THESIS

UNDERSTANDING *MYCOBACTERIUM ABSCESSUS* IN CYSTIC FIBROSIS MICE

Submitted by

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ABSTRACT

UNDERSTANDING *MYCOBACTERIUM ABSCESSUS* IN CYSTIC FIBROSIS MICE

Cystic fibrosis (CF) is caused by mutation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, which normally encodes an ABC transporter-class ion channel protein that allows chloride and thiocyanate ions transport across epithelial cell membranes. Thus, CFTR plays an important role in airway homeostasis. Mutations of CFTR in patients with CF leads to a defect in transport of chloride and thiocyanate ions by epithelial cells, resulting in a multi-system disorder that affects the respiratory tract, gastrointestinal tract, the endocrine system, among others. The epithelial cell dysfunction in the lungs of CF patients also leads to an impaired pulmonary defense mechanism, resulting in decreased bacterial clearance and chronic inflammation. In CF patients, lung disease due to non-tuberculous mycobacteria (NTM) — an environmental organisms found in soil, water, and biofilms — is one of the most feared complications. Among the NTM, the rapidly-growing *Mycobacterium abscessus* is particularly notorious given its intrinsic resistance to many antibiotics. The transmission of *M. abscessus* to humans occurs by wound contamination, airborne transmission, or ingestion. Despite the fact that *M. abscessus* infection is increasing worldwide, little is known about how *M. abscessus* causes disease.

To improve our understanding of *M. abscessus* in CF patients, we set out to investigate the progression of *M. abscessus* infection in a CF mouse model by developing a reinfection mouse model using three different strains of “CF mouse” —

Beta-ENaC mice, Cftr^{tm1UNC}TgN(FABPCFTR) mice and CFTR^{tm1UNC}/ CFTR^{tm1UNC} mice — to track the bacterial burden and organ pathology. Our results support the hypothesis that repeated infection with *M. abscessus* is more likely to result in disease progression and increased pathogenesis of the disease in CF mouse models. The high bacterial burden persisted in the lung after four infections in β-ENaC transgenic mice and CFTR^{tm1UNC}/CFTR^{tm1UNC} mice and maintained in the organs by day 30. Cftr^{tm1UNC}TgN(FABPCFTR) mice tended to show slowly increasing bacterial burden in all organs. In summary, we demonstrate that reinfection of the CF mouse models with *M. abscessus* is more likely to result in a sustained infection in the lungs associated with increased pulmonary pathology.

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DEDICATION

This thesis is dedicated to all Cystic Fibrosis patients and

those who have suffered infections with non-tuberculosis mycobacterium.

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CHAPTER 1 – LITERATURE REVIEW

Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disorder due to mutations of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, resulting in a defect in the transport of ions by epithelial cells in multiple organ systems, including the respiratory tract, gastrointestinal tract, reproductive tract, various exocrine organs, and the immune system [5, 6, 7]. CF has been observed in children ranging from one month old — the most life-threatening period for infants who have signs of malnutrition and poor weight gain — to adolescents [1]. CF has a significant impact on the patient's quality of life. The incidence of CF for Caucasian populations is 1 in 2,500 whereas it is 1 in 10,900 for Native Americans, 1 in 15,000 for Africans, and 1 in 35,000 for Asians [2, 3, 15]. However, the clinical expression of CF is not differentiated by race but instead depends on age and genotypic CF genes [15].

Bacterial infection is a serious problem in CF patients as it is estimated that 80% to 95% of CF patients will eventually acquire chronic bacterial lung infections and resultant tissue-damaging pulmonary inflammation [3]. While lung infections with pyogenic bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* are more common infections in the lungs of CF patients, lung disease due to non-tuberculous mycobacteria (NTM) is particularly feared because of their intrinsic drug resistance. Among the NTM, *Mycobacterium abscessus* is particularly notorious because it is highly difficult to completely eradicate with available antibiotics [4]. Most

children with CF who recover from *M. abscessus* have a high risk of reinfection, disease progression, and mortality.

The immune system, both in cellular and humoral immunity, as it relates to CF is of great interest to scientists and clinicians. This thesis is focused on evaluating the bacterial burden and pulmonary organ pathogenesis in CF mouse models exposed to repeated infections with *M. abscessus*. Additionally, it is intended that these experiments will provide improved understanding of the potential *M. abscessus* pathogenesis due to reinfection in the CF host.

Cystic Fibrosis Characteristics

Under normal conditions, the CFTR gene is located on the apical membrane epithelial cells, encodes the CFTR protein channel which controls chloride ion (Cl^-) transportation in the body. The CFTR gene results in an ABC transporter-class ion channel protein that normally allows for chloride and thiocyanate ions to cross epithelial cell membranes. The CFTR protein channel, during normal regulation will secrete Cl^- out of the cell. CFTR also regulates the sodium ion (Na^+) and thiocyanate ion and associates with the epithelial sodium channel (ENaC) in pulmonary epithelium to reabsorbs Na^+ into the cell. ENaC is a heteromultimeric protein composed of three subunits (α, β, and γ), located on the cilial surface, has an important role in modulating the airway surface liquid (ASL) clearance and essential for cilial transport of mucus in the respiratory tract [84]. The defective CFTR-mediated Cl^- secretion and increased ENaC-mediated Na^+ absorption contribute to the pathogenesis of CF airway disease

which loss of normal regulation by CFTR and alter the airway ion transport [81-82]. Overexpression of ENaC shows hyperabsorption of Na^+ and ASL depletion leads to increased mucus accumulation to airway surface and increased airway inflammation [81].

The regulation of CFTR and ENaC interaction in the respiratory airway has been studied because of the CF involvement. It was indicated that the indirect effect of CFTR genes influences ENaC activity by coexpressed with cyclic adenosine monophosphate (cAMP)-dependent pathway resulting in increased intracellular Cl^- concentration and correlated to the inhibitory effects of CFTR on ENaC [83-85]. Mutation in the CFTR gene changes the regulation of ion homeostasis in the cell. Dysfunction of CFTR in CF patients leads to decreased inhibition of ENaC by lacking of chloride and thiocyanate ions crossing the epithelial cell membranes and encouraged ENaC activity resulting in excessive highly viscous mucus formation and secondary ciliary dysfunction in multi-system, including respiratory tract, gastrointestinal tract and endocrine system [3, 8, 9, 84].

The recent studies found the novel cell populations, pulmonary ionocytes, the rare specialized pulmonary cells that regulate ion transport and hydration in the epithelial surface, are a major source of CFTR activity in the respiratory tract which express higher levels of CFTR genes than other lung cell types. The pulmonary ionocytes co-expresses highly CFTR and Foxi1 expression, which regulates the expression of multiple subunits of V-ATPase. V-ATPase is necessary for ion transportation and controls fluid pH. These new cell type in the airway epithelium have been discovered only 1-2% of the cell population in mouse trachea and primary human

bronchial tissue by using the single-cell RNA sequencing technology to analyze Foxi1 expression in the individual cells. This new discovery shows that Foxi1$^+$ CFTR$^+$ ionocytes are located in multiple layers of the respiratory tract and appears to play a critical role in CFTR expression which is responsible for the pathogenesis of CF [86-87].

To date, approximately 1,600 mutations have been identified in the *CFTR* gene [10]. The severity of the defects are highly related to the type of gene mutations. The CFTR mutations can be categorized into six classes based on the mechanism of the channel function defect. These CFTR mutation classes are associated with either the absence, decreased expression, or dysfunction of the CFTR protein, resulting in diverse physiological phenotypes in CF patients [8, 10].

A class 1 mutation is the premature insertion of the stop codon into the CFTR gene which affects protein expression. This CFTR mutation will express degraded CFTR proteins in the endoplasmic reticulum (ER). The most common mutation of the CFTR gene, a Class 2 mutation, is the missense mutation wherein phenylalanine at position 508 of the CFTR protein is deleted (ΔF508). The ΔF508 allele is found in 70% of all CFTR mutations [3]. These mutations cause misfolded CFTR protein, resulting in CFTR protein being degraded in the endoplasmic reticulum (ER). As the result, CFTR proteins are not expressed at the apical surface of epithelial cells. Class 3 mutation, known as a gating defect, is a missense mutation result in blocking Cl^- channel. Class 4 mutations reduce Cl^- channels. Class 5 mutations reduce functional CFTR synthesis, and class 6 mutations decrease the half-life of CFTR proteins. All of these defects cause Cl^- ion channel transportation impairment which can affect the ENaC at the epithelial cells throughout the body [3, 8, 10].

The symptoms and signs of CF vary from individual to individual. In some cases, CF can be diagnosed with in the first month of life. However, the disease may not progress or develop until adulthood. Eventually, pulmonary issues become more obvious and serious in the majority of CF patients. Normally, the mucociliary function can clear all mucus, debris, and thus help airway bacterial infections. However, in CF patients, non-functional mucociliary function results in viscous mucus secretion that are unable to be cleared, chronic airway and parenchymal inflammation, and finally manifesting as bronchiectasis and lung fibrosis.

Approximately 80% of patients with CF will eventually acquire chronic bacterial infections in the lungs that include *P. aeruginosa*, *S. aureus*, *Haemophilus influenza* and NTM [11]. One mechanism for host immune evasion by these organism is through production of biofilms. The prevalence of NTM isolation from CF patients has been reported to be 4% to 24%, depending on the geographical region and patient population [12]. More importantly, the most prevalent NTM that has been isolated is *M. abscessus* [4]. Chronic *M. abscessus* infection and inflammation cause bronchial wall damage and dilation (bronchiectasis) leading to respiratory failure from repeated CF exacerbations, ultimately causing respiratory insufficiency and death.

Although respiratory problems related to CF are still the major problem, an extrapulmonary clinical manifestation also can be present in CF patients, such as the impairment of gastrointestinal tract function. Meconium ileum due to increased viscosity of intestinal mucus may cause intestinal obstruction, occurs in 13% to 17% of CF newborns [1]. Malnutrition, poor weight gain, and growth failure may occur in early childhood due to pancreatic insufficiency from the thick secretions that block the

pancreatic ducts. As a result, pancreas-derived digestive enzymes are inadequately secreted into the intestinal tract, impairing absorption of nutrients. Furthermore, the retained digestive enzymes in the pancreas and the local inflammation in the pancreas lead to further damage and fibrosis of the pancreas, culminating in a vicious cycle of pancreatic insufficiency.

Mutation of CFTR also impacts the reproductive system. Infertility is found in more than 95% of male CF subjects [13]. CFTR functions in association with the HCO_3^- channel to play an important role in sperm capacitation. The mutation of the CFTR gene causes malformation of the vas deferens, further reducing fertility. Infertility in women has also been observed in CF patients. Mutated CFTR, in association with sex hormone abnormality, and viscous mucus production in the female reproductive tract, involving the cervix, ovary, oviduct, and uterus. Reduced fertility due to mutated CFTR may also be due to inadequate fluid regulation in the female reproductive tract, the small size of female reproductive tissues, decreased ovulation rates, and abnormality of the estrous cycle [14].

Cystic Fibrosis Immune Dysregulation

CFTR dysfunction also affects many components of both cellular and humoral immunity, contributing to many of the medical problems seen in CF patients [7, 9]. Chronic lung disease due to CF is the most serious and challenging management problem and adversely affects the quality of life and lifespan [7]. Affected infants may not show any signs or symptoms at birth, but then later manifest the symptoms of CF

pulmonary disease. Excessive inflammatory reaction in the airway caused by the immunostimulatory pathogen-associated molecular patterns (PAMPs) results in increased proinflammatory cytokines and chemokines (TNF and CXCL-8), and increased polymorphonuclear neutrophil (PMN) accumulation [16-17]. Other studies found that increased bacterial presence in CF airways induces biofilm formation, as well as increased recruitment of leukocytes into the lungs [18-22]. Neutrophils are rapidly recruited into the inflammation site and response to PAMPs for clearing the bacterial infection. However, the ongoing chronic inflammation in CF patient and the neutrophil recruitment still continue over time. The isolated neutrophil from CF and COPD patients in the pulmonary airways is impaired to response by LPS stimulation and impaired antibacterial killing capacities. This sustained and excessive neutrophil accumulation in lung tissue contributes to excessive lung inflammation and lung tissue damage [18, 23].

The CFTR defect results in increased NF-κB signaling and downstream pro-inflammatory cascades, including Ca^{2+}-dependent signaling, nuclear factor of activated T-cell (NFAT) transcription, and mitogen-activated protein kinase (MAPK)-dependent activation of activator protein-1 (AP-1) [24-28].

Another PAMP receptor, TLR4 is displayed on the airway epithelial cell surfaces and via MyD88, activates the Trif-dependent pathway. Trif signaling increases the production of type I interferon (IFNs) [29], including IFN-α and IFN-β, that are mainly involved in the infection clearance in the airway. However, these processes seem to be interfered in CF cells. TLR4 is expressed at lower levels on the CF cells surface and lack of antigen recognition leads to a weak signaling to MyD88 and Trif signaling [23].

Decreased Trif-dependent effectors cells impacted by dysfunctional CFTR genes may contribute to poor bacterial clearance and chronic infection of CF lungs [23].

Studies evaluating the CF airway demonstrated defects in cell function of neutrophils, macrophages, dendritic cells and T cells [30-32]. The excessive PMN migration to the lungs caused impaired phagocytosis and delayed apoptosis of neutrophils [30-32]. Excessive neutrophil accumulation in the airway also resulted in protease-rich degranulation, and neutrophil extracellular traps (NET) [34-36] contributing to exaggerated inflammatory tissue damage [16, 33, 37-38] and decreased lung function.

Alveolar macrophages play an important role in the anti-bacterial host defense, as well as removal of apoptotic cells. The number of alveolar macrophages in CF patients has shown to be higher than in non-CF individuals [39], correlated with the increased concentration of CCL-2 [39-40]. In the innate inflammatory process, there are two subpopulations of macrophages acting in multifunctional capacities. Classically activated macrophages secrete high concentrations of pro-inflammatory cytokines and anti-microbial products. In contrast, some forms of alternatively-activated macrophages have more immunosuppressive function, albeit this paradigm is getting much more complex. Studies evaluating the macrophage polarization in CF patients showed that in the CF pulmonary airway without any inflammatory stimulant there is excessive expression of classical activation leading to a pro-inflammatory state [41] and inducing a hyper-inflammatory response in the CF airway leading to pulmonary tissue damage through activation of the NF-κB and MAPK signaling pathways [42-44].

Classification of Nontuberculous Mycobacterial strains

Globally, NTM infection is increasing in CF patients [47]. *M. abscessus* is a rapid-growing NTM species that causes infection affecting many different organ types, including the central nervous system infections, skin and soft tissues, eyes, disseminated diseases and bacteremia in those who are severely immunocompromised such as those with untreated AIDS, but is found relatively frequently as a respiratory infection in susceptible individuals such as those with CF [45]. It is an acid-fast bacillus that is commonly found in soil and water. Due to their intrinsic multidrug resistance (MDR), they may cause recalcitrant infections in either the lungs or extra-pulmonary sites [12]. *M. abscessus* complex can be divided into three subspecies: *M. abscessus* subspecies *massiliense, M. abscessus* subspecies *abscessus,* and *M. abscessus* subspecies *bolletii* [46]. Laboratory identification of *M. abscessus* complex relies on the phenotypic method, such as biochemical testing, and molecular sequencing to differentiate between subspecies. *M. abscessus* is a quickly evolving, emerging human pathogen whose impact has grown considerably in the past decade [47]. Due to *M. abscessus* is a rapid-growing NTM, the genetic mutation for resistance to antibiotics is commonly occur in *M. abscessus* strains. The global distribution of *M. abscessus* strains from CF individuals found that the most dominant clones are *M. abscessus* clustered [78]. Moreover, Macrolide and aminoglycoside resistance in *M. abscessus* has been identified by the whole genome sequencing from this *M. abscessus* clustered [88].

Pulmonary disease is caused by inhalation of aerosolized *M. abscessus* transmission or direct contact with contaminated material. Additionally, infections caused by *M. abscessus* have been reported in contaminated materials and improper

surgical procedures, such as during cosmetic surgery, or the use of contaminated surgical equipment. *M. abscessus* may also be found in wound infections of post-operative patients [45]. While immunocompromised hosts are susceptible to *M. abscessus* infection, immunocompetent hosts may also be infected with *M. abscessus* [48]. Abnormal lung structure and pulmonary environments induce host susceptibility to *M. abscessus* infection [47, 49].

M. abscessus subspecies are inherently drug resistant. The Clinical and Laboratory Standard Institute (CLSI) list effective agents against *M. abscessus* complex as clarithromycin, amikacin, and cefoxitin. Mycobacteria have a complex cell wall made up of peptidoglycolipid containing mycolic acid and peptidoglycan which likely contributes to the mechanism of drug resistance and pathogenic virulence. Also, the recognition of functional erythromycin ribosome methyltransferase (*erm*) gene leads to inducible macrolide resistance through the 23s ribosomal RNA methylation [45, 50]. Additionally, biofilm formation allows *M. abscessus* to colonize and persist in the pulmonary environment. *M. abscessus* complex is resistant to many antimicrobial drugs that require prolonged treatment resulting in toxicity and poor therapeutic treatment outcomes [50].

Aims

The purpose of my thesis work is to investigate if repeated infection of various CF mouse models with *M. abscessus* influences the bacterial burden and organ pathology. **We hypothesize that repeated infections with *M. abscessus* is more**

likely to result in increased pathogenesis of disease in CF mouse models, as evinced by increased and sustained bacterial burden as well as worse organ pathology than a single infection. If our hypothesis is supported by experimental evidence, it may further increase public health measures to limit infection since NTM are ubiquitous in the environment and exposure to them are likely pervasive. We will test this hypothesis with the following Aims:

Aim 1: Develop in CF mice a model of reinfection with sub-optimal doses of *M. abscessus* and quantify the bacterial burden.

Aim 2: Analyze the lung histopathology of CF mice reinfected with *M. abscessus*.

CHAPTER 2 – MATERIALS AND METHODS

Preparation of *M. abscessus* OM194 stock

M. abscessus used in this study was an outbreak strain obtained from Dr. Andres Floto (University of Cambridge, UK): *M. abscessus* subsp. *massiliense* OM194. *M. abscessus* OM194 is an outbreak strain that has a rough colony morphology and produces biofilms *in vivo*. This OM194 strain is a clinical isolate of the subspecies *massiliense*. *M. abscessus* were grown in 7H9 broth with Glycerol, Dubos Oleic Albumin Complex (OADC), and Tween 80 to an optical density (OD) of 1.0, or a concentration of approximately 1 x 10^8 CFU/mL. The culture was then centrifuged at 2,800 g for 10 minutes at 23°C. The bacterial cell pellet was resuspended in fresh 7H9 broth with glycerol, OADC, and Tween 80. This solution was again centrifuged for 5 minutes at 2,800 g at 23°C. The supernatant was discarded, and the remaining pellet saved. Finally, 1.5 mL of bacterial culture was pipetted into glass vials, which were plugged and sealed. A titer was obtained for each final culture, and labelled vials were stored in cryovial boxes at -80°C.

Animal Model

Infection experiments were performed in three Cystic Fibrosis mouse models (1) $Cftr^{tm1UNC}/Cftr^{tm1UNC}$ mice, (2) $Cftr^{tm1UNC}$TgN(FABPCFTR) mice, and (3) β-ENaC transgenic mice. These mouse models were obtained from the Jackson Laboratories.

(1) $CFTR^{tm1UNC}$/ $CFTR^{tm1UNC}$ mouse model was backcrossed onto the C57BL/6 genetic background, called CFTR Knockout, inserted the stop codon into exon 10 of the CFTR coding sequence (S489X mutation) which generated null mutation of CFTR gene [6].

(2) $Cftr^{tm1UNC}$TgN(FABPCFTR) mouse model was knockout for murine CFTR gene but expressed CFTR gene into the intestinal villous epithelial cells under the control of rat intestinal fatty acid binding protein (FABP) promoter. These transgenic mouse models can prevent the gut obstruction and intestinal pathology from the defective CFTR gene, and improve the survival rate in CFTR-Knockout mouse model [6, 54].

(3) β-ENaC transgenic mouse model was an overexpressing of the β subunit of airway-specific epithelial Na^+ Channel (β-ENaC) mouse model, which allow to mimic increased ENaC activities in CF airways and develop the cystic fibrosis-like lung disease [67, 81].

Animal Infection

CF mice were infected with an intratracheal infection using a microsprayer of 35 µL containing 1.0 x 10^6 CFUs of *M. abscessus* OM194. The *M. abscessus* inoculum was prepared by thawing the bacterial vial. Thereafter, the mycobacterial suspension was obtained from the vial with a 1-ml tuberculin syringe fitted with a 26.5-gauge needle and expelled back into the vial. This procedure was repeated 20 times without removing the needle to mix the suspension and break up any small clumps of bacilli.

Mice were exposed to bacteria by intra-tracheal infection once, twice, thrice, and four times to establish a progressive infection. The time duration between infections is 24 hours. A group size of n=4 was sufficient given the power analysis referenced in section 2.6. We and staff at Colorado State University's Laboratory Animal Resources (LAR) monitored the mice daily for signs of distress indicating sickness from the bacterial infection. If mice became distressed, they were humanely euthanized by CO_2 inhalation, and their whole lungs, livers, and spleens were harvested.

Bacterial Burden

On day 5, day 15, and day 30 post-infection with the *M. abscessus* subsp. *massiliense* OM194, 4 mice from each group were humanely euthanized by using 20% carbon dioxide (CO_2) euthanasia and their whole lungs, spleens, and livers were harvested to determine the bacterial burden baseline at each time point. Organs were homogenized in 5 mL of phosphate-buffered saline (PBS), and serial dilutions were plated on nutrient 7H11 agar and TSA for 1 week at 30°C, and thereafter CFU were enumerated.

Organ Histology

The whole lungs and spleens from *M. abscessus* subsp. *massiliense* OM194 infected mice were fixed in 10% formalin, and left inside a biosafety level 3 (BSL-3) barrier at CSU for 2 weeks at 4°C. The organs were then transported to a biosafety

level 2 (BSL-2) laboratory at CSU and the 10% formalin was discarded from the organs. Then the organs were placed in 70% ethanol at 4°C for at least 2 days.

Next, the 70% ethanol was discarded from the organs, and the organs were immersed in 1XPBS at 4° C and sent to the Experimental Pathology Core Facility at CSU Veterinary Diagnostic Laboratories and Diagnostic Medicine Center for histopathological analysis with Hematoxylin and Eosin (H&E) stains and Ziehl-Neelsen stain (Acid Fast Bacillus (AFB) stains).

Statistical Analysis

Bacterial burdens in the *M. abscessus* subsp. *massiliense* OM194 infected animal organs were analyzed with GraphPad Prism version 4 (GraphPad Software, San Diego, CA), using analysis of variance (ANOVA) and Dunnett and Tukey multiple comparison tests. Data are presented using the mean values (n = 4) plus or minus the standard error of the mean (SEM). Significance was considered below a P value of $p<0.050$.

CHAPTER 3 – ANIMAL MODEL DEVELOPMENT

Development of Cystic Fibrosis Mouse Models

Recent research has found that among wild-type animal models and the defective animal models are all have different susceptibility to *M. abscessus* infection, many are able to clear bacterial infection quickly, do not achieve acute infection, and develop the progressive disease, as shown in Table 1 [68]. Several genetically modified mouse strains have been developed for use as animal models of Cystic Fibrosis [6, 51]. These CF mouse models allow to exhibit a high level of *M. abscessus* infection and become a good model to study the bacterial infection in the CF patient.

Generating the CF mouse models designed simply to disrupt the functional CFTR gene expression to create the null mutation or produce low level of CFTR mRNA, as shown in Table 2. The primary phenotypes from developing the CF animal models, including airway and intestinal electrophysiological defects, reduced fertility, and intestinal obstruction, which can mimic the CF in human. However, the characteristic of CFTR mutant mouse models appeared an abnormal immune profile which fails to thrive and increased the mortality rate in the early period of life. The null CFTR expression mouse model generated the loss of CFTR function, which can not detect CFTR mRNA in these mice [8]. The severity of intestinal pathology was affected to the survival rate of null mouse models. The hypomorphic strains and point mutation strains mouse models have been developed to improve nutrient absorption and increase lifespan in CF mice. These CF mouse models expressed low level of the CFTR mRNA [6]. The generation of transgenic CF mouse models

Table 1. Animal models were screened by using intravenous infection (i.v.) for susceptibility to *M. abscessus* infection. Snimal models which maintained a high level of infection are in blue, animal models which cleared the bacterial infection are in red, and models which have not been tested yet are in black [68].
Key: GKO, Gamma-Interferon knockout; MyD88, Myeloid differentiation primary response gene 88; SCID, Severe Combined immunodeficient; NOS2, Nitric Oxide Synthase 2; SOD, Superoxide Dismutase; TLR, Toll-like Receptor; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor; TNFR, Tumor Necrosis Factor Receptor; IL, Interleukin; Lep, Leptin

Structural Defects	Immune Defects	Immunocompetent
$CFTR^{tm1UNC}/CFTR^{tm1UNC}$	GKO-/-	C57/BL6
$Cftr^{tm1Unc}$-TgN (FABPCFTR)	MYD88-/-	C3HeB/FeJ
ßENaC transgenic	Nude/Beige	Lep^{ob}/Lep^{ob}
	SCID	
	NOS2-/-	
	SOD-/-	
	TLR2/4 -/-	
	TLR2/4/9 -/-	
	GM-CSF-/-	**Cutaneous**
Other Models	TNFR-1-/-	Nude/Beige
Guinea Pigs	IL-1-/-	CCL17 transgenic
Rabbits	Beige (*M. avium*)	Beige

High level of infection
Bacterial clearance
Not completed

Table 2. Genetically modified mouse strains have been developed for use as animal models of cystic fibrosis [6, 51].
Key: R, Replacement; I, Insertion; Ex, Exon; M, Methionine; Y, Tyrosine; F, Phenylalanine; G, Glycine; D, Aspatic acid; R, Arginine; H, Histidine
*Tg(FABPCFTR) strain is a gut-corrected CFTR-knockout mouse model; FABP, Rat Intestinal Fatty Acid Binding Protein; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator.
**Tg(CCSPScnn1b) strain is also referred to as β-ENaC overexpression mouse model; CCSP, Clara Cell Secretory Protein; Scnn1b, Sodium Channel Epithelial 1 Beta Subunit.

CF mouse strain	Mutation
Null mutation	
Cftr^{tm1Unc}	S489X Ex10 R
Cftr^{tm1Cam}	R487X Ex10 R
Cftr^{tm1Hsc}	M1X Ex1 R
Cftr^{tm3Bay}	Ex2 R
Cftr^{tm3Uth}	Y122X Ex4 R
Hypomorphic mutation	
Cftr^{tm1Hgu}	Ex10 I
Cftr^{tm1Bay}	Ex3 I
F508del mutation	
Cftr^{tm2Cam}	F508del R
Cftr^{tm1Kth}	F508del R
Cftr^{tm1Eur}	F508del
Other point mutations	
Cftr^{tm2Hgu}	G551D R
Cftr^{tm3Hgu}	G480C
Cftr^{tm2Uth}	R117H R
Transgenes	
Tg(FABPCFTR)*	CFTR
Tg(CCSPScnn1b)**	Scnn1b

was developed to increased survival rate of CF mouse models, which can mimic the CF lung disease in CF individuals [6, 51].

While some mouse models were discovered that demonstrate a susceptibility to infection and maintain a high level of *M. abscessus* group strains, including the CF mouse models with structural defects, $CFTR^{tm1UNC}/CFTR^{tm1UNC}$ mice, $Cftr^{tm1UNC}$TgN(FABPCFTR) mice, and β-ENaC transgenic mice [68]. The CF mouse model immune defects in the lung are shown in Table 3.

$CFTR^{tm1UNC}$ knockout mouse model shows abnormal cAMP-mediated chloride ion transport of epithelial tissue in the respiratory tract and gastrointestinal tract, similar to CF patients [8, 52]. Severe intestinal complications, including intestinal obstruction, develop in $CFTR^{tm1UNC}$ knockout mice leading to low survival rate in this CF mouse strain. Therefore, $Cftr^{tm1UNC}$TgN(FABPCFTR) mice which have a corrected lethal intestinal defect by using the rat intestinal fatty acid-binding protein (FABP) gene promoter for expression in intestinal epithelial cells [6, 51, 54]. This mouse model shows the expression of the intestinal villi and can rescue CF intestinal pathology [6, 51, 53].

Lastly, the β-ENaC transgenic mice are Cystic Fibrosis-like mouse models that are genetically modified to produce overexpression the β-subunit of epithelial sodium channels (ENaC). The ENaC overexpression in mice resulting in deplete the airway surface layer, reduce mucus clearance, and develop CF ion transportation pathophysiology in the lung [67], similar to the typical features of early lung disease in CF individuals [55]. These three CF mouse models have been screened in development of a reinfection model.

Table 3. Cystic Fibrosis mouse models screened to develop a reinfection model. Three CF mouse models were used in this study, including (1) $CFTR^{tm1UNC}/CFTR^{tm1UNC}$, (2) $Cftr^{tm1UNC}$TgN(FABPCFTR), and (3) β-ENaC transgenic mice.

Mutation	Genotype	Immune Effects in the Lung
$CFTR^{tm1UNC}/CFTR^{tm1UNC}$	CFTR KO (S489X mutation)	• Massive neutrophils infiltration [57] • Impaired maturation of the phagolysosome [35, 56-57, 61] • Delayed apoptosis in neutrophils [57] • Increased pro-inflammatory cytokines; IL-1β, IL-6, TNF-α, and increased chemokines; CXCL-1, CCL-2, CCL-3 [57-59, 64] • Decreased levels of anti-inflammatory cytokines; IL-10 [58, 61] • Decrease iNOS expression [62] • CFTR-mediated state of airway epithelial hyperinflammation [64]
$Cftr^{tm1Unc}$-TgN (FABPCFTR)	CFTR KO + transgenic CFTR intestinal fatty acid binding protein promotor (gut-corrected S489X mutation)	
βENaC over-expressing (βENaC-Tg)	Over expression of Epithelium Sodium Channel (ENaC)	• Macrophages and neutrophils accumulation in the airways [63] • Increased the neutrophil-attracting chemokines CXCL-1, CXCL-2 in BAL fluid [63] • Reduced/inactivated antimicrobial molecules; defensins, lysozyme, lactoferrin [65-66]

In this study, using sub-optimal doses of *M. abscessus* 1.0 x 10^6 CFU/mouse, Figure 1 shows a low bacterial load in the lungs, spleen and liver after one infection between day 5 and day 15 in the Cftr^{tm1UNC}TgN(FABPCFTR) mice. Also, in the CFTR^{tm1UNC}/CFTR^{tm1UNC} mice and β-ENaC transgenic mice, the bacterial burden tends to decrease after primary infection (Figure 1). Thus, additional infections (two infections) were administered to the CF mouse models using, resulting in an early higher bacterial burden in all three CF mouse models, which overtime resulted in bacterial clearance between 15 and 30 days in the spleen and liver. Although, it must be noted the bacterial burden in the lung of CFTR^{tm1UNC}/CFTR^{tm1UNC} mice and β-ENaC transgenic mice was still persistent (Figure 2). The CF models were exposed to 3 infections resulting in showed a higher bacterial burden between 5 and 25 days, however bacterial clearance resulted on day 30 in the Cftr^{tm1UNC}TgN(FABPCFTR) mice and CFTR^{tm1UNC}/CFTR^{tm1UNC} mice. However, the β-ENaC transgenic mice demonstrated bacterial persistance (Figure 3).

Of the three CF mouse models, β-ENaC transgenic mice exhibited the highest levels of infection in the lung after four times infections with *M. abscessus* subsp. *massiliense* OM194. Figure 4 shows a steady increase in bacterial load in all three mouse models. Cftr^{tm1UNC}TgN(FABPCFTR) mice tended to show slowly increase bacterial burden in all organs. CFTR^{tm1UNC}/CFTR^{tm1UNC} mice and β-ENaC transgenic mice tend to exhibit increased CFUs, especially in the lung. Hence, the multiple reinfections resulted in progressive *M. abscessus* pulmonary infection.

Pathogenesis of *M. abscessus* in Cystic Fibrosis Mouse Models

Lung pathology of CFTR^{tm1UNC}/CFTR^{tm1UNC} mice, Cftr^{tm1UNC}TgN(FABPCFTR) mice, and β-ENaC transgenic mice infected with four infections of *Mycobacterium abscessus* OM194 strain were stained with hematoxylin and eosin-stained (H&E) and were determined at day 5, day 15 and day 30 after reinfection.

As early as 5 days post-infection, as shown in figure 5, the influx of cells to the sites of infection are evident in all three CF mouse models. We observed small and moderate inflammatory cells influx into the site of infection in Cftr^{tm1UNC}TgN(FABPCFTR) and CFTR^{tm1UNC}/CFTR^{tm1UNC} mouse, respectively. The β-ENaC transgenic mouse exhibited the largest influx of inflammatory cells than the other two mice.

As the disease progressed on day 15, as shown in figure 6, increased inflammatory cell infiltration in the site of infection and granuloma formation are observed. A significant increase in foci of inflammatory cells are evident and clearly increased granuloma development in the CFTR^{tm1UNC}/CFTR^{tm1UNC} mice and β-ENaC transgenic mice.

Infection with *M. abscessus* OM194 strain for 30 days resulted in increased size and numbers of granuloma formation in all three CF mouse models, as shown in figure 7. The severity of pulmonary pathology was less in the Cftr^{tm1UNC}TgN(FABPCFTR) and CFTR^{tm1UNC}/CFTR^{tm1UNC} mouse models than compared to the β-ENaC transgenic mice at 30 days after reinfection. *M. abscessus* infected in β-ENaC transgenic mice exhibited more severe lung consolidation and increased size and number of granulomatous tissue upon examination of lung histology due to coalescence of granulomas in the lungs and increased inflammation.

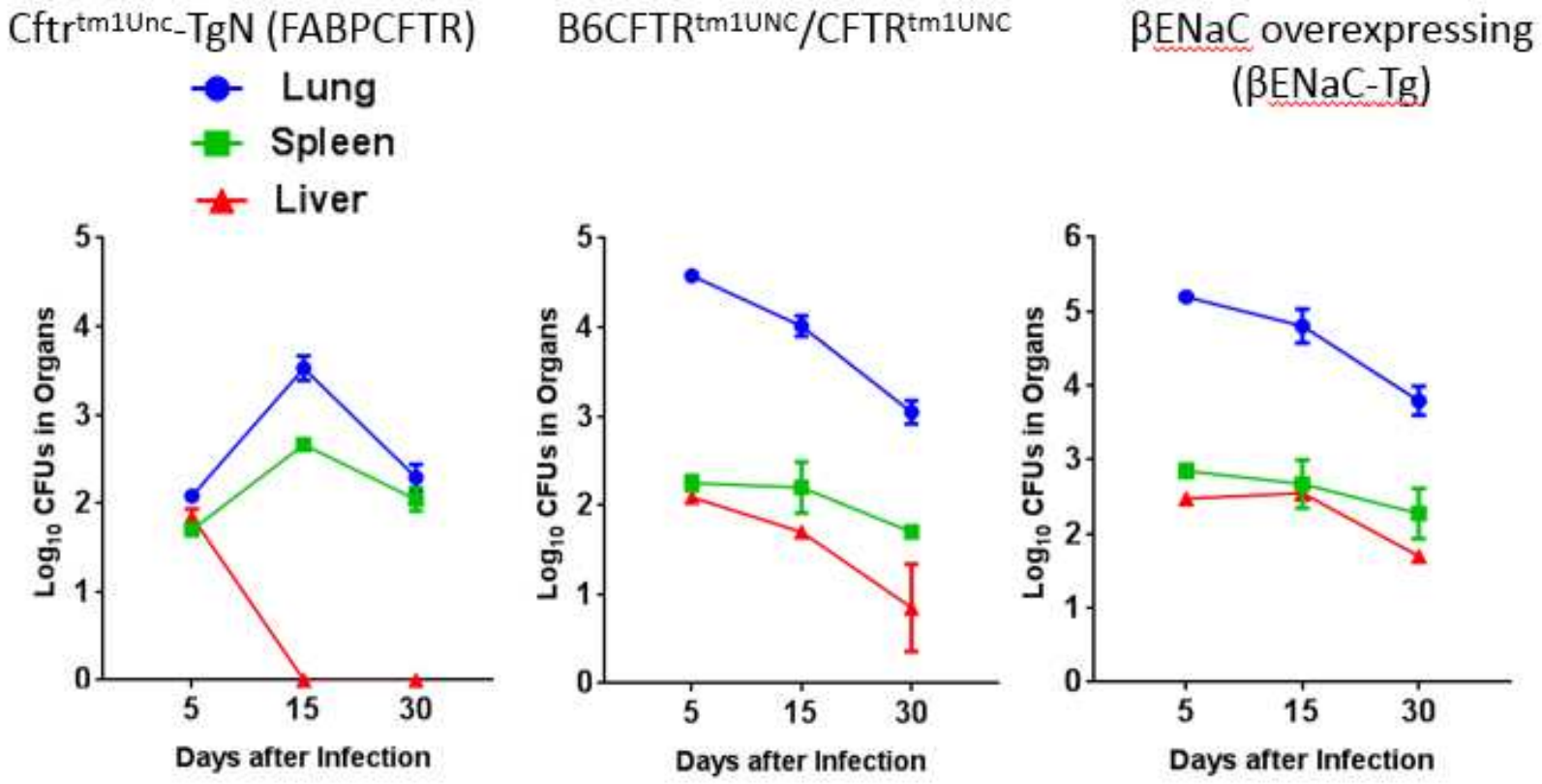


Figure 1. Bacterial Burden after One Infection. Primary infection results in lower bacterial load and clearance.

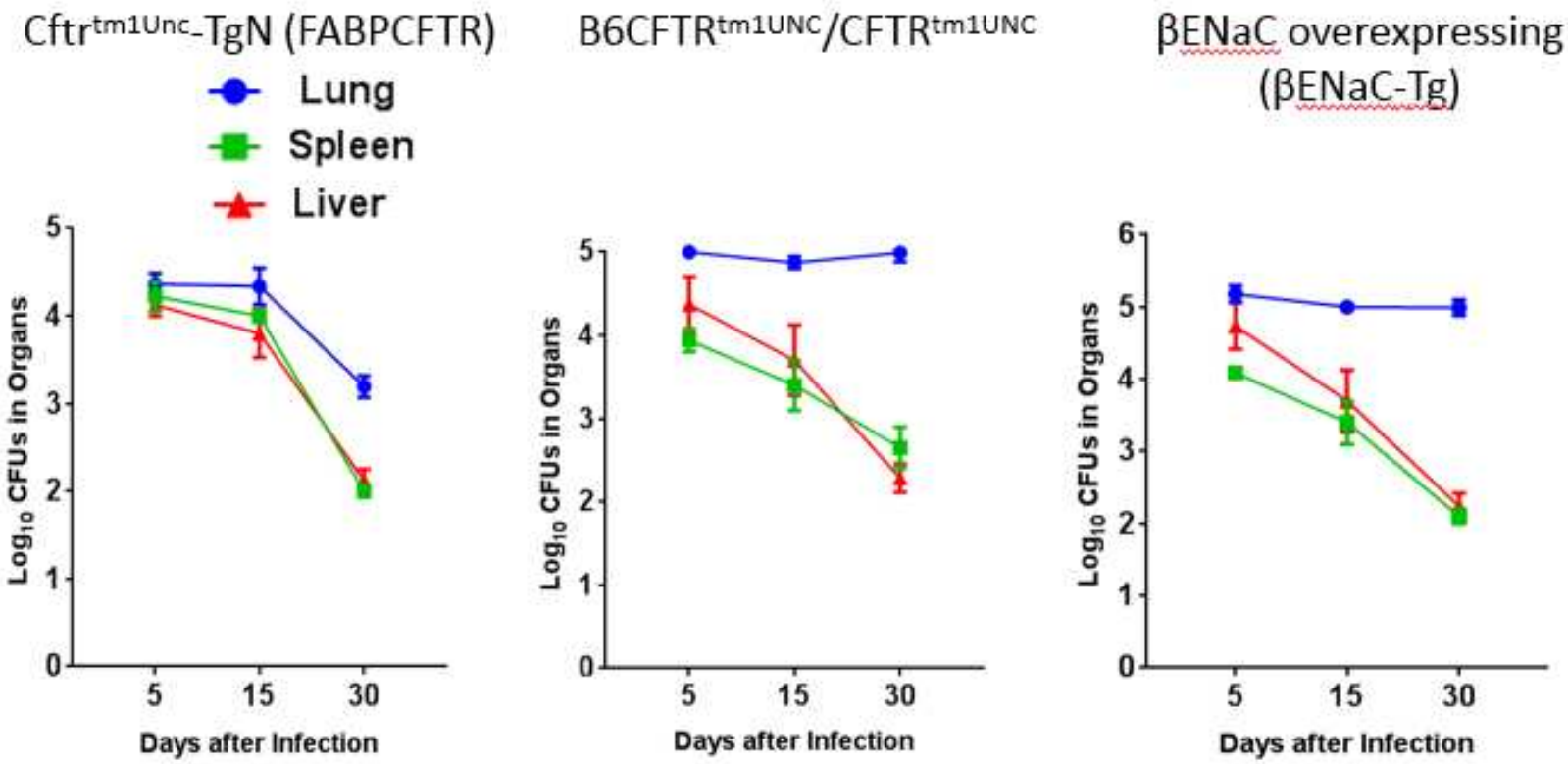


Figure 2. Bacterial Burden after Two Infections. Two infections result in an early higher bacterial load and clearance in spleens and livers.

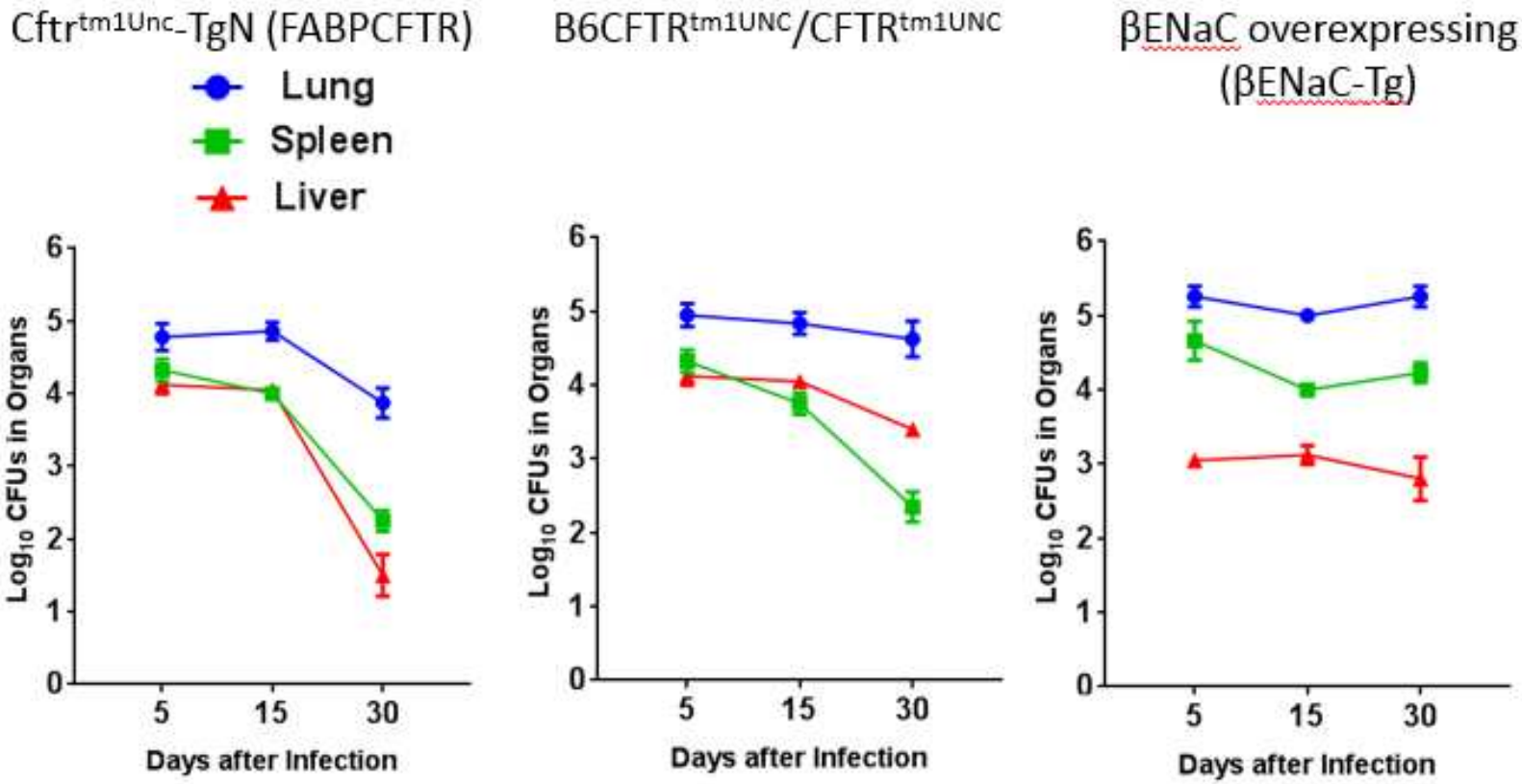


Figure 3. Bacterial Burden after Three Infections. Three infections result in higher lung bacterial burdens and clearance in spleens and livers.

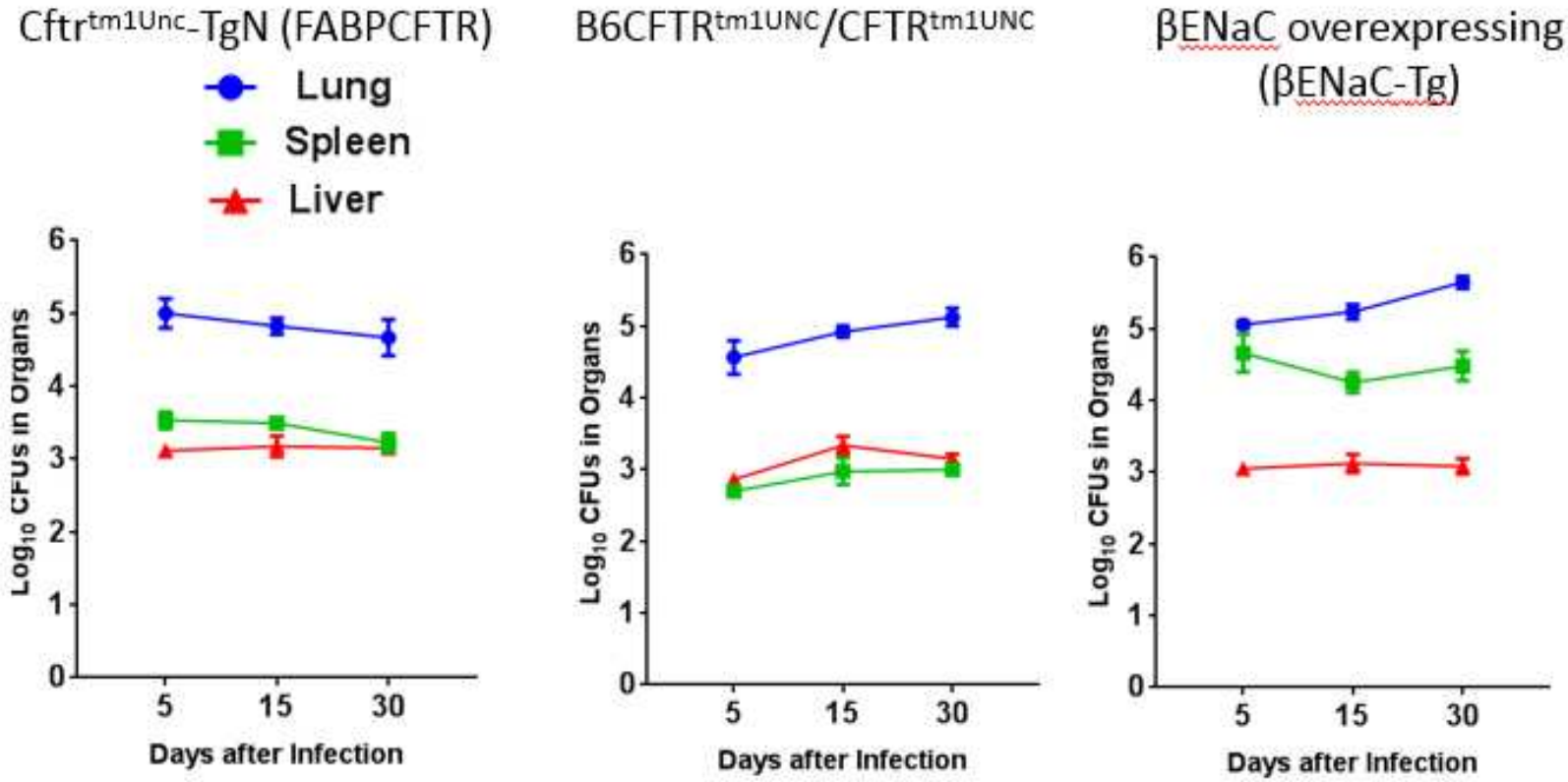
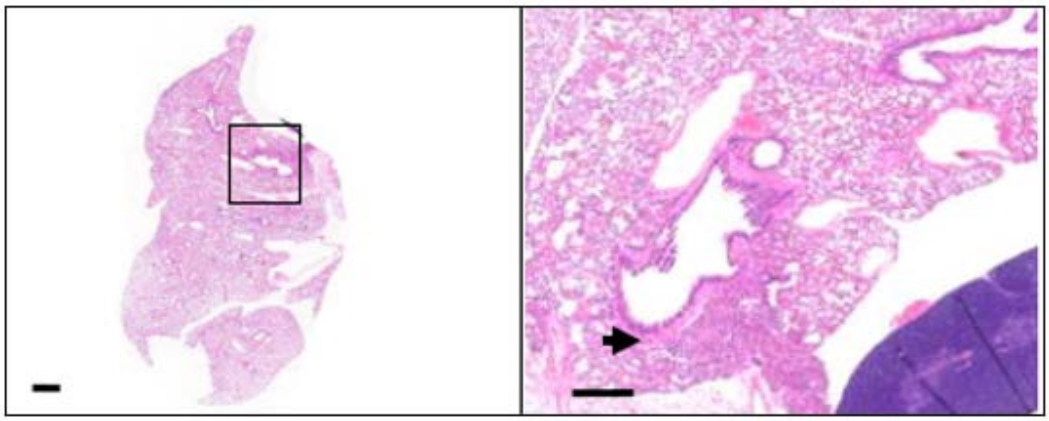
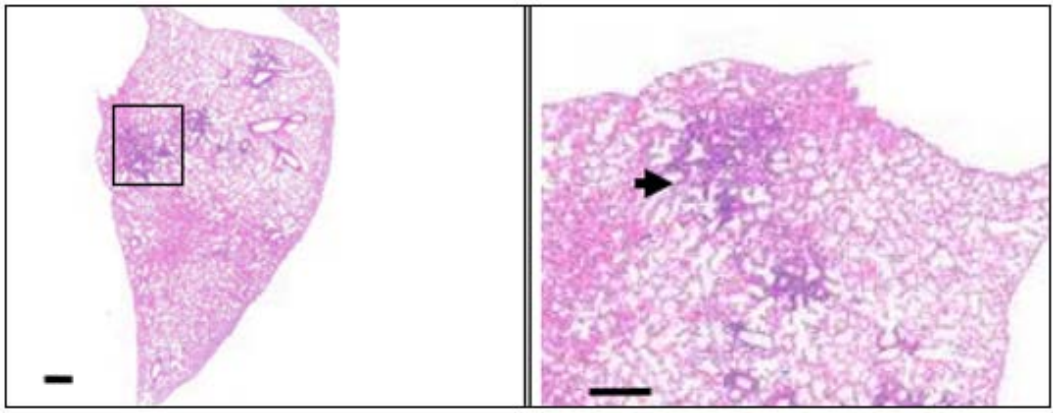


Figure 4. Progressive Infection after Four Infections. Multiple reinfections result in progressive pulmonary infection.

CFTR^{tm1UNC}-TgN (FABPCFTR)



B6CFTR^{tm1UNC}/ CFTR^{tm1UNC}



βENaC overexpressing

(β-ENaC Tg)

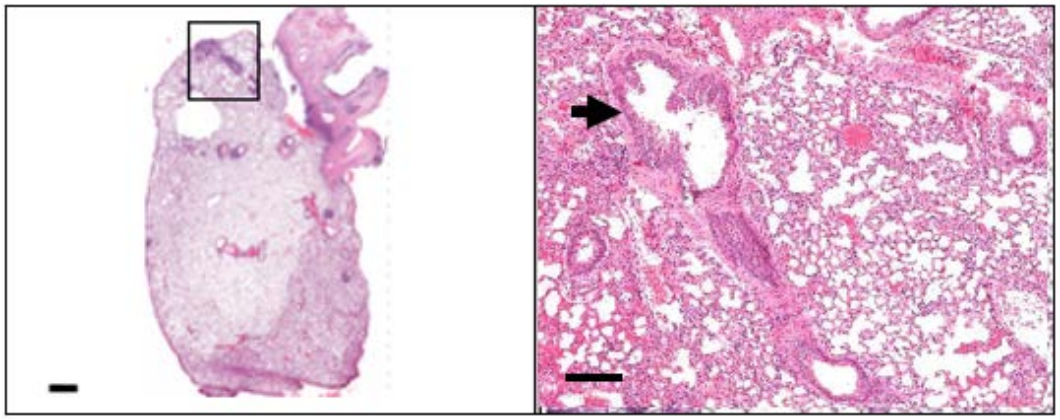
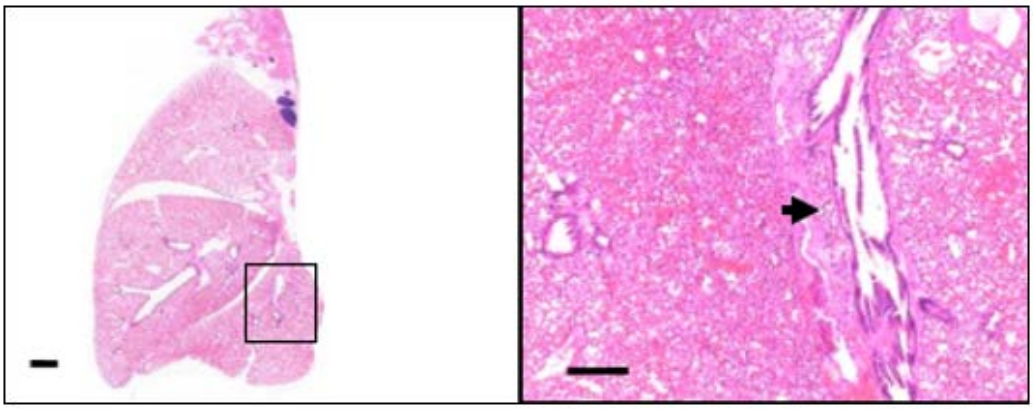
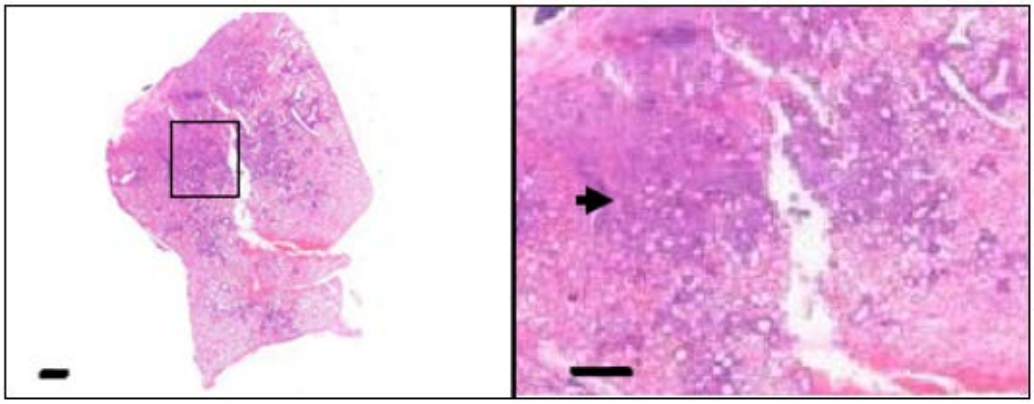


Figure 5. Organ Histology analysis of pulmonary tissue from *Mycobacterium abscessus* reinfection in cystic fibrosis mice at day5; Magnifications, 1X (left), and 20X (right).

CFTR^{tm1UNC}-TgN (FABPCFTR)



B6CFTR^{tm1UNC}/ CFTR^{tm1UNC}



βENaC overexpressing

(β-ENaC Tg)

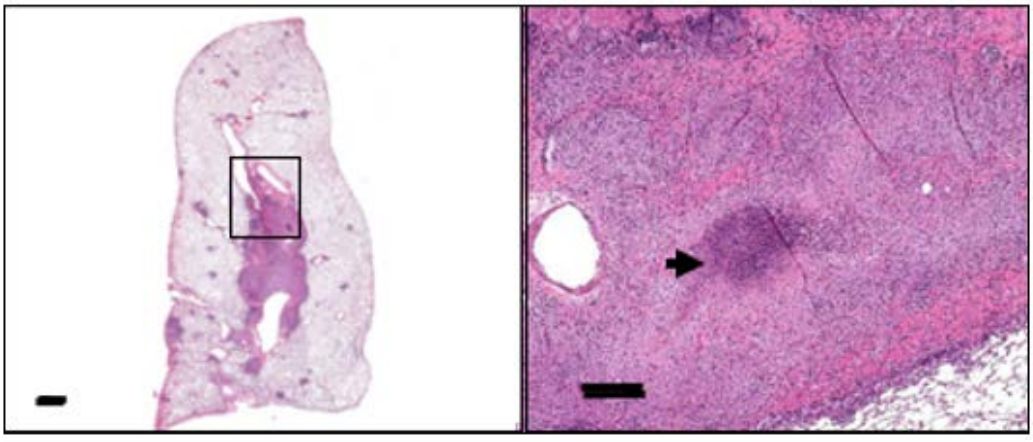
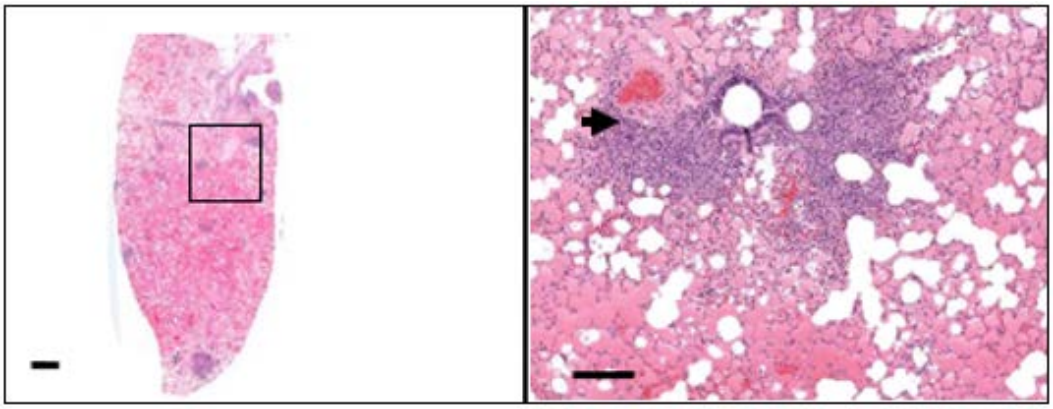
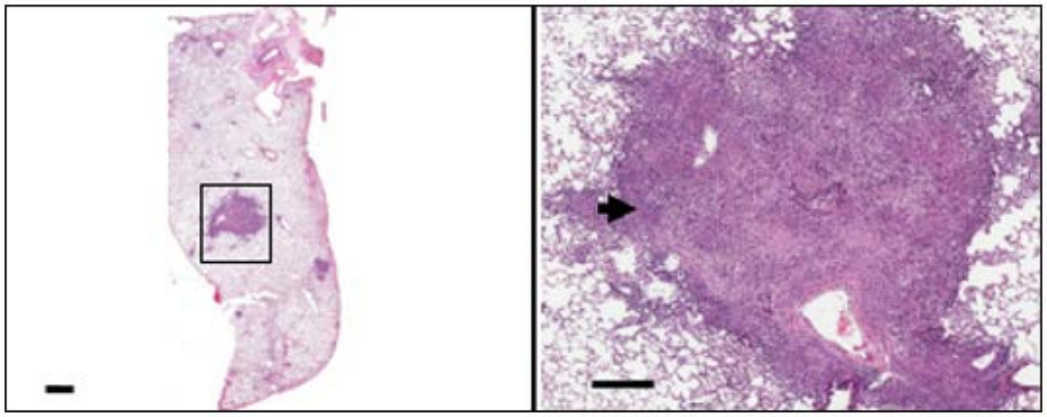


Figure 6. Organ Histology analysis of pulmonary tissue from *Mycobacterium abscessus* reinfection in cystic fibrosis mice at day15; Magnifications, 1X (left), and 20X (right).

CFTR^{tm1UNC}-TgN (FABPCFTR)



B6CFTR^{tm1UNC}/ CFTR^{tm1UNC}



βENaC overexpressing

(β-ENaC Tg)

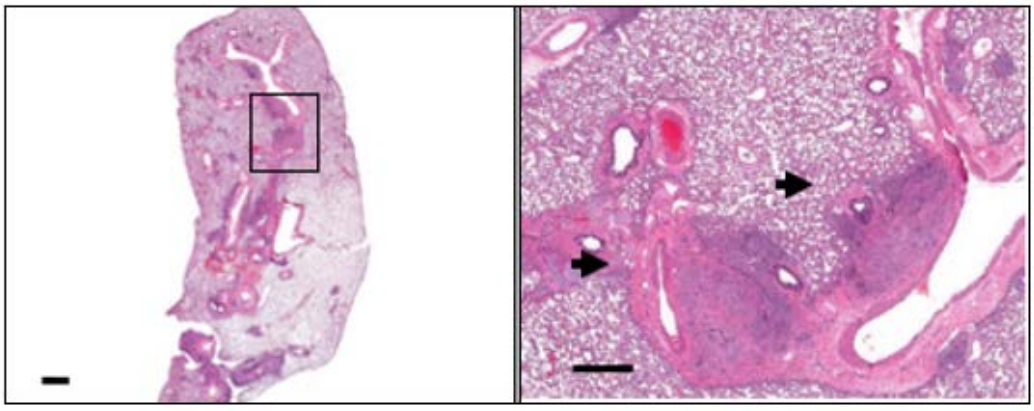


Figure 7. Organ Histology analysis of pulmonary tissue from *Mycobacterium abscessus* reinfection in cystic fibrosis mice at day30; Magnifications, 1X (left), and 20X (right).

CHAPTER 4 – CONCLUSION

To investigate the numbers of sub-optimal *M. abscessus* pulmonary infections required to develop progressive disease in CF mouse models, we set out to screen three CF mouse strains to model *M. abscessus* reinfection in CF individuals. We observed in all three Cystic Fibrosis mouse models that were exposed to four sequential pulmonary infections of *M. abscessus* OM194 resulted in progressive pulmonary disease which supports our hypothesis that reinfection of NTM from the environment is more likely to result in disease progression in the CF mouse model. In this study, we found that four sequential exposures with sub-optimal numbers of *M. abscessus* OM194, the $CFTR^{tm1UNC}/CFTR^{tm1UNC}$ mice, $Cftr^{tm1UNC}$TgN(FABPCFTR) mice, and β-ENaC transgenic mice developed a progressive infection denoted by increased bacterial burden in lungs, spleens and livers and pulmonary pathology denoted by increased number and size of the granulomatous inflammation.

Over the last ten years, the NTM species have resulted in quickly evolving and emerging serious pulmonary infections, especially among patients that are immunocompromised and in patients with structural lung disease such as chronic obstructive pulmonary disease, bronchiectasis, and cystic fibrosis. This becomes a major threat to the patient's health. The detection rate of NTM-positive culture from individuals with CF has increased remarkably over time in USA and Europe [47, 69-72]. The human environment, natural inhabitants of natural waters, engineered water systems and soils, have been observed as a major sources of transmissible pathogenic NTMs, and thus it is generally accepted that NTM infection was acquired through

environmental contamination [73-77]. Moreover, in the previous study by Bryant, *et al.* strong evidence was found of indirect human-to-human transmission of *M. abscessus* within CF treatment facilities, indicating that the long-distance spread of *M. abscessus* are being transmitted by fomites [78]. Also, Dr. Jackson and Dr. Ordway studied the virulence and pathogenicity of *M. abscessus* subsp *massiliense* epidemic strain, Brazilian BRA100 strain *M. massiliense* CRM-0019, which was shown that the outbreak strain of *M. abscessus* subsp *massiliense* is particularly virulent in part because of increased resistance to the treatment [79]. Consequently, repeated exposure of exogenous infections by contact with pathogenic NTMs from the environment may allow for the bacterial genetic adaptation, and influence the dissemination and development of NTMs to become a lung pathogen and lead to progressive pulmonary disease in CF individuals [80]. It is important to have CF mouse models to study the quantities of repeated inhaled *M. abscessus* required for development of progressive infection and ultimately disease resulting in severe lung tissue pathology, and pulmonary inflammation. Also, these could be helpful for testing experimental therapies prior to clinical trials.

In our study, we found that β-ENaC transgenic mice achieved the highest level of susceptibility to a progressive disease after four infections. The high bacterial burden persisted after four infections in β-ENaC transgenic mice and maintained in the organs by day 30, indicating that these mice are unable to control *M. abscessus* with adaptive or innate immune response. This implies that β-ENaC transgenic mice are the best model to mimic CF individuals with NTM infection. β-ENaC transgenic mice are a functional candidate for testing the novel anti-mycobacterial drug for CF patients

because of the high-level of disease progression in β-ENaC transgenic mice after four infections.

Additionally, we found that the β-ENaC transgenic mice and $CFTR^{tm1UNC}/CFTR^{tm1UNC}$ mice developed lung pathology in the disease and progressed on day 15 and day 30 post-reinfections. Since humans develop pulmonary infection in both necrotizing and non-necrotizing granulomas, the β-ENaC transgenic mouse and $CFTR^{tm1UNC}/CFTR^{tm1UNC}$ mouse models are useful to study the organ histopathology observed in CF patients.

According to the National Jewish Health, the survival rate of CF patients has been increased in over the past three decades [89] since the CF animal model has been developed to improve quality of life and help to the development of the novel treatment for CF individuals. Animal models of CF are important for human diseases which is help to the further understanding in the mechanism of CF disease progression and CF pathology. However, the use of CF mouse models is limited by short life span of CF mice, which may not develop the chronic bacterial infection or chronic lung disease, unlike CF individuals. In human, chronic bacterial infection involves in the progressive and irreversible damage in the respiratory system remain the most prominent cause of elevated morbidity and mortality in CF. Also, the recurrent bacterial infection due to the antibiotic resistance is problematic for CF, which fails to cure pulmonary exacerbations and infections [3]. These problems remain to be life-threatening implications that might be solved by the novel therapeutic treatment.

The impact of the bacterial reinfection in CF patients is an urgent question in the *M. abscessus* field. Our recent development of a reinfection CF mouse model indicates

that the reinfection process is able to inhibit host immunity to control the infection and allow for *M. abscessus* to grow and persist in the lung. The exogenous reinfection contributes to the development of increased bacterial burden and organ pathology. This thesis is intended to understanding *M. abscessus* pulmonary infection in CF mouse models which might also lead to new therapeutic treatments and the better understanding of the interaction between CF immune system and the progression of bacterial infection, and how they contributed to the chronic inflammation leading to other CF conditions.

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LIST OF ABBREVIATIONS

AFB	Acid Fast Bacillus
ANOVA	Analysis of variance
AP-1	Activator Protein-1
ASL	Airway Surface Liquid
BAL	Bronchoalveolar lavage
BSL-2	biosafety level 2
BSL-3	biosafety level 3
C57bl/6	C57 black 6
cAMP	Cyclic adenosine monophosphate
CCL-2	C-C motif chemokine ligand 2
CCL-3	C-C motif chemokine ligand 3
CXCL-1	C-X-C motif chemokine ligand 1
CXCL-2	C-X-C motif chemokine ligand 2
CXCL-8	C-X-C motif chemokine ligand 8
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFTR KO	Cystic Fibrosis Transmembrane Conductance Regulator knockout
CFU	Colony forming units
CO_2	Carbon dioxide
Ca^+	Calcium ion
Cl^-	Chloride ion

CLSI The Clinical and Laboratory Standard Institute

ER Endoplasmic Reticulum

erm Erythromycin Ribosome Methyltransferase

ENaC Epithelium Sodium Channel

ER Endoplasmic Reticulum

FABP Fatty acid-binding protein gene

Foxi1 Forkhead box I1

GKO Gamma-Interferon knockout

GM-CSF Granulocyte-Macrophage Colony-Stimulating Factor

HCO_3^- Bicarbonate ion

H&E Hematoxylin and Eosin

IFNs Interferons

IFN-α Interferon- α

IFN-β Interferon-β

IL-1β Interleukin-1β

IL-8 Interleukin-8

IL-10 Interleukin-10

iNOS Inducible nitric oxide synthase

LAR Lab Animal Resources

Lep Leptin

LPS Lipopolysaccharide

MAPK Mitogen-activated protein kinase

MCP-1 Monocyte chemoattractant cytokine-1

MDR Multidrug resistance

MyD88 Myeloid differentiation primary response gene 88

Na^+ Sodium ion

NET Neutrophil extracellular trap

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells

NFAT Nuclear factor of Activated T cells

NOS Nitric Oxide Synthase

NTM Non-tuberculous Mycobacteria

OADC Dubos Oleic Albumin Complex

OD Optical density

PAMPs Pathogen-associated molecular patterns

PBS Phosphate Buffered Saline

PMN Polymorphonuclear neutrophils

rpm Revolutions per minute

rRNA Ribosomal RNA

SCID Severe Combined Immunodeficient

SEM Standard Error of the Mean

SOD Superoxide Dismutase

TLRs Toll-Like Receptors

TLR4 Toll-Like Receptor 4

TNF- α Tumor Necrosis Factor alpha

TNFR Tumor Necrosis Factor Receptor

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

TSA Tryptic Soy Agar

V-ATPase Vacuolar Proton-ATPase