect on systemic proinflammatory cytokines or IL-10 production compared to placebo-treated mice.

Anti-inflammatory therapy using prednisone and ibuprofen in patients with CF has been successful, providing evidence that the inflammatory response in the CF lung is

excessive relative to the burden of bacterial infection [101, 103]. In CFTR knockout mice that were infected with *P. aeruginosa*, increased local concentrations of proinflammatory mediators, more severe weight loss, and increased mortality resulted compared to their wild-type counterparts, but there was no increase in bacterial burden [220]. These results further suggest that inflammation is dysregulated and excessive to bacterial infection in the CF lung. The reduction in inflammation mediated by IL-10 is similar to corticosteroids and ibuprofen, suggesting that IL-10 may have similar benefit in patients with CF.

Because decreased IL-10 levels may contribute to high levels of proinflammatory cytokine production in CF, we hypothesized that administration of IL-10 as a therapeutic would ameliorate the excessive response. In a chronic infection model of *P. aeruginosa* in IL-10T mice, we previously characterized AAV5.Cβ-mIL10 based on the inherent benefits of IL-10 gene transfer from AAV: long-term gene expression, a proven safety profile and the ability to elicit a minimal inflammatory response in comparison to other gene transfer agents [17, 19, 143, 155, 158, 182, 240]. Additionally, we optimized lung delivery of *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids in CD-1 mice to create a model of chronic infection using a non-invasive technique. In CD-1 mice, a dose of $5x10^6$ colony forming units (cfu) was used for *P. aeruginosa*-laden bead administration by aspiration challenge with surfactant phospholipids. CFTR knockout mice are generally more susceptible to *P. aeruginosa* infection, associated with higher mortality [220, 224]; in this report, we conduct dosing studies of *P. aeruginosa*-laden beads to determine the optimal dosage for inflammation in gut-corrected CFTR knockout mice. Then, we characterize AAV5.Cβ-mIL10 in a gut-

corrected CFTR knockout mouse model of chronic *P. aeruginosa* lung infection to determine whether IL-10 gene delivery and expression specifically reduces the proinflammatory response in the lung.

5.2 Materials and Methods

Mice

Breeding pairs of stock CFTR S489X(-/-); FABP-hCFTR(+/+), denoted gut-corrected CFTR knockout mice, were received from the University of Florida, originally obtained from The Jackson Laboratory (Bar Harbor, MN). These mice were bred at the Medical University of South Carolina in a specific pathogen-free facility. All caging supplies were cleaned with an automated cage wash and autoclaved prior to use in animal rooms. Mice were fed autoclaved or irradiated Harlan Teklad Sterilizable Rodent Blox 8656 (Harland Teklad, Indianapolis, IN) and bedded on autoclaved 1/8 inch corn cob bedding and corn husk nesting material (Harland Teklad, Indianapolis, IN). Mice were housed, 2-4, in Micro-Isolator Top Flow Ventilated cages (Tecniplast, Exton, PA) in ventilated racks. Mice were placed in fresh microisolator cages immediately after instillation of AAV or *P. aeruginosa*-laden agarose beads. Gut-corrected CFTR knockout mice used in these studies ranged from age 10 wks to 24 wks for *P. aeruginosa* dosing experiments. At the onset of the AAV5.Cβ-mIL10 experimental studies, the mice ranged from age 8 wks to 15wks with an average age of 10.6 $\pm$ 2.5 wks. The procedures were approved by the Institutional Animal Use and Care Committee (IACUC) at MUSC.

Surfactant phospholipid liposomes preparation

DPPC and POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine) phospholipids were purchased (Avanti Polar Lipids, Alabaster, AL) with stock concentrations of 20 mg/ml (DPPC in chloroform) and 10 mg/ml (POPC in chloroform). For every ml of final liposome suspension, 17.5 mg DPPC (0.875 ml stock) and 7.5 mg POPC (0.75 ml POPC stock) were added in a clean, sterile 13x100 mm glass tube. The tube was capped and mixed well via vortex. The solvent was removed via nitrogen stream, with the tube submerged in 40°C water bath, and mixed during the drying process. After the samples were dry, the tubes were transferred to a lyophylization flask and vacuum-dried overnight without additional heating. PBS (1.0 ml) was added to each tube, and then the tubes were heated at 45°C for 15 min, vortexed for 30 sec, and sonicated for 10 sec. This step was repeated 3 times to ensure the liposome preparation had consistent viscosity and did not contain large liposome aggregates. The liposomes were transferred to sterile screw-cap plastic cryovials using sterile pipette tips and stored at 4°C. The stock phospholipid liposome concentration was 7:3 (mol/mol) DPPC/POPC at 25 mg/ml total phospholipid. For administration, a concentration of 4 mg/ml phospholipid liposomes was used.

Administration of AAV vector

A recombinant AAV vector carrying IL-10 was used for these studies. AAV.Cβ-mIL10 (see **Figure 5.1**) contains AAV-2 inverted terminal repeats (ITRs), a CMV/chicken-beta-actin hybrid (Cβ) promoter including an intron, a full length murine IL-10 sequence, and the SV40 polyadenylation region. AAV.Cβ-mIL10 was cloned,

pseudotyped with the AAV-5 capsid, and titered using previously published methods [167, 243]. The pseudotyped packaged vector was then designated as AAV5.Cβ-mIL10.

Aspiration challenge was conducted using previously published methods [248, 249] with minor modifications, including the addition of surfactant phospholipids. Mice were anesthetized through inhalation of a mixture of oxygen and isoflurane for 5 min in a closed chamber. Mice were removed from anesthetization chamber and suspended by their front incisors in a supine position on an acrylic platform at a 60^{o} angle. Coated forceps were used to extend the tongue from blocking the trachea. Using a p200 pipette, 0.040 ml containing PBS or 4mg/ml surfactant phospholipid liposomes with $8.21x10^{9}$ vg of AAV5.Cβ-mIL10 was instilled at the back of the oral cavity above the tracheal opening. Immediately after, the mice aspirated the liquid, evidenced by the mouse gulping. Mice were monitored for recovery which occurred within 5 min of the administration.

Figure 5.1

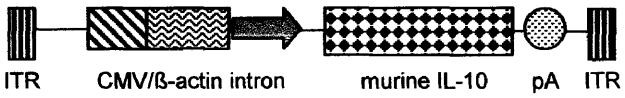


Figure 5.1 Schematic of the AAV5.Cβ-mIL10 vector. AAV5.Cβ-mIL10 contains the CMV/chicken-beta-actin hybrid promoter including an intron, the murine IL-10 coding sequence, and the SV40 polyadenylation region. Adapted from [243].

***Pseudomonas aeruginosa*-embedded bead preparation and infection**

The mucoid *Pseudomonas aeruginosa* isolate, PA 15719, was obtained from the sputum of a patient with CF using a protocol approved by the Institutional Review Board (IRB) at MUSC. Bacteria were maintained as glycerol stocks and stored at -80°C. Bacteria-impregnated agar beads were prepared as described by van Heeckeren et al [217, 220] with minor modifications. An inoculation loop was used to collect a sample of frozen mucoid *P. aeruginosa* stock, streaked for isolation on a tryptic soy agar (TSA) plate, and incubated at 37°C overnight. One colony was picked from the plate and used to inoculate 50 ml tryptic soy broth (TSB) in a 125 Erlenmeyer flask, which was placed, shaking, overnight at 37°C. A 0.1 ml aliquot of the culture was used to inoculate a second flask of 50 ml TSB in a 125 Erlenmeyer flask. Bacteria were grown to late log phase, shaking, in a 37°C incubator. A 2 ml aliquot from was added to warm (50°C) 2% low melting agarose (Type 1 low EEO; Sigma) in PBS. The bacteria-agar mixture was added to warm (50°C) heavy mineral oil (0122-4, Fisher) containing a stir bar, and stirred rapidly at room temperature for 6 min, followed by cooling with slow additions of ice over a 10-min period. The agar beads were transferred to a separatory funnel; the mineral oil removed, and washed one time with 0.5% sodium deoxycholate (SDC), one time with 0.25% SDC, and four times with PBS. The *P. aeruginosa*-laden agarose beads were collected and allowed to settle. Additional liquid was removed to obtain a final volume of 75% beads and homogenized using a Kinematica Polytron PT10/35 homogenizer for 4 min at high speed to obtain beads predominantly 100–120 μm in diameter. The size of the beads was measured microscopically before and after homogenization. Using serial dilutions, quantitative bacteriology was performed on an aliquot of the homogenized bead

slurry to determine bacterial concentration in colony forming units (cfu) per milliliter. The homogenized bead slurry was prepared the day before inoculation of the mice, stored at 4°C, and concentration of bacteria calculated on the day of inoculation.

Mice were infected with $1x10^5$ cfu *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids and monitored for recovery which occurred within 5 min of administration. Mice were sacrificed after 4 days.

Assessment of weight loss

Body weight was measured in all mice before PBS or AAV5.Cβ-mIL10 administration, before infection with *P. aeruginosa* (6 weeks later), and at time of sacrifice (4 days after infection). A record was made of any hunching, poor grooming, fatigue, or increased respiratory effort following inoculation with agarose beads. At 4 days following *P. aeruginosa*-laden bead inoculation, mice were anesthetized with isoflurane and sacrificed by cardiac puncture. Blood was obtained from all mice.

Bronchoalveolar lavage collection

Following sacrifice, bronchoalveolar lavage fluid (BALF) was collected. The trachea was isolated by blunt dissection and an incision made in the upper portion of the trachea, which was cannulated with a 21-gauge shielded I.V. catheter (BD Bioscience, San Hose, CA) attached to a 5 ml syringe. The alveoli were washed 3 times, each with 1 ml sterile PBS. Approximately 1.5-3ml of bronchoalveolar lavage fluid (BALF) was recovered and pooled.

BALF cell type count and percentages

The BALF samples were centrifuged at 2000 rpm for 10 min at 4°C, the supernatant transferred to a new tube, and stored at -80°C. The BALF cell pellet was resuspended in 1 ml PBS and a hemacytometer used to count the number of cells. A 500 µl aliquot of the cell suspension was transferred to a microscope slide using a cytospin (Shandon Cytospin® 4 Cytocentrifuge, Thermo Scientific, Waltham, MA) by centrifuging at 1000 rpm on medium speed for 5 min. The fluid on the slide was allowed to dry, then stained with hematoxylin and eosin (H&E) using standard techniques. A manual count of approximately 300 cells was performed, identifying the cell type as alveolar macrophages, neutrophils, or lymphocytes. The percentage of cells for each cell type was calculated and multiplied by the absolute cell count to get the total cells of that cell type in the BALF.

Lung histopathology

Lungs were inflated and fixed with 4% paraformaldehyde. After 48 hr, the lungs were embedded in paraffin, cut in 150-200 µm step-sections, and stained with H&E using standard techniques. Histopathologic scoring parameters were based on published criteria [217] with the following designations: 0=within normal limits; 1=darker red than normal; 2=0-2 lymphocytic infiltrated nodules present; 3=3-6 lymphocytic infiltrated nodules and <¼ lung consolidated; 4=¼ to ½ lung consolidated; and 5=½ to ¾ lung consolidated.

Lung homogenate collection, quantitative bacteriology, and cytokine analysis

Lungs were collected and placed on ice. The same day of sacrifice, the lungs were weighed (OHAUS Adventurer balance, 1.000 g sensitivity) and washed in 0.5 ml cell wash buffer (Bio-Rad, Hercules, CA). Using surgical scissors, lungs were cut into small pieces, 2 ml of PBS was added, and the lungs were homogenized for 60 seconds at high speed using Kinematica Polytron PT10/35 homogenizer (Brinkmann Instruments Co., Westbury, NY).

For quantitative bacteriology, the lung homogenate was diluted with PBS in ten-fold serial dilutions, spread on TSA plates, and incubated at 37°C overnight. Colonies per mouse lung were calculated. The spleens were also removed from mice whose lungs were designated for quantitative bacteriology, homogenized in 1 ml sterile PBS, and plated at a dilution of 1:100 to determine if bacteremia was present.

For cytokine analysis, the homogenized lungs were further processed by freezing at -80°C, thawing on ice, and sonicating in ice-cold water for 45 sec. The processed lung homogenates were centrifuged at 1500xg at 4°C for 10 min, supernatants collected, and stored at -80°C. Cytokine levels were measured using a customized Bio-Plex cytokine kit (Bio-Rad) for detection of IL-1β, IL-6, TNF-α, KC, and MIP-1α. Analysis was conducted using the Bio-Plex multiplex suspension array system (Bio-Rad) following the company's protocol. Each sample was performed in duplicate. Cytokine concentrations were standardized for lung weight by multiplying the cytokine value by the volume of sample (2ml) and dividing by the lung weight to obtain final values in picogram (pg) per gram of lung tissue.

Statistical analysis

Data are presented as average $\pm$ SD. Change in body weight is expressed as the percentage of weight lost from the original weight three days before inoculation. Statistical analysis of percentage weight loss, cytokine levels, H&E scoring, and differential cell count and percentages were determined by unpaired, two-tailed Student's t test using the Graph Pad InStat statistics software program (GraphPad Software, San Diego, CA). Welch correction was applied when standard deviations were significantly different. For all data sets, p values less than 0.05 were determined to be statistically significant.

5.3 Results

Gut-corrected CFTR knockout mice have high mortality and inflammatory cytokine production in dosing studies with *P. aeruginosa*-laden bead by aspiration challenge with surfactant phospholipids

Anesthetized mice were either infected with $5x10^5$, $1x10^6$, and $5x10^6$ cfu *P. aeruginosa*-laden beads or not infected and sacrificed 3 days later.

In mice infected with $5x10^5$ cfu *P. aeruginosa*-laden beads (n=6), the average pre-infection weight was 26.0 g with mice losing an average 27.1% of their body weight following infection (**Table 5.1**). There was 33.3% mortality at this dose. Mice receiving the $1x10^6$ cfu dose (n=12) had an average initial weight of 23.1 g, losing an average 26.5% of their body weight. There was also 33.3% mortality at this dose. The average pre-infection weight of the mice in the $5x10^6$ cfu dose group (n=4) was 23.4 g; all mice died 2-3 days after infection. Mice that were not infected with *P. aeruginosa* (n=4) had an average initial weight of 19.8 g with mice gaining an average body weight of 0.3%.

Proinflammatory cytokine levels in the lung homogenates were compared between mice infected with the $5x10^5$ and $1x10^6$ cfu *P. aeruginosa* dose (**Figure 5.2**). There was not a dose-dependent difference with cytokine levels; however, all cytokines in infected mice were greater than in uninfected mice (**Figure 5.2** and **Table 5.1**). These studies suggested that a higher dose of *P. aeruginosa* did not correlate with higher proinflammatory cytokines, which could be achieved with a lower dose of infection. Due to the high mortality (33.3%) associated with $5x10^5$ and $1x10^6$ cfu of *P. aeruginosa*-laden beads, we decided to perform our experimental studies with a lower dose of $1x10^5$ cfu.

Figure 5.2

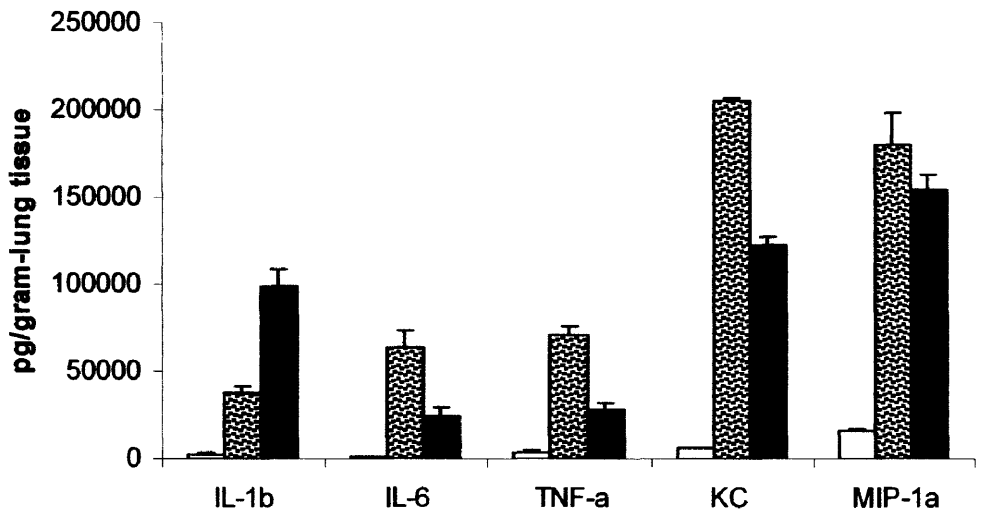

250000
200000
150000
100000
50000
0
pg/gram-lung tissue
IL-1b
IL-6
TNF-a
KC
MIP-1a

Figure 5.2 Higher dose of *P. aeruginosa*-laden beads does not correlate to higher pro-inflammatory cytokine levels in gut-corrected CFTR knockout mice.
Anesthetized mice were either not infected with *P. aeruginosa* or received $5x10^5$ or $1x10^6$ cfu *P. aeruginosa*-laden agarose beads by aspiration challenge with surfactant phospholipids. Mice were sacrificed 3 days later. Lungs were collected, homogenized, and cytokine levels measured. Levels of IL-1β, IL-6, TNF-α, KC, and MIP-1α were measured for uninfected mice (□) and mice infected with $5x10^5$ (▨) or $1x10^6$ (■) cfu *P. aeruginosa*-laden agarose beads. Data shown are the average ± standard deviation for 4 mice in the uninfected group and 2 mice in each of the infection groups and 8 mice in the intratracheal injection group from 2 experiments. Statistical analysis could not be performed due to the small sample sizes.

Table 5.1 Cumulative data from dosing study of *P. aeruginosa*-laden beads by aspiration challenge with surfactant phospholipids in gut-corrected CFTR knockout mice.

Parameter	*Uninfected*	*P. aeruginosa* dose (cfu)		
		$5x10^5$	$1x10^6$	$5x10^6$
Initial body weight (g)	19.8±0.6 (4)	26.0±2.2 (6)	23.1±3.2 (12)	23.4±2.0 (4)
Average percentage change in body weight[§]	-0.3%±2.5 (4)	27.1%±3.4 (4)	26.5%±2.9 (8)	NA
Mortality	0% (0/4)	33.3% (2/6)	33.3% (4/12)	100% (4/4)
Cytokine levels (ng-gram lung tissue)	(n=4)	(n=2)	(n=2)	
IL-1β	2.9±0.5	38.3±3.6	0.5±10.0	ND
IL-6	0.7±0.1	63.2±10.3	24.8±4.3	ND
TNF-α	4.0±0.8	70.2±5.0	28.2±3.6	ND
KC	5.5±0.3	204.4±1.1	121.4±5.7	ND
MIP-1α	15.7±1.4	178.7±18.6	153.9±8.8	ND

Data shown is the average ± standard deviation. Body weight data are combined from 2 different experiments with $5x10^5$ and $1x10^6$ cfu doses and 1 experiment with $5x10^6$ cfu and uninfected mice, which were censored by death. Anesthetized gut-corrected CFTR knockout mice were either not infected with *P. aeruginosa* or received $5x10^5$, $1x10^6$, or $5x10^6$ cfu *P. aeruginosa*-laden agarose beads by aspiration challenge with surfactant phospholipids. Mice were sacrificed 3 days later. Mice were weighed before infection and before sacrifice. Lungs were collected, homogenized, and cytokine levels measured. Statistical analysis could not be performed due to the small sample size. [§]Change in body weight is expressed as the average percentage change of weight lost from the original weight 3 days before infection.

AAV5.Cβ-mIL10 significantly reduces percentage weight loss in mice infected with *P. aeruginosa*-laden beads.

Gut-corrected CFTR knockout mice received $8x10^9$ vector genomes (vg) of AAV5.Cβ-mIL10 (n=17) or PBS (n=16) by aspiration challenge with surfactant phospholipids. Six weeks later, they were infected with $1x10^5$ cfu *P. aeruginosa*-laden beads administered by aspiration challenge with surfactant phospholipids. Additionally, there was a group of PBS (n=6) and AAV5.Cβ-mIL10 (n=6) that were not infected with *P. aeruginosa*. Mice were sacrificed after 4 days.

The first assessment for the therapeutic effect of IL-10 gene transfer was percentage weight loss. To ensure that AAV5.Cβ-mIL10 did not impact mouse weight, the mice were weighed before administration with AAV5.Cβ-mIL10 or PBS and 6 wks later before *P. aeruginosa*-laden bead infection. There was no significant difference in the percentage weight gain between the groups ($p>0.05$).

The mice were weighed a second time before administration with *P. aeruginosa*-laden beads and 4 days later before sacrifice (**Figure 5.3**). The average pre-infection weight of the mice receiving AAV5.Cβ-mIL10 (n=17) was 23.2 g with mice gaining an average of 0.3% of their body weight. Mice that were not infected with *P. aeruginosa* (n=6) had an average weight of 23.2 g, and a similar average weight gain of 2.0%. The average weight of the mice in the PBS group infected with *P. aeruginosa* (n=16) was 24.1 g with mice losing an average body weight of 5.7%.

There was no significant difference in the percentage weight loss between uninfected mice and infected mice that received AAV5.Cβ-mIL10 treatment ($p>0.05$)

while infected mice treated with PBS had a significant increase in percentage weight loss ($p<0.05$) (**Figure 5.3**). There was no mortality at either dose of *P. aeruginosa* infection.

Figure 5.3

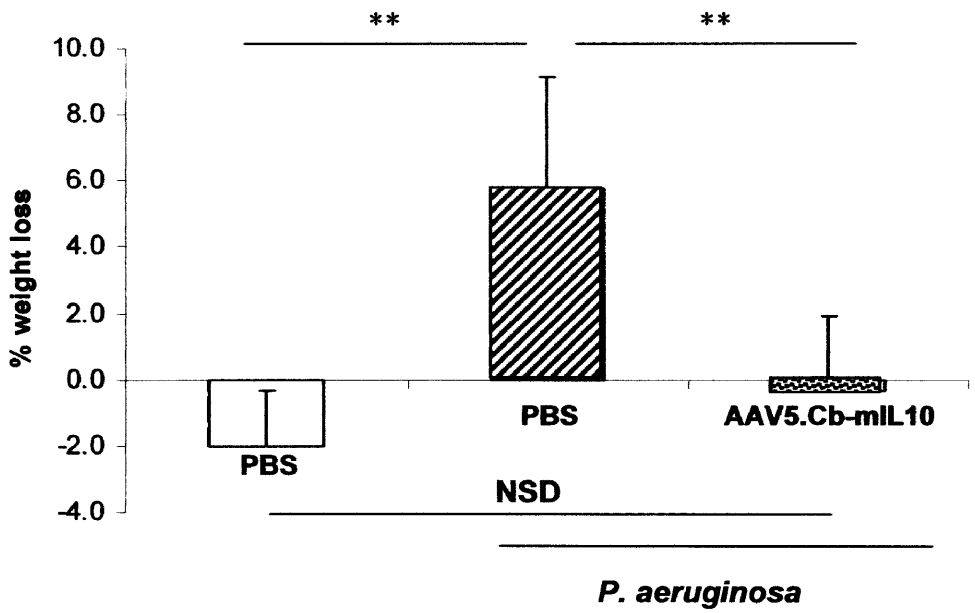

**
**
10.0
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
% weight loss
PBS
PBS
AAV5.Cb-mIL10
NSD
P. aeruginosa

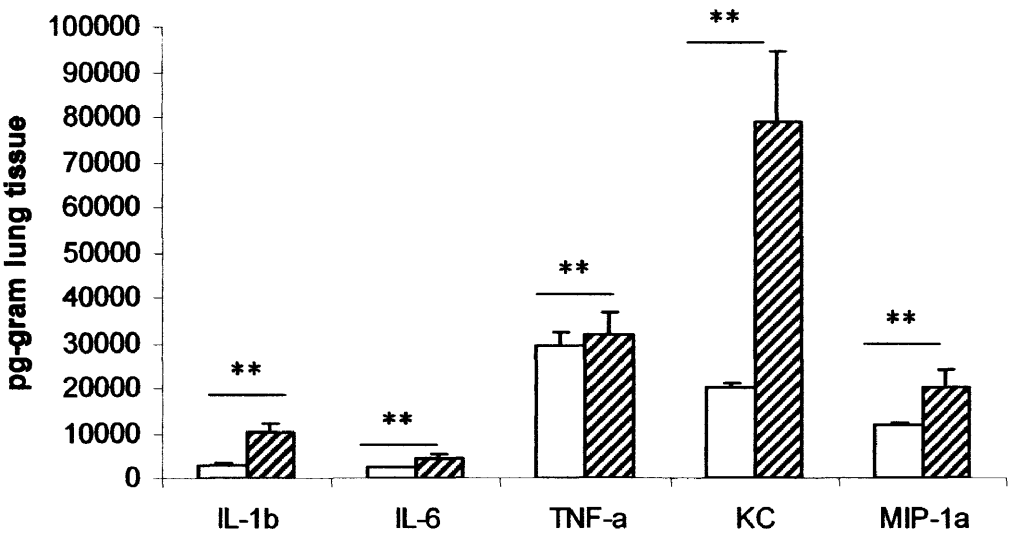
Figure 5.3 AAV5.Cβ-mIL10 treatment correlates with gain in percentage weight that is similar to uninfected mice. Anesthetized mice received PBS or AAV5.Cβ-mIL10 by aspiration challenge with surfactant phospholipids. After 6 weeks, AAV5.Cβ-mIL10-treated mice were infected with $1x10^5$ cfu *P. aeruginosa*-laden beads while PBS-treated mice were infected with *P. aeruginosa*-laden beads or not infected. Mice were sacrificed 4 days later. Change in body weight was obtained by the average percentage of weight lost from the original weight 4 days before inoculation for uninfected PBS-treated mice (□), PBS-treated mice infected with *P. aeruginosa* (▨), and AAV5.Cβ-mIL10-treated mice infected with *P. aeruginosa* (▩).**$p<0.005$ and NSD is not significantly different ($p>0.05$). Data shown are average ± standard deviation for 6 animals in the uninfected PBS-treated group, 16 animals in PBS-treated infected with *P. aeruginosa* group, and 17 animals in AAV5.Cβ-mIL10-treated infected with *P. aeruginosa* group from 1 experiment.

Inflammatory cytokines, but not IL-10, increase with *P. aeruginosa* infection.

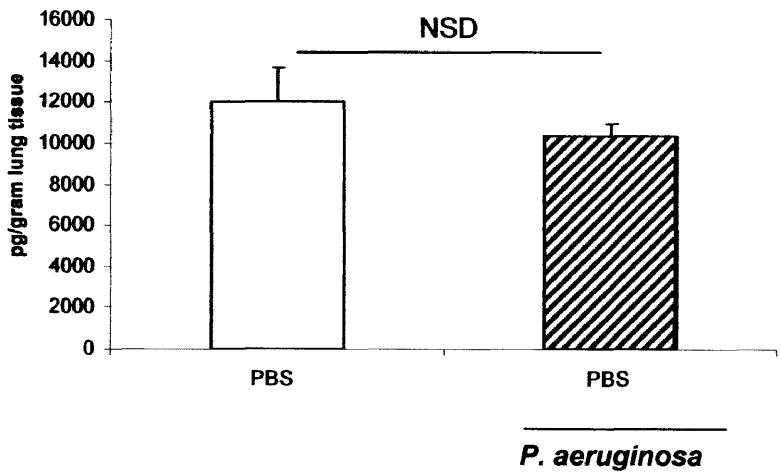
Pro-inflammatory cytokine expression with or without administration of *P. aeruginosa* was analyzed to determine the change in cytokines, including IL-10, with infection of gut-corrected CFTR knockout mice. Mice were inoculated with $1x10^5$ cfu *P. aeruginosa*-laden agarose beads by aspiration challenge with surfactant phospholipids or were not infected. Four days later, the mice were sacrificed, lungs collected, and cytokines levels measured in the lung homogenate.

Pro-inflammatory cytokine levels (IL-1β, IL-6, TNF-α, KC, and MIP-1α) in the lung homogenates of infected mice were significantly greater than the mice that were not infected (**Figure 5.4A**). However, the level of IL-10 with *P. aeruginosa* infection remained the same (average, 10,000 pg/gram-lung) when compared to IL-10 levels in the absence of infection (average, 12,000 pg/gram-lung) (**Figure 5.4B**).

Figure 5.4


100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
pg-gram lung tissue
**
**
**
**
**
IL-1b
IL-6
TNF-a
KC
MIP-1a

A.


16000
14000
12000
10000
8000
6000
4000
2000
0
pg/gram lung tissue
NSD
PBS
PBS
P. aeruginosa

B.

Figure 5.4 ***P. aeruginosa*** **infection causes a significant rise in lung homogenate inflammatory cytokine levels, but not IL-10.** Anesthetized mice received PBS by aspiration challenge with surfactant phospholipids. After 6 weeks, PBS-treated mice were infected with *P. aeruginosa*-laden beads or not infected. Mice were sacrificed 4 days later. Lungs were collected and homogenized. Cytokines **(A)** IL-1β, IL-6, TNF-α, KC, and MIP-1α and **B)** IL-10 were measured in uninfected (☐) and infected groups (▨). **$p<0.005$ and NSD is not significantly different ($p>0.05$). Data shown are the average ± standard deviation for 5 mice in each group from 1 experiment.

AAV5.Cβ-mIL10 produces significant levels of IL-10 protein in the lung.

IL-10 levels following AAV5.Cβ-mIL10 administration to the lungs of gut-corrected CFTR knockout mice infected with *P. aeruginosa* were measured to verify gene transfer and expression. Gut-corrected CFTR knockout mice were either not infected or infected with $1x10^5$ cfu *P. aeruginosa*-laden agarose beads by aspiration challenge with surfactant phospholipids. Four days later, the mice were sacrificed, lungs collected, and homogenized. Homogenized lungs were analyzed to determine IL-10 levels.

AAV5.Cβ-mIL10-treated mice produced IL-10 at 59,000 ng/gram of lung tissue (**Figure 5.5**). IL-10 production was also measured in the serum to determine whether production was systemic. Serum levels of IL-10 were very low (<30 pg/gram of lung tissue) and not significantly different in the treatment groups (data not shown), thus confirming localized secretion of IL-10 from the AAV5.Cβ-mIL10 vector.

Figure 5.5

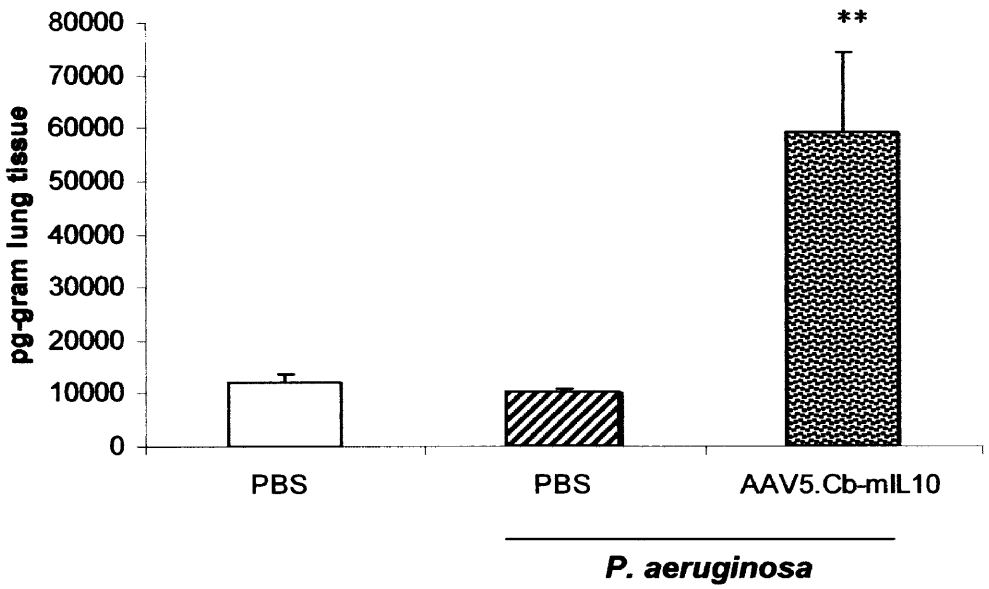

80000
70000
60000
50000
40000
30000
20000
10000
0
pg-gram lung tissue
**
PBS
PBS
AAV5.Cb-mIL10
P. aeruginosa

Figure 5.5 Successful administration of AAV5.Cβ-mIL10 to lung using aspiration challenge with surfactant phospholipids. Anesthetized mice received $8x10^9$ vg of AAV5.Cβ-mIL10 or PBS by aspiration challenge with surfactant phospholipids. After 6 weeks, AAV5.Cβ-mIL10-treated mice were infected with $5x10^6$ cfu *P. aeruginosa*-laden beads while PBS-treated mice were infected with *P. aeruginosa*-laden beads or not infected. Mice were sacrificed 4 days later and lungs collected. IL-10 protein levels were measured in the lung homogenate of PBS-treated uninfected mice (□), PBS-treated *P. aeruginosa*-infected mice (▨), and AAV5.Cβ-mIL10-treated *P. aeruginosa*-infected mice (▩). **$p<0.005$. Data shown are average ± standard deviation for 5 or more animals in each group from 1 experiment.

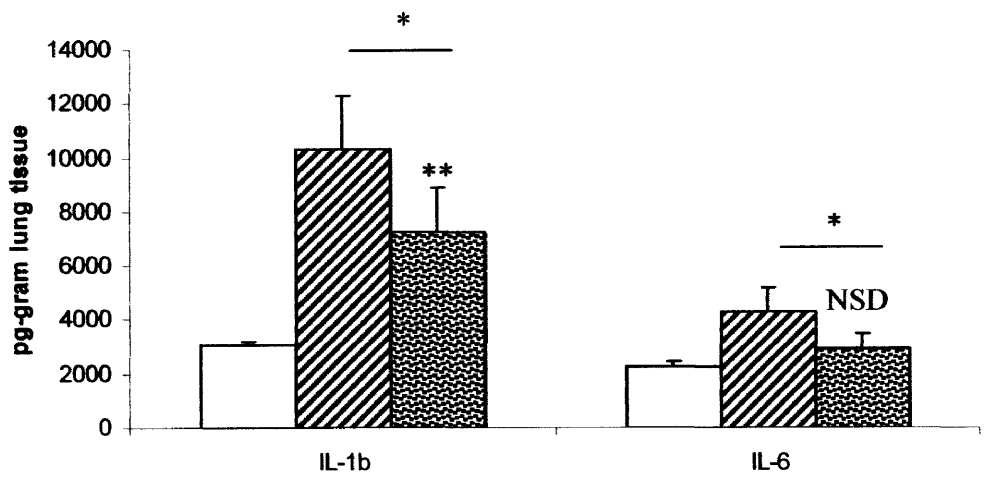
AAV5.Cβ-mIL10 decreases proinflammatory cytokine levels in the lungs of gut-corrected CFTR knockout mice.

The lung homogenates were also analyzed for pro-inflammatory cytokine levels following *P. aeruginosa* infection of gut-corrected CFTR knockout mice receiving AAV5.Cβ-mIL10 or PBS. AAV5.Cβ-mIL10 reduced chemokine levels: a 1.6-fold reduction in KC and a 1.3-fold reduction in MIP-1α compared to PBS treatment (**Figure 5.6**). There was also a decrease in proinflammatory cytokine levels: 1.4-fold reduction in IL-1β, 1.7-fold reduction in IL-6, and a 2 fold reduction in TNF-α.

While a decrease in proinflammatory cytokine levels upon *P. aeruginosa* infection is desired, a decrease below uninfected levels could be damaging because this effect could be immunosuppressive or immunostimulatory [244]. Cytokine decrease by AAV5.Cβ-mIL10 was not below the cytokine levels in uninfected mice, except in the level of TNF-α (**Figure 5.6**). Additionally, to determine if the reduction in lung proinflammatory cytokines had a systemic effect, we measured circulating proinflammatory cytokine concentrations in the serum and did not observe a decrease ($p>0.05$).

Figure 5.6

A.


pg-gram lung tissue
14000
12000
10000
8000
6000
4000
2000
0
*
**
*
NSD
IL-1b
IL-6

B.

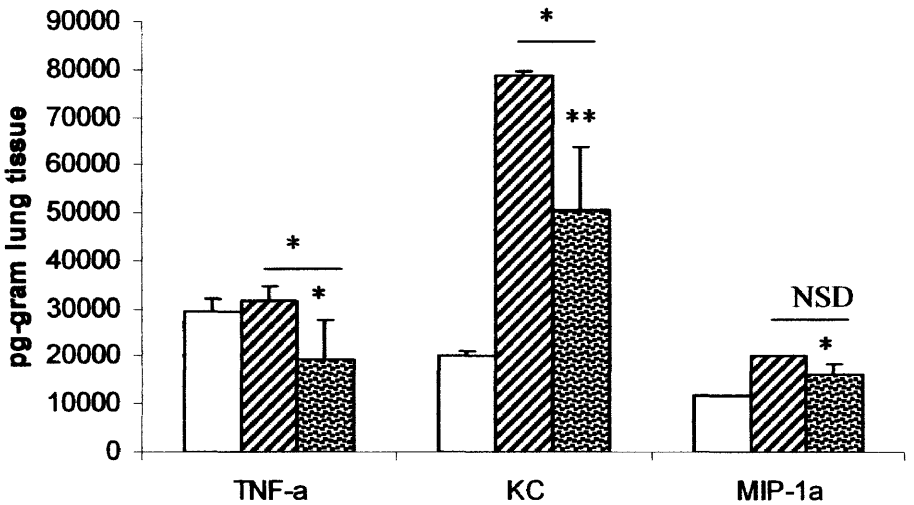

pg-gram lung tissue
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
*
*
*
**
NSD
*
TNF-a
KC
MIP-1a

Figure 5.6 AAV5.Cβ-mIL10 decreases proinflammatory cytokine levels in the lung. Anesthetized mice received $8x10^9$ vg of AAV5.Cβ-mIL10 or PBS by aspiration challenge with surfactant phospholipids. After 6 weeks, AAV5.Cβ-mIL10-treated mice were infected with $5x10^6$ cfu *P. aeruginosa*-laden beads while PBS-treated mice were infected with *P. aeruginosa*-laden beads or not infected. Mice were sacrificed 4 days later. Lungs were collected and homogenized. Cytokines **(A)** IL-1β and IL-6 and (B) TNF-α, KC, and MIP-1α were measured in PBS-treated uninfected mice (□), PBS-treated *P. aeruginosa*-infected mice (▨), and AAV5.Cβ-mIL10-treated *P. aeruginosa*-infected mice (▩). *$p<0.05$, **$p<0.005$ and NSD is not significantly different ($p>0.05$). Indication of significance between infected mice receiving AAV5.Cβ-mIL10 and uninfected mice is shown above the AAV5.Cβ-mIL10 bar. Data shown are the average ± standard deviation for 5 or more mice in each group from 1 experiment.

IL-10 expression from AAV5.Cβ-mIL10 decreases inflammation-associated lung pathology.

Lung sections in the IL-10 treatment group that were infected with *P. aeruginosa* contained fewer neutrophil and lymphocyte infiltration, primarily found in discrete nodules that were fewer in number (**Figure 5.7A, B**). In contrast, histological examination of lung sections obtained from mice in the PBS-treatment group infected with P. aeruginosa, revealed more inflammatory nodules and lung consolidation (**Figure 5.7C, D**). Histology sections taken from uninfected mice did not exhibit any inflammatory changes (**Figure 5.7E, F**). Histological scoring was used to show that inflammatory changes were diminished in AAV5.Cβ-mIL10-treated mice (**Table 1**).

This pathology corroborates our cytokine findings and supports our hypothesis that AAV5.Cβ-mIL10 can effectively reduce inflammation in *P. aeruginosa*-infected lungs. Moreover, despite fairly high IL-10 levels in the lung, there was no indication of systemic infection or immunosuppression modulated by IL-10.

Figure 5.7

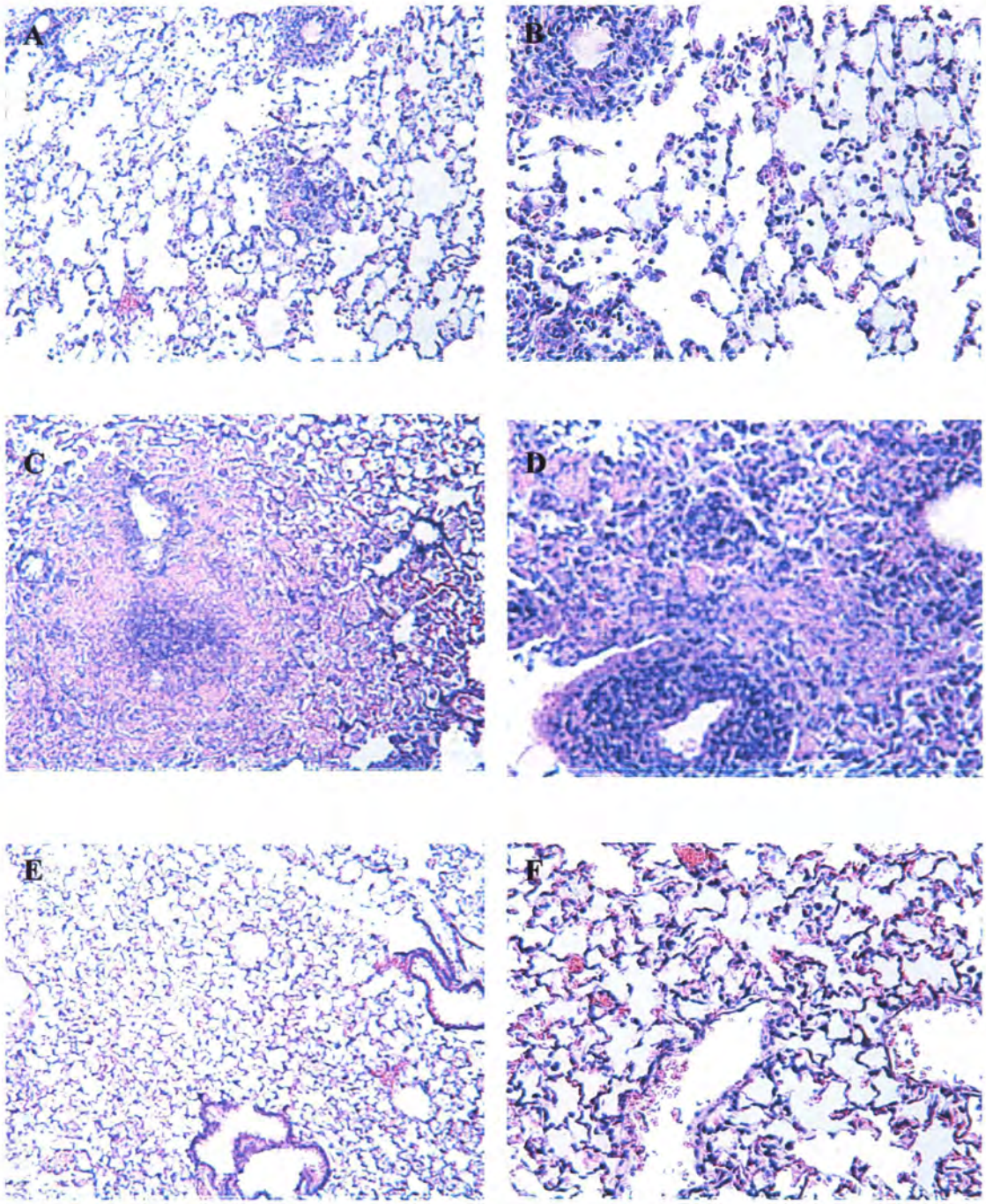


Figure 5.7 AAV5.Cβ-mIL10 reduces inflammation-associated pathology in *P. aeruginosa*-infected mice when compared to similarly-infected mice treated with PBS. Anesthetized mice received $8x10^9$ vg of AAV5.Cβ-mIL10 or PBS. After 6 weeks, AAV5.Cβ-mIL10-treated mice were infected with $1x10^5$ cfu *P. aeruginosa*-laden beads while PBS-treated mice were infected with *P. aeruginosa*-laden beads or not treated. Mice were sacrificed 4 days later. Lungs were collected and fixed in paraformaldehyde, then sectioned and H&E stained. (**A, B**) AAV5.Cβ-mIL10 with *P. aeruginosa*-laden beads, (**C, D**) PBS with *P. aeruginosa*-laden beads, and (**E, F**) PBS. Magnifications are 10x (A, C, E) and 20x (B, D, F). Observations represent the findings from 1 animal in the uninfected group and 5 animals in the infected group and are from 1 experiment.

Table 5.2 Histolopathologic scores of lung sections from gut-corrected CFTR knockout mice infected with *P. aeruginosa* following PBS or AAV5.Cβ-mIL10 administration.

P. aeruginosa-*infected treatment group*	*Number of mice*	*Score*	*Average± SD*
PBS	5	2.5 2.5 3 2.5 2.5	2.6 ± 0.2
AAV5.Cβ-mIL10	5	2 2 2 1 1	1.6 ± 0.5
p value			**p>0.005**

Anesthetized mice received $8x10^9$ vg of AAV5.Cβ-mIL10 or PBS. After 6 weeks, anesthetized mice were infected with $1x10^5$ cfu *P. aeruginosa*-laden beads and sacrificed 4 days later. Lungs were collected and fixed in paraformaldehyde, then sectioned and H&E stained. Sections were graded by a blinded pathologist.

Histopathologic scoring parameters were based on previously published criteria [217].
0=within normal limits
1=darker red than normal
2=0-2 lymphocytic infiltrated nodules present
3=3-6 lymphocytic infiltrated nodules, <¼ lung consolidated
4=¼ to ½ lung consolidated
5=½ to ¾ lung consolidated
6=>¾ lung consolidated

Bacterial burden is similar in mice receiving PBS or AAV5.Cβ-mIL10 treatment.

Gut-corrected CFTR knockout mice treated with PBS or AAV5.Cβ-mIL10 and, 6 weeks later, were infected with $1x10^5$ cfu *P. aeruginosa*-laden agarose beads. Four days later, the mice were sacrificed, lungs and spleen collected, and homogenized. To determine if treatment with AAV5.Cβ-mIL10 resulted in any change in bacterial burden, quantitative bacteriology was performed on lung homogenates. There was no different in the amount of *P. aeruginosa* recovered from the lungs of AAV5.Cβ-mIL10-treated mice when compared to PBS-treated mice ($p<0.05$). No bacteria grew in cultures of spleen homogenates in AAV5.Cβ-mIL10-treated or PBS-treated mice.

Neutrophil migration is decreased with AAV5.Cβ-mIL10 administration.

Gut-corrected CFTR knockout mice were inoculated with $1x10^5$ *P. aeruginosa*-laden agarose beads 6 weeks after receiving AAV5.Cβ-mIL10 or PBS. Four days later, the mice were sacrificed and BALF was collected. BALF cells were obtained through centrifugation, resuspended in PBS, and cytospun onto a slide which was H&E stained. BALF cells were counted and identified on the H&E slide as alveolar macrophages (AM), neutrophils (PMN), or lymphocytes. While the percentage of individual cell types in the BALF was not significantly different between the treatment groups in the presence of *P. aeruginosa* infection, the absolute number neutrophils was decreased 2.5-fold ($p<0.05$) in AAV5.Cβ-mIL10-treated mice compared to mice that received PBS (**Figure 5.8**). There was also a trend toward a decrease in the percentage of neutrophils with AAV5.Cβ-mIL10 treatment (data not shown).

Figure 5.8

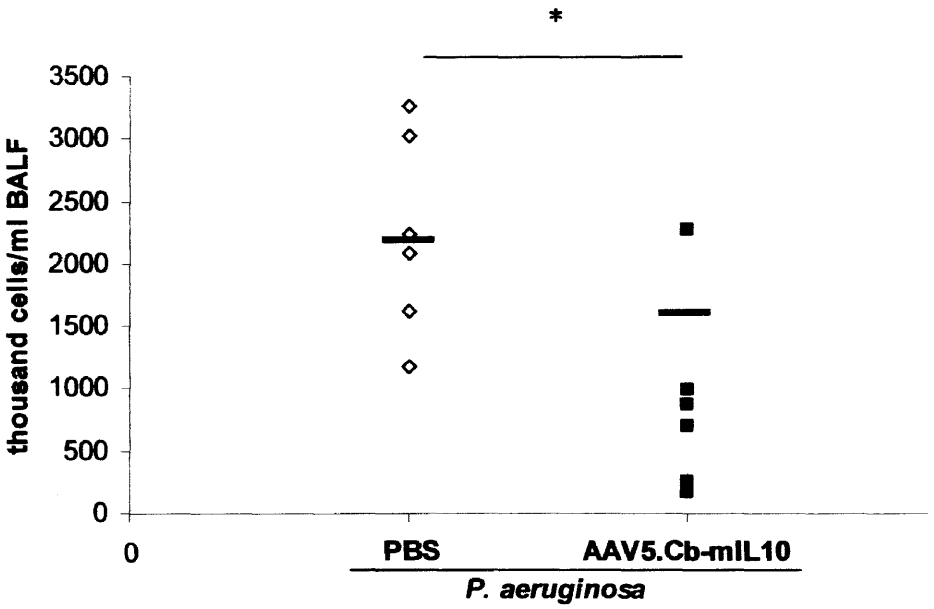

*
3500
3000
2500
2000
1500
1000
500
0
thousand cells/ml BALF
0
PBS
AAV5.Cb-mIL10
P. aeruginosa

Figure 5.8 AAV5.Cβ-mIL10 significantly decreases neutrophil migration to the lungs of gut-corrected CFTR knockout mice infected with *P. aeruginosa*. Anesthetized mice received PBS or AAV5.Cβ-mIL10. After 6 weeks, anesthetized mice were infected with $1x10^5$ cfu *P. aeruginosa*-laden agarose beads. Mice were sacrificed 4 days later. BALF was collected and analyzed for cell number and type. *$p<0.05$ Data points are neutrophil cell counts for 6 mice in each group from one experiment; the black line is the average of each group.

5.4 Discussion

Several lines of evidence suggest that in CF lung disease, inflammation is excessive relative to bacterial infection [35, 267]. Infants with CF have large quantities of IL-8, neutrophils, and neutrophil elastase (NE) in their BALF, compared to non-CF infants, which occurs in the presence and absence of detectable bacterial infection [64]. When compared to normal mice that were infected with the *P. aeruginosa*-laden beads, CF mice demonstrated greater weight loss, a more marked inflammatory response, and higher mortality despite having the same bacterial load as their non-CF counterparts [220, 223]. Furthermore, treatment of patients with CF using anti-inflammatory therapeutics slows the progression of lung disease without exacerbating infection [96-100]. Taken together, these studies suggest that inflammation is dysregulated and disproportionately severe to the bacterial infection, resulting in a pathologic response in the CF lung. In addition to excessive levels of proinflammatory cytokines, there are lower levels of anti-inflammatory cytokine, IL-10, in the airways of patients with CF, compared to healthy subject controls [30]. IL-10 deficiency and corresponding excessive inflammation are related as shown in studies with IL-10-deficient transgenic (IL-10T) mice [139, 141, 236]. Furthermore, many of the pathologies noted with *P. aeruginosa* infection were reversed by administration of IL-10 in wild-type mice [139, 141] and CFTR knockout mouse models [37, 140].

Previous use of IL-10 as a therapeutic for inflammation in a chronic lung infection model has administered the recombinant protein, systemically, leading to a corresponding systemic reduction in proinflammatory cytokines [139]. Because dysregulated inflammation occurs exclusively in the CF lung, we were interested in lung-targeted delivery and expression of IL-10 using adeno-associated virus (AAV)-based gene

transfer. The use of AAV vectors to deliver therapeutic genes in CF has been studied extensively in animal studies and clinical trials. These vectors have a proven safety profile and are capable of producing long-term gene expression with a minimal inflammatory response [17, 19, 182, 240].

We found that treatment with AAV5.Cβ-mIL10 resulted in reduced inflammation in a model of chronic *P. aeruginosa* lung infection in gut-corrected CFTR knockout mice. First, we performed a *P. aeruginosa*-laden bead dosing study in gut-corrected CFTR knockout mice to determine the amount of bacteria to administer by aspiration challenge with surfactant phospholipids. Our finding that lower doses of *P. aeruginosa* result in higher morbidity in gut-corrected CFTR mice, compared to previous studies with CD-1 mice, is supported by the literature [220, 224]. Then, gut-corrected CFTR knockout mice were treated with PBS or AAV5.Cβ-mIL10. Six weeks later, the mice were infected with $1x10^5$ cfu *P. aeruginosa*-laden agarose beads and sacrifice after 4 days. While proinflammatory cytokines (IL-1β, IL-6, TNF-α, KC, and MIP-1α) were significantly increased with *P. aeruginosa* infection, IL-10 levels remained the same ($p>0.05$). Treatment with AAV5.Cβ-mIL10 corresponded with high levels of lung-specific IL-10 expression, decreased proinflammatory cytokine levels, inflammation-associated lung pathology and neutrophil migration with no effect on bacterial burden ($p>0.05$). This last point is important because of concerns regarding the administration of an anti-inflammatory agent during an active bacterial infection. Like corticosteroid and ibuprofen use in patients with CF, modulation of inflammation mediated by IL-10 does not exacerbate bacterial infection in our chronic model. Additionally, blood levels of proinflammatory cytokines and IL-10 from mice receiving AAV5.Cβ-mIL10 were not

significantly different from those measured in the PBS-treated mice (data not shown). The most significant finding was the percentage weight loss between the groups of mice. *P. aeruginosa*-infected mice treated with AAV5.Cβ-mIL10 had the same percentage weight loss as uninfected mice ($p>0.05$); both groups were significantly lower in percentage weight loss compared to infected mice treated with PBS ($p<0.005$).

Our data agree with the findings of others who used related animal models and exogenous IL-10 treatment. In a chronic *P. aeruginosa* infection in CD-1 mice, intraperitoneal administration of recombinant IL-10 (rIL-10) resulted in increased survival, fewer neutrophils in the BAL, and decreased area of lung inflammation when compared to placebo-treated mice; there was no decrease in bacterial burden [139]. Recombinant IL-10 was also administered, subcutaneously, in an acute model using intratracheal LPS challenge in CF mice and resulted in a significant decrease of PMNs and TNF-α, which correlated with decreased NFκB activity and increased IκBα [37]. The anti-inflammatory activity of IL-10 most likely occurs by stabilizing IκBα, an inhibitor of NFκB, reducing the ability of NFκB to induce proinflammatory cytokine expression and secretion by airway epithelial cells [70].

In this study, there was no infection with an AAV vector expressing a reporter gene or inert protein to determine the effect of AAV on proinflammatory cytokine expression and neutrophil migration, separate from the effects of IL-10. While AAV-mediated gene transfer leads to the development of antibodies against the vector capsid which can prevent vector re-administration, the lack of inflammatory responses documented in numerous *in vivo* gene transfer models employing AAV vectors suggest that these agents do not interact significantly with the innate immune system [143].

Studies in non-human primates with AAV-CFTR showed the vector did not result in an increase in proinflammatory cytokines and neutrophil influx as measured in the BALF [143]. Additionally, studies in murine models have shown that AAV-GFP has the same response as PBS when measuring percentage weight loss and neutrophil migration (Dr. Tom Ferkol, unpublished studies), supporting our use of PBS as a negative control.

Future studies should analyze the duration of AAV-mediated IL-10 gene expression, shown for some transgenes to persist for 1.5 years in animal models [159-162]. This would be important for clinical applications of AAV5.Cβ-mIL10 because the anticipated dosage could be administered less frequently than most CF medications (typically, multiple times per day or week), potentially increasing patient adherence, currently an obstacle in CF [245].

In conclusion, we have demonstrated gene transfer of IL-10 reduces local inflammation without modulating a systemic cytokine decrease or promoting an increase in bacterial burden. Our results support the hypothesis that decreased IL-10 levels are linked to high levels of proinflammatory cytokine production in CF and that exogenous administration of IL-10 may reduce the pathogenesis of the excessive inflammatory response to the chronic endobronchial *P. aeruginosa* infection in patients with CF.

Chapter 6.

Implications

Although the life expectancy of patients with CF has significantly improved during the past 30 years, the beneficial effects of antibiotics, airway clearance, and improved nutrition appears to have reached a plateau. Despite the initial success *in vitro* for treatments aimed at modifying the basic CFTR defect, current attempts in clinical trials have not been successful in ameliorating the CF phenotype. Additionally, while targeting the CFTR may improve the clinical outcome of young patients, it may not benefit patients with extensive irreversible lung damage resulting from years of chronic infection and inflammation. Therapeutics targeting the inflammatory cascade and slowing disease progression offer the most benefit for the majority of patients with CF.

The airways of patients with CF are proinflammatory, due to innate features of the CF immune system caused by CFTR mutations, multiple acute infections with bacteria, and/or persistent, chronic bacterial infections. Upon bacterial colonization, components of *P. aeruginosa* (LPS, flagellin) interact with toll-like receptors (TLR) expressed from alveolar macrophages, leading to activation of NFκB, production of proinflammatory cytokines, and recruitment of inflammatory cells. Supporting the recruitment of B and T cells during this response are the presence of circulating IgG subclass antibodies against *P. aeruginosa*; however, the airway is predominantly characterized by large quantities of neutrophils. These neutrophils are recruited to the airway and release reactive oxidant species, but are not effectively phagocytosed, contributing to the viscous secretions lining the airway.

The production of regulatory cytokines and antioxidants and apoptosis promote tissue repair (fibrosis) and resolution of the inflammatory response. Patients with CF appear to have an inflammatory response that is not effectively resolved because the modulators of the immune response, including IL-10, IκBα, and AAT, are produced at low levels, rapidly degraded, and/or overwhelmed by the levels of proinflammatory cytokines, reactive oxidant species, and proteases produced. Fibrosis occurs in the CF airway, but the lungs are continuously in a cycle of tissue injury and repair.

Our therapeutic, AAV-directed IL-10 gene transfer, could prove beneficial in patients with CF. Using an administration technique such as aerosolization or single-lobe-lung delivery of IL-10, the vector will efficiently transduce the airway epithelia, as was shown in our animal studies and previous human clinical trials. AAV vectors have been found to predominantly persist in episomal form and, over a period of 6 weeks, mediate maximal expression of IL-10 protein. Our animal studies showed IL-10 was produced from alveolar cells, and because the protein is secreted, IL-10 may also demonstrate its anti-inflammatory effects in sites removed from the successfully transduced cells, including the upper and lower respiratory tract.

Therapeutic IL-10 protein expression decreased production of cytokines IL-1β, IL-6, and TNF-α and chemokine IL-8 (KC and MIP-1α), which are elevated in the CF lung, and their reduction may aid in resolution of the excessive inflammatory response in the human lung as was observed in our animal studies. Decreased cytokine expression led to decreased inflammation-associated lung pathology and neutrophil recruitment, supporting the finding that IL-10 facilitates resolution of lung inflammation, decreases myeloid-derived cells (neutrophils), and prevents cell-to-cell communication between

macrophages and T cells, resulting in decreased production of proinflammatory cytokines and cytotoxic factors. Bacterial killing was not affected with IL-10 treatment in our studies, suggesting that the host response was not decreased enough to cause an increase in bacterial proliferation. In patients with CF, IL-10 treatment is also not likely to cause bacterial proliferation because prior treatment with anti-inflammatory therapeutics (such as ibuprofen and glucocorticoids) have not caused an increase in bacterial burden because the inflammatory response is decreased from a heightened state, but not decreased to a state of immunosuppression. The goal of anti-inflammatory treatment in patients with CF is not to reduce *P. aeruginosa* infection, but to decrease the pathologic response that results in tissue injury and respiratory failure.

Our studies also support the use of surfactant as a carrier for non-invasive lung delivery of gene transfer agents in animal models and may improve clinical delivery of AAV to produce a clinical outcome, not yet demonstrated in clinical trials [16, 18]. Surfactant has been instilled into the lungs of critically ill adults and children, supporting the use of this vehicle as safe and well-tolerated [268]. Because obtaining high titers of AAV vectors is a current limitation for clinical trials, dosing of AAV5.Cβ-mIL10 should be conducted in animal and human studies to determine if the addition of surfactant phospholipids will increase gene transfer.

Currently, an obstacle in CF treatment is patient adherence to medicine regiments because patients are typically on several medications, administered multiple times per day or week. AAV5.Cβ-mIL10 could be administered less frequently than most CF medications due to the ability of AAV-based vectors to convey long-term gene expression of therapeutic proteins. Future studies should analyze the duration of AAV-

mediated IL-10 gene expression, shown for some transgenes to persist for 1.5 years in animal models [159-162].

In conclusion, we demonstrated gene transfer of IL-10 reduces local inflammation without modulating a systemic cytokine decrease or promoting an increase in bacterial burden. Our results support the hypothesis that decreased IL-10 levels are linked to high levels of proinflammatory cytokine production in CF and that exogenous administration of IL-10 may reduce the pathogenesis of the excessive inflammatory response to the chronic endobronchial *P. aeruginosa* infection in patients with CF. These studies add to a significant body of research that suggests inflammation in CF is pathologic, and regulation of this response aids in resolving excessive cytokine release, neutrophil migration, and lung destruction. In addition, we developed a technique for non-invasive *P. aeruginosa* infection and gene transfer that will be beneficial to CF researchers working with *in vivo* models. Both contributions further develop AAV-based gene transfer as a promising therapeutic against inflammation in CF.

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EDUCATION

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- Travel Scholarship, Graduate Student Association
- Abstract Award, South Carolina Association for Public Health Conference
- Selected for oral presentation, American Thoracic Society International Meeting
- Service and Leadership Award, College of Graduate Studies
- *Presidential Scholar*
- First Place, MUSC Research Day

Wofford College

Senior Research and Thesis *'Origin of Life' Experiments, continued study of Chummy – a unique fungus growing amoung us – and production of acetone and butanol by Clostridium acetobutylicum*
'Special Topics in Biology' Honor Seminar
Physical Chemistry Report published as a model in *Journal of Chemical Education*

RESEARCH PUBLICATIONS

Woraratanadharm, J., S. Rubinchik, H. Yu, F. Fan, **S.M. Morrow**, and J.Y. Dong. 2004. Highly specific transgene expression mediated by a complex adenovirus vector incorporating a prostate-specific amplifcation feedback loop. *Gene Therapy* (2004) 11, 1399-1407.

RESEARCH PRESENTATIONS

S Morrow Buff, S Caldwell, H Yu, J Baatz, T Ferkol, T Flotte, and I Virella-Lowell. Oral Presentation. *Delivery of IL-10 by an AAV vector to the lungs of CFTR knockout mice reduces inflammation upon chronic infection with P. aeruginosa.* To be presented at the ASGT Conference. 2007.

S Morrow Buff, H Yu, J Baatz, and I Virella-Lowell. Poster Presentation. *Administration of AAV5.CB-AAT via aspiration challenge with surfactant is less invasive than intratracheal injection and produces similar vector distribution.* 2006. 20th Annual North American Cystic Fibrosis conference. Pediatric Pulmonology. Supplement 29. A234. 2006.

S Morrow Buff, H Yu, T Ferkol, T Flotte, and I Virella-Lowell. Oral Presentation. *AAV5.CB-IL10 administration attenuates inflammation in pseudomonas-infected IL-10 knockout mice.* ATS International Conference. Section C84 - Novel Approaches to the Assessment and Treatment of CF Lung Disease. A722. 2006.

H Yu, **S Morrow Buff**, J Baatz, and I Virella-Lowell. Poster. *Administration of Pseudomonas using aspiration challenge with surfactant: a promising alternative to Intratracheal Injection and Traditional Aspiration Models.* ATS International Conference. A409. 2006.

Scotty Morrow Buff, Hong Yu, John Dong, Tom Ferkol, Terence Flotte, and Isabel Virella-Lowell. Poster, Presenter. *Intratracheal AAV5-IL-10 significantly reduces the airway pro-inflammatory response in pseudomonas-infected IL-10 knockout mice.* 19th Annual North American Cystic Fibrosis conference. Pediatric Pulmonology. Supplement 28. A259. 2005.

Scotty M. Morrow, Frank Nocken, Horst Fischer, Min Luo, Semyon Rubinchik, Peisheng Zhang, Roger C. Young, Neil A. Bradbury, Raymond A. Frizzell, and John Y. Dong. Poster Presentation. *Analysis of chloride channel conductance by optimized half-CFTR modules reveals novel strategy for utilization of AAV vectors.* 18th Annual North American Cystic Fibrosis Conference. Pediatric Pulmonology. Supplement 27. A122. 2004.

SM Morrow, S Rubinchik, H Yu, and Jian-yun Dong. Poster Presentation. *Novel approaches and strategies for the production of high-titer clinical-grade AAV vectors.* American Society for Microbiology. 2004.

Scotty M. Morrow, Frank Nocken, Min Luo, Peisheng Zhang, Roger Young, Horst Fischer, and Jian-yun Dong. Poster Presentation. *Efficient Transfer of Half-CFTR Modules with a Strong Promoter in AAV Vectors.* Conference of the American Society of Gene Therapy. A128. 2002.

S.M. Morrow, F. Nocken, M. Luo, P. Zhang, H. Fischer, R. Young, and J-Y Dong. Poster Presentation. *Efficient CFTR Transfer with AAV Vectors Containing Modular CFTR Components.* 15th Annual North American Cystic Fibrosis conference. Pediatric Pulmonology. Supplement 24. A223. 2001.

IN-HOUSE SEMINARS AND PRESENTATIONS

Scotty M. Buff. *Reducing Inflammation in a Cystic Fibrosis Mouse Model with AAV-IL10 Therapy.* Microbiology and Immunology Departmental Seminar. Spring 2007.

Scotty M. Buff. *AAV5.Cb-IL10 Attenuates Inflammation in* Pseudomonas*-Infected IL-10 Knockout Mice.* Cystic Fibrosis Foundation Site Visit Presentations. Fall 2006.

Scotty M. Buff. *AAV-mediated Anti-Inflammatory Gene Therapy in an Optimized Cystic Fibrosis Mouse Model.* Microbiology and Immunology Departmental Seminar. Fall 2005.

Scotty M. Buff. *Development of AAV vectors for gene therapy of CF.* Microbiology and Immunology Departmental Seminar. Spring 2005.

Scotty M. Morrow, Frank Nocken, Semyon Rubinchik, Neil Bradbury, Ray Frizzell, and Jian-yun Dong. *YFP imaging assay detects chloride conductance mediated by AAV vectors carrying half-CFTR modules.* MUSC Student Research Day, Charleston, SC. A084. 2004.

Scotty M. Morrow, Semyon Rubinchik, Hong Yu, and Jian-yun Dong. *Production of high titer clinical-grade AAV Vectors: Novel approaches and strategies.* MUSC Student Research Day, Charleston, SC. A139. 2003.

Scotty M. Morrow. *Advancing AAV as a Gene Therapy Vector for Cystic Fibrosis: Characterization and Optimization of Half-CFTR Modules and Development of a Novel Strategy for recombinant AAV Production.* Microbiology and Immunology Departmental Seminar. Fall 2003.

Scotty M. Morrow, Frank Nocken, Semyon Rubinchik, Neil Bradbury, Ray Frizzell, and Jian-yun Dong. *Functional Analysis of Differentially Truncated CFTR Proteins for Gene Therapy of Cystic Fibrosis.* MUSC Student Research Day, Charleston, SC. A122. 2002.

Scotty M. Morrow. *Delivery of the CFTR for Gene Therapy of CF.* MUSC Clinical and Basic Science CF Presentations. Spring 2002.

Scotty M. Morrow. *Towards effective gene therapy of cystic fibrosis: Evaluation of CFTR activity following transfer with AAV vectors containing half-CFTR modules.* Microbiology and Immunology Departmental Seminar. Fall 2001.

Scotty M. Morrow, Frank Nocken, Min Luo, Peisheng Zhang, Roger Young, and Jian-yun Dong. *Gene Therapy of Cystic Fibrosis Using Half-CFTR Modules is Efficacious and Overcomes Packaging Space Limits in AAV Vectors.* MUSC Student Research Day. A118. 2001.

Scotty M. Morrow. *Presentation of Cystic Fibrosis Research at MUSC.* Cystic Fibrosis Foundation Site Visit Presentions. Spring 2001.

Scotty M. Morrow. *Phenotypic Analysis of Cyototoxic T Lymphocytes Using MHC-Peptide Tetramers.* Microbiology and Immunology Departmental Seminar. Spring 2001.

RESEARCH EXPERIENCE

Pediatric Pulmonology
Darby Children's Research Institute
Medical University of South Carolina
Charleston, South Carolina, 2005-present

Mentor: Isabel Virella-Lowell, M.D.
Anti-inflammatory gene therapy of cystic fibrosis

- Conduct animal research
- Supervise mouse breeding
- Analyze samples with BioPlex
- Wrote three grants

Department of Cell Biology and Physiology
Basic Science Tower
University of Pittsburgh
Pittsburgh, Pennsylvania, Nov-Dec 2002

Collaborators: Raymond Frizzell, Ph.D. and Neil Bradbury, Ph.D.
YFP imaging and patch-clamp analysis of half-CFTR modules

- Learned YFP and bisoxonal imaging procedures
- Further trained in patch-clamp technique and analysis

Department of Microbiology and Immunology
Basic Science Building
Medical University of South Carolina
Charleston, South Carolina, 2000-2005

Mentor: John Y. Dong, M.D., Ph.D.
Functional analysis of half-CFTR modules and development of a novel strategy for high-titer AAV

- Established collaboration with Micheal Bowman, M.D., Ph.D.
- Attended cystic fibrosis educational sessions with third and fourth year medical students
- Established collaboration with Roger Young, M.D., Ph.D. to learn patch-clamp analysis technique
- Successfully patched positive and negative controls, and found novel activity from N-terminal module
- Weekly attended CF clinic briefings in MUSC Pediatric Comprehensive CF Center
- Initiated development of SPQ imaging assay
- Collaborated with Raymond Frizzell, Ph.D. and traveled to Pittsburg to conduct functional analysis

- Successfully demonstrated function by all half-CFTR co-expression systems by imaging assay and found novel activity of C-terminal module
- Established collaboration with William Guggino, Ph.D. (Johns Hopkins)
- Established collaboration with Richard Boucher, M.D. and Jude Samulski, Ph.D. (UNC-Chapel Hill)
- Traveled to UNC-Chapel Hill to pursue other possible collaborations
- Edited, wrote, and submitted numerous grants on cystic fibrosis and infectious disease vaccines

ACTIVITIES

Medical University of South Carolina

Fellow, *Presidential Scholars*
Student Academic Subcommittee, Student Government Association
Representative, Student Diversity Advisory Committee
Student Handbook Chairperson, Graduate Student Association

Wofford College

Secretary, American Chemical Society
Tutor, General Chemistry
Laboratory Assistant and Instructor, General Chemistry

Community

Assistant Coach, AYSO Soccer
Girl Scout counselor and leader, Camp Mary Elizabeth

VOLUNTEER EXPERIENCE

Founder and Director
'Junior Doctors of Health'
Wilmot J. Fraser Elementary School
Charleston, South Carolina, 2004-present

- Initiated and implemented Nutrition, Exercise, and Mentoring program in inner-city school
- Ensured program's sustainability
- Received monetary donations and grant funding
- Established multiple collaborations
- Recruited and gave orientations for MUSC student volunteers
- Implemented teacher exercise program
- Incorporated new activities to expand program each year
- Coordinated book drive raising in excess of 300 books and $200 monetary donations

Extramural Grant Awards

Buff (Awardee) $1,500
Coastal Community Foundation, Cooper River Bridge Run, 2007
MUSC-Junior Doctors of Health
This project is to fund Wilmot J. Fraser Elementary School students and their parents to attend the Cooper River Bridge Kids Run to promote family exercise in a healthy environment.
Role: Coordinator/Co-PI

Intramural Grant Awards

Buff (Awardee) $2,500
MUSC, President's Office, 2006-2007
Junior Doctors of Health
This project is a mentorship based program between MUSC students and Wilmot J. Fraser Elementary School (grades 2nd-6th) where MUSC students teach about the importance of nutrition, exercise, and leading a healthy lifestyle. It has expanded to include 7th grade and now includes information about safety.
Role: Coordinator/PI

Stevens (PI) $2,480
MUSC Family Fund, YES Grant, 2004-2005
MUSC-based Clinics, Diabetes Awareness (Project II)
This project was to provide Wilmot J. Fraser Elementary School with MUSC students to teach about healthy eating and exercise. Presentations were also given to parents.
Role: Coordinator

Related Presentations

Scotty Buff. Oral Presentation. *"Junior Doctors of Health"*. MUSC Board of Trustees, Education subcommittee. 2007

Scotty Buff. Oral Presentation. *Combating Pediatric Obesity with "Junior Doctors of Health"*. Society for Pediatric Nurses. 2007

Scotty Buff, Pamella Gibbs, Rachel Rozansky, Ashley Costa, and Brittany Ray. Oral Presentation. *Enhancing Diabetes and Obesity Awareness by Implementing a Healthy Eating/Exercise Program in a Local Elementary School - "Junior Doctors of Health"*. South Carolina Association for Public Health Conference. 2006

MUSC President Raymond Greenberg, M.D., Ph.D., Rosetta Swinton, R.N., and **Scotty Morrow**. Oral Co-presentation. *Community Interventions for Obesity: A Heavyweight Solution.* College of Medicine Dean's Prevention Lecture. Fall 2004.

MUSC President Raymond Greenberg, M.D., Ph.D. and **Scotty Morrow**. Oral Co-presentation. *Making a Difference: Think Globally, Act Locally.* MUSC Presidential Scholars' Fellow Lecture. Fall 2004.

S Morrow, D Klos, T Johnson, Sav Gunasinghe, K Kelleher, O Peck, S Johnson, and L Egede. Poster Presentation. *Enhancement of Diabetes Education by Implementing a Healthy Eating/Exercise Program in Two Local Elementary Schools, "Junior Doctors of Health".* MUSC Presidential Scholars Day, Charleston, SC. 2004.

Medical University of South Carolina

- United Methodist Relief Center
- Happy Days and Special Times
- Alterra Clare Bridge
- American Cancer Society
- Habitat for Humanity
- Voter Registration Drive

Wofford College

Co-organizer, Art in Action

Community

Event Organizer, Cystic Fibrosis Foundation
Tutor, Summer of Success

ADDITIONAL WORK EXPERIENCE

- Ticket and concession sales, Regal Converse Cinema 6
- Surgeon assistant and animal care, Smith Animal Hospital
- Waitress, Denny's
- Chemical inventory and MSDS, Wofford College
- Waitress, Cracker Barrel

MEETINGS ATTENDED

- American Society of Gene Therapy, Seattle (Oral), 2007
- 20th Annual North American Cystic Fibrosis Conference, Denver (Poster), 2006
- South Carolina Association for Public Health Conference, Myrtle Beach (Oral and poster), 2006
- American Thoracic Society International Conference, San Diego (Oral and poster), 2006
- 19th Annual North American Cystic Fibrosis Conference, Baltimore (Poster), 2005
- American Society of Gene Therapy Stakeholder's Meeting, Arlington, 2004
- 18th Annual North American Cystic Fibrosis Conference, St. Louis (Poster), 2004
- American Society for Microbiology, Clemson (Poster), 2004
- 17th Annual North American Cystic Fibrosis Conference, Anaheim, 2003
- American Society of Gene Therapy, Boston (Poster), 2002

- 15th Annual North American Cystic Fibrosis Conference, Orlando (Poster), 2001
- American Society of Gene Therapy, Seattle, 2001
- International Society of Cancer Gene Therapy, San Diego, 2000

PROFESSIONAL MEMBERSHIPS

American Society of Microbiology