THESIS

# UNDERSTANDING THE PROCESS OF *MYCOBACTERIUM ABSCESSUS* REINFECTION ON HOST IMMUNITY

Submitted by

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ABSTRACT

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*Mycobacterium abscessus is a bacterial pathogen emerging in humans. The bacterium is difficult to treat, and it results in increased mortality. The patient case rates of diseases associated with this bacterium are increasing. Mycobacterium abscessus pathogenesis is poorly understood which limits our understanding of M. abscessus. The bacterium is part of the diverse family of nontuberculous mycobacterial species (NTM), and it is classified as a member of rapidly growing mycobacteria (RGM). These are environmental pathogens and they are found in tap water, showerheads, natural water sources, and soil.*

*M. abscessus* can infect individuals by breathing in aerosolized water particles, contact with surface fomites and transmission from infected patients. Individuals with human immunodeficiency syndrome (HIV) and are immunocompromised are more susceptible to infection with *M. abscessus*. Also, individuals with cystic fibrosis, chronic obstructive pulmonary disease or using tumor necrosis factor α (TNF- α) inhibitors are also susceptible to becoming infected with *M. abscessus*. Surprisingly, immunocompetent, tall slender Caucasian, non-smoking women also show an increasing rate of being infected with *M. abscessus.*

To gain a better understanding *M. abscessus* pathogenesis and immune modulation, we developed βENaC over-expressing mouse models (βENaC-Tg). The main goal for this study was to discover the re-infection with the bacterium to study immune responses induced by *M. abscessus*. This is particularly important since the *M. abscessus* is an environmental pathogen and from birth to death we are all suspected to it. The reinfection process can have serious consequences in immune-compromised patients causing immune exhaustion, or in other words where the immune system is unable to fight against the bacteria after several times of infection. Our *M. abscessus* immunological results have shown after βENaC-Tg mice that are exposed to 4 subsequent aerosols of *M. abscessus* intracheal infections their CD4+ T cells show increased expression of the immune exhaustion marker PD1+ and CD11b+ macrophages show increased expression of another immune exhaustion marker CTLA4+. Together, these results support the idea that repeated environmental exposure to *M. abscessus* through environmental sources can potentially result in immune exhaustion leading to lack of effective function of T cells and macrophages to kill *M. abscessus*.

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I am very grateful to my family and friends, for being encouraging and supportive.

# DEDICATION

This work is dedicated to all patients infected with *Mycobacterium abscessus*, and/ or with every type of Nontuberculous Mycobacteria, which are difficult to treat.

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# Chapter 1, Introduction

## 1.1 Historical and Current Relevance of *Mycobacterium abscessus* and classification of NTB

### *Mycobacterium strains*

The family of Mycobacteria is divided into two main groups: *Mycobacterium tuberculosis* complex and Non-tuberculous mycobacteria (NTM), which includes all the other mycobacteria species. [5]. *M. abscessus* is a complex of, rapidly growing acid-fast bacillus (AFB) NTM which is an environmental pathogen and is found in tap water, showerheads, and in soil, [6, 7]. *Mycobacterium abscessus* was isolated from a knee abscess in 1951 [5]. In the 1950s Ernest Runyoun found a way to classified nontuberculous mycobacteria, called the Runyon classification system, which is based on the rates of growth and pigmentation [4, 8]. Until 1992 *M. abscessus* subspecies abscesses was in the same species with *bolettii*. However, due to differences in drug susceptibility patterns between, *M. abscessus subspecies* and *M. abscessus subspecies bolettii*, was re-classified as a new subspecies.[5]. Currently, three subspecies of *M. abscessus* are known: *M. abscessus sbsp massilliense, sbsp. abscessus*, and *sbsp. bolletii* shown in Table 1 [9]

*Mycobacterium abscessus* (MABS) group strain pulmonary infections, particularly in patients with chronic obstructive pulmonary disease (COPD), bronchiectasis, and cystic

fibrosis (CF), are increasing in prevalence throughout the world (1). The quantities of inhaled MABS and the number of exposures required for infection and ultimately progression of disease remain unknown. Studying whether reinfection plays a potential role in the pathogenesis of nontuberculosis mycobacterial (NTM) lung disease is highly relevant since reinfection most likely occurs given the ubiquitous nature of NTM in the environment. The missing knowledge gaps in understanding the stages of MABS infection, colonization, dissemination, and disease progression are due to the intractability of transient infection in human patients and the lack of a reinfection animal model. A greater understanding of NTM immunopathogenesis is vital for the development of rational and effective treatment approaches for patients with chronic obstructive pulmonary disease, bronchiectasis, and cystic fibrosis.

*Mycobacterium abscessus* is an emerging human pathogen, and the case rate associated with the bacterial infection has increased during past decades [10, 11]. As mentioned above, there are three subspecies of *M. abscessus* including *M. abscessus subspecies abscessus, M. abscessus subspecies bolletii*, and *M. abscessus subspecies massilliense* [9]. New species of NTM were discovered after the development of 16S rRNA gene sequencing [4, 8]. It is known that the 16S rRNA gene of mycobacteria is very conserved [8]. That is why, when there is a difference in sequencing 1% or more it shows a new species [8]. Nevertheless, to distinguish *M. abscessus* from *M. chelonae* this sequencing is not a reliable option, since these two species differ by only 4 pair 9bp0 within the 16S rRNA genes [8].

It is important to have species-level identification of NTM clinical examples since antimicrobial sensitivity defines treatment options, [8].

Two criteria are considered while doing phenotypic testing of NTM species; growth rate and pigmentation [4, 8]. When identifying NTM species by the growth rate, the bacteria is being classified as either slowly growing mycobacteria, or rapidly growing mycobacteria (RGM) [8]. RGM are described as NTM clinical example which is forming colonies in seven days in subculture [8]. *Mycobacterium abscessus*, *M. chelonae,* and *M. fortuitum* are examples of RGM. In contrast, for slowly growing mycobacteria more than seven days are required to create a colony upon subculture [8]. *Mycobacterium intracellular* and *M. avium* belong to slowly growing mycobacteria. To identify mycobacterium species pigmentation is not used as frequently. However, it is well-known that *M. tuberculosis* strains are lacking pigmentation and have rough colony morphology [8]. That is why when there is pigmentation, it is an indication of a strain of NTM [8]. For slowly growing Mycobacteria identification high-performance liquid chromatography (HPLC) is an established method, but this does not work for RGM [8].

It has been suggested by the Infectious Diseases Society of America (IDSA) and American Thoracic Society (ATS) that since *Mycobacterium avium* complex and *Mycobacterium intracellulare* are currently clinical identical they do not need to be differentiated from one another, also because treatment will not be modified based on species-level identification [8]. Furthermore, IDSA and ATS recommended in order to differentiate between *M. abscessus*, *M. chelonae*, and *M. fortuitum* PCR restriction

endonuclease assay (PRA) should be used [8]. Additionally, it has been suggested by ATS and IDSA that in order to obtain species-level identification for *M. abscessus*, *M. chelonae*, and *M. fortuitum* one can use the susceptibility of RGM to cefoxitin, amikacin, clarithromycin, ciprofloxacin, doxycycline, linezolid, sulfamethoxazole, and tobramycin [8]. Aside from those two leading research companies, recent literature suggested that identification of *M. abscessus* can be done even to the subspecies level with rpoB and erm gene sequencing [5] That means that the rpoB and erm gene sequencing will determine if the clinical isolate is drug resistant to macrolides, such as *M. abscessus subspecies abscessus*, and *M. abscessus subspecies bolletii* which have the inducible erm gene while *M. abscessus subspecies massilliense* lacks an erm gene and doesn't result in macrolide drug resistance [12]. Thus, it is important to know the subspecies specificity of each *M. abscessus* strain to differentiate which strains have an increased ability to express then erm gene and result in a drug resistant macrolide disease.

Epidemiology of *Mycobacterium abscessus*

*M. abscessus's* pulmonary or disseminated forms are caused by inhalation of aerosolized bacteria, it even can be transmitted by person to person [13, 14]. In contrast, the cutaneous form of *M. abscessus* can be a result of non-sterile tattooing practices, unclean needles used for cosmetic surgery [15-17]. For a long time, it has been believed that *M. abscessus* can affect only immunocompromised individuals. However, recent studies have shown that it can affect immunocompetent individuals as well [13, 18]. Although it can affect immunocompetent individuals, those people who are immunocompromised such as those who have HIV or AIDS, patients with a lung

transplant, patients who take steroids have increased susceptibility to *M. abscessus* [13, 18]. Those individuals who suffer from Cystic Fibrosis are at high risk for infection with *M. abscessus* [19, 20]. Patient with bronchiectasis and other lung abnormalities results an increased susceptibility to *M. abscessus* disease [14, 21].

It has been thought for a while that not only immunocompromised individuals are susceptible to the bacteria. Recent studies show that non-smoking, immunocompetent, post-menopausal caucasian, tall, slender females have increased risk of disease with *M. abscessus* [22]. The possible explanation is that in tall, slender females, elongation of the chest cavity (pectus cavatus) may result in their increased susceptibility [22]. Additionally, slender individuals have more adiponectin and less leptin, which may also increase the risk of infection with *M. abscessus*. Recent, studies showed that leptin deficient mice are more susceptible to *M. abscessus* disease and supports this hypothesis. Moreover, this study also revealed that low levels of leptin decrease expression of T helper 1 (Th1) cell IFN-gamma production, which is responsible for activating macrophages harboring the bacterial leading to killing of *M. abscessus*. In contrast, the high level of adiponectin upregulates IL-10 and IL-1R expression, and lower expression of TNF-α [19]. Also, another possible explanation for this hypothesis is that most likely women cough less effectively compared to men due to social reasons, sometimes described as “Lady Windermere syndrome” [4, 19]. This phenomenon itself can make the bacteria clearing from the lungs more challenging. Finally, low levels of estrogen in post-menopausal females may increase risk because estrogen binding to the estrogen receptors on macrophage enables more efficient phagocytosis [19]. All

these potential risk factors for *M. abscessus* infection points to the urgent need for improved treatments and additional studies to understand pathogenesis.

According to the Centers of Disease Control and Prevention (CDC) in 2019 in the United States, the annual prevalence of NTM infection increased to 39.6 cases/100,000 population in 2016 and annual incidence to 19.0 cases/100,000 population. Overall prevalence for the study period was higher in the elderly. Interestingly, the highest prevalence in the United States was observed in Hawaii. It has 396 cases per 100000 people annually. Pulmonary infection is 1.4 times higher in women over 65 than in men over 65. CDC is stating that People age 60 years or older, annual prevalence of NTM infection increased from 19.6 cases per 100,000 people between 1994 and 1996 to 26.7 cases per 100,000 people between 2004 and 2006 [7].

Drug Treatment against NTMs

*Mycobacterium abscessus* can cause a chronic pulmonary infection, that needs to be treated for up to 2 years [12]. Drug treatment against NTM*s* contains a macrolide, such as an azithromycin or clarithromycin, and an aminoglycoside such as amikacin or azithromycin, and cefoxitin as cephalosporin or a fluoroquinolone such as levofloxacin [23, 24] (Table 2).

Fluoroquinolones interrupt the synthesis of nucleic acid [25]. Cephalosporins are β-lactams and interrupt the synthesis of the cell wall [25, 26]. To inhibit protein synthesis by *M. abscessus,* macrolides bind to 50S ribosomes and aminoglycosides bind to 30S ribosomal subunits [25]. To interfere protein synthesis macrolides also bind to the 23S ribosomal RNA (rRNA) [27]. *Mycobacterium abscessus* acquires the aminoglycoside

resistance by a p55 efflux pump [4]. Erythromycin ribosome methyltransferase (41) genes (erm (41) genes) were found in the isolated *M. abscessus subspecies abscessus* and *M. abscessus subspecies massiliense* species [28]. These erm(41) genes methylate the 23S rRNA causing macrolide resistance in these pathogens [28, 29]. These multiple resistance factors expressed by clinical isolates of *M. abscessus* results in the potential for high levels of drug resistance and cause a challenge to treat patients infected with these pathogens [30].

The majority of individuals infected with *M. abscessus* take these antibiotics for a long duration and sadly the treatment doesnt result in a cured disease [31]. *Mycobacterium abscessus* is an intracellular pathogen and it is capable to survive within macrophages [32, 33]. This characteristic gives the bacteria the ability to "hide" from other immune cells that can potentially kill the pathogen resulting in pathogen survival. Biofilm production also protects the bacteria from exposure to bactericidal compounds. Biofilm production also contributes to the chronic nature of the infection [33]. The long exposure to antibiotics leads to multi-organ toxicity in many patients [23]. The chronic *M. abscessus* infection leads to an individual's undergoing lung resection to save their life [34-36]. Experiencing recurrence of disease is common even after successful drug therapy [34]. For example, some patients after a lung transplant when diagnosed with *M. abscessus* infection may result in subsequent successful disease cure. Nevertheless, a month after disease cure reinfection with *M. abscessus* occurred and the patient was diagnosed with deep gluteal abscesses caused by recurrence of the bacteria, and ultimately developed bronchiolitis obliterans [34]. That is why it is urgent to

find new more effective drug treatments in order to prevent recurrence-and re-infection. In Section 1.3 we will extend further on the drug resistance and re-infection.

There are significant differences between immune response in humans and the mouse [37]. For instance, the pro-inflammatory interleukin-32 (IL-32) cytokine, which plays an important role in human immune response, is not produced in mice [38, 39]. In humans, the immune response against *M. abscessus* infection not was well characterized or understood [22]. However, the understanding of *M. abscessus* pathogenesis in humans is growing. As an example, recently it has been shown that *M. abscessus* is less likely to survive inside of macrophages unlike slow-growing mycobacteria, also, it is more likely to consume macrophages which leads to the injure release of reactive oxygen species [18]. *Mycobacterium abscessus* human infection leads to changes in the morphology of rough and smooth morphology of the strains overtime during treatment which remains poorly understood [40]. MyD88 (myeloid differentiation primary response gene 88) is an adaptor protein which has found on toll-like receptors (TLRs), during the infection with *M. abscessus* of alveolar cells, which activates NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, which induces cytokines' production which inflammation [10].

## 1.2 Cystic Fibrosis and Human Immunity

Cystic fibrosis is a result of the mutation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), which is affecting multi-systemic organs, including the respiratory system, digestive system, reproductive system, and immune system [10, 11]. CFTR was discovered in CF patients from the 1950s [9]. The ions homeostasis in the cell is changed after the CFTR gene mutation. In a normal state, the CFTR gene expresses at the CFTR protein channel, and this protein is responsible for the transportation of Chloride ($Cl^-$) ion over the body. CFTR's function also refers to other ions production, such as Sodium ($Na^+$) ion. Nevertheless, CFTR proteins defected was observed when there is a mutation of the CFTR gene in the CF patients. The mutation results in ion dysregulation, thus $Cl^-$ ion cannot get out of the cell as it happens during the normal phase. Also, hyper-absorption of $Na^+$ ions is caused by the high level of $Cl^-$ ion inside of the cell, which leads to thickened mucus since there is no water inside [4, 41, 42] (Figure 1).

Autosome recessive genetic disorder caused the mutation of CFTR. There were founds of almost 1600 mutations of this gene [40]. Depending on the type of gene mutations the defects vary. Based on the effect on the channel function these mutations are systematized into 6 classes. These classification criteria are based on several things such as absence of CFTR protein, less expression of CFTR protein the expression of the dysfunctional CFTR protein [40, 41].

The symptoms of CF are individual for every patient. In some rare cases, the disease might be diagnosed during the first month of life. However, the disease might stop progressing and will appear later during adulthood. The pulmonary disease has

more obvious characteristics and is more serious for patients. In healthy patients, the function of the mucociliary process results in clearing all mucus, bacteria, and debris, to prevent the airways from the infection. In contrast, in CF patients there are issues with their lungs, due to non-functional mucociliary clearance and thick secretions causing airway plugging and promotes bacterial colonization.

After the defective effect of CF in the respiratory tract there still can be a bacterial infection and inflammation. In fact, for approximately 80% of CF patients, it is common to get a secondary infection by other organisms. There are several most common problematic pathogens, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus, Hemophilus influenza*, and non-tuberculosis mycobacteria to cause infection in CF patients [43]. Those are evading the host immune defense by making biofilm. Depending on the CF patient's region and the population there have been 3.7-24% nontuberculosis mycobacteria isolated from those patients [44]. Bronchial wall damaged is caused as a result of chronic bacterial infection and inflammatory, also, it is leading to respiratory failure from repeated CF, which itself is causing death.

As mentioned above, the mutation of CFTR gene mutation causes CFTR dysfunction, which is affecting the immunity, both in cellular and humoral immunity [11, 42]. Cystic Fibrosis is one of the most difficult pulmonary diseases to manage, and it highly affects the patient's life [11]. As the first sign of CF can be the dense mucus, which also can increase the colonization of bacteria and infection in airways. Pathogen-associated molecular patterns (PAMPs) have an immunostimulatory effect and it causes

excessive inflammation in the airway. PUMPs enhances the pro-inflammatory mechanisms by increased production of chemokines (IL-8 and TNF) and cytokines as well as a high level of polymorphonuclear neutrophils (PMN) [14]. Several studies showed that the bronchoalveolar lavage fluid which was contained from the infant with CF, showed a high level of IL-8 and TNF-alpha, which means that there is an increased secretion [15]. Enhanced bacterial level in the airways and the attachment to the epithelial membrane induces biofilm production to protect bacteria, which leads to the expression of PAMPs. Thereafter, the local immune response is increasing, and more leukocytes are recruiting to the infection sites [16-18, 20, 21].

A study showed an increase in NF-κB signaling and pro-inflammatory cascade, which includes $Ca^{2+}$ dependent signaling, mitogen-activated protein kinase (MAPK) – dependent activation of activator protein, and activated T-cell transportation's nuclear factor in CFTR deficiency cases which was compared to wild-type CFTR [28]. It is well-known that NF-κB signaling plays a great role in the regulation of immune response to infection, and how CFTR mutation affects this pathway has been shown in several studies [23-25, 29]. In a healthy condition, TLR4 is expressed on the surface of the epithelial cells in airways in response to MyD88, it activated Trif-dependent signaling. Trif pathway is induced Interferon's (IFNs) production, such as IFN-α and IFN-β, that play an important role in clearing the airway. In contrast, these mechanisms seem distracted in CF patients. The dysfunctional CFTR genes cause increased activation of the NF-κB pathway as a result of TLR-MyD88 signaling activation, as well as decreased Trif-dependent signaling [22]. Expression of TLR4 is decreased in CF patients and it

has lower sensitivity to LPS stimulation and MyD88 and Trif signaling [22]. A primary function of Tregs is to mitigate the potentially harmful effects of inflammation [45-47]. While Tregs may play a host-defense role in various mycobacteria, viral, parasitic, and fungal infections [48] as well as help limit inflammation and pathologic damage in disease, a reduced influx or exhaustion of Tregs to the lungs, particularly before MABS infection is under control, is known to result in increased inflammation and worsen mycobacterial disease in experimental animals and primary human cells ]. Tregs induce macrophages toward an alternative “deactivated” phenotype (also known as M2 macrophages) M1 macrophages are essential for killing mycobacteria [46, 49, 50].

### 1.3 Drug Resistance

As mentioned above, drug resistance is remaining as a major issue in *M. abscessus* treatment. It has been shown that all anti-tuberculosis drug treatments are not helpful for all clinical isolates of *M. abscessus* [8]. This bacterium is a multi-drug resistant [5]. Resistance to clarithromycin can be caused by the mutations in the 23S rRNA gene of *M. abscessus* [8]. Furthermore, resistance to amikacin can be gain due to mutation in the 16S rRNA dene of *M. abscessus* [8]. The study showed that in vitro the bacteria is resistant to clarithromycin, amikacin, cefoxitin, clofazimine, and imipenem [8]. Nevertheless, when treating pulmonary *M. abscessus* there are no reliable drug regimens that can alter the salvia from culture-positive to culture-negative over a certain period [8]. Surgical resection of the damaged lung tissue and at least a year antibiotic combination treatment such as clarithromycin, amikacin, and cefoxitin are the most effective treatment for pulmonary *M. abscessus* [8]. Pulmonary infection in most cases have chronic nature and is incurable [8].

NTM cause many challenges for effective antibiotic treatments [4, 28, 29]. The hydrophobic cell envelop of Mycobacteria is serving as a great protection to the entry of lipophilic antimycobacterial [41, 51, 52][41, 51, 52][38, 48, 49][38, 48, 49][38, 48, 49][38, 48, 49][41, 51, 52][41, 51, 52][41, 51, 52][41, 51, 52][41, 51, 52][41, 51, 52][41, 51, 52][41, 51, 52][38, 48, 49][38, 48, 49][38, 48, 49][38, 48, 49][38, 48, 49][38, 48, 49] agents. Additionally, β-lactamases conferring resistance to β-lactams with other antibiotic inactivation mechanisms plays a significant role for NTM drug resistance [25, 26, 53]. Mycobacteria produce aminoglycoside phosphotransferases and aminoglycoside acetyltransferases; those are responsible for the resistance to amikacin and to other aminoglycosides [54]. Mycobacteria has a unique natural resistance to rifamycin, pyrazinamide ethambutol, and isoniazid [55-57]. Ethambutol is a component to which most rapidly growing mycobacteria are resistant in general [58]. The, resistance to aminoglycosides and to tetracyclines is responsible the pp55 efflux pump [4]. As already have been mentioned, the expression of erythromycin ribosome methyltransferase gene was observed in all isolates *of M. abscessus sbsp. abscessus* [4, 28]. Due to methylation of the 23S rRNA, these genes erm [41] arrogate causative macrolide resistance in these pathogens [4, 28, 29]. However, in *M. chelonae* these genes are not found [4]. To detect the stimulation of these genes and macrolide resistance, we cannot use the traditional testing since it takes prolonged incubation observation [59] Clarithromycin contributes more to causative macrolide resistance than Azithromycin [60] (Figure 2).

Another challenge in developing effective treatment is the fact that the *in vitro* susceptibility assays works differently, then it does *in vivo* [61, 62]. Additionally, we have limited understanding of antibacterial treatments pharmacokinetics at the infection site [62, 63]. The pharmacokinetics and pharmacodynamics have an issue to develop and test the effect for each compound at the infection sites, since there is a great level of variability in the drugs loading into the macrophages liposomes [64]. A very few antibiotics were showed to have as good in vivo effect as they do in vitro for NTM, after all these resistance mechanisms paired with MIC and minimum bacterial concentration (MBC) [55-60, 62].

### 1.4 Aims

We hypothesize that after four times of reinfection there will be expression of markers of immune exhaustion on CD4+T cell expression of PD1+ cells and CD11b+ macrophages expression of CTLA4 in reinfected CF βENaC-Tg mouse model. The objective of this project is to understand how the re-infection of *M. abscessus* impacts the immune response in cystic fibrosis (βENaC-Tg) mouse model.

Thus, our aims are as follows:

Aim1: Does *M. abscessus* re-infection (4 times) result in the expression of markers of immune exhaustion PD-1 on T cells in cystic fibrosis mice? Experiments will be conducted exposing an aerosol infection 4 times to the (βENaC-Tg) mice and evaluating the lungs for using flow cytometry to evaluate the immune responses of T cell expression of PD-1 day 5, 15, 30 after reinfection.

Aim 2: Does *M. abscessus* re-infection (4 times) result in expression of markers of immune exhaustion CTLA4 on CD11b+ macrophages in cystic fibrosis mice? Experiments will be conducted exposing an aerosol infection 4 times to the (βENaC-Tg) mice and evaluating the lungs for using flow cytometry to evaluate the immune responses of macrophage expression of CTLA4 on day 5, 15, 30 after reinfection.

Chapter 2, Material and Methods

## Chapter 2 Material and Methods

### 2.1 Animal Infection

Mice were infected using a micro-spray aerosol with *M. abscessus* OM194, 40 µL containing 1 x $10^6$ colony forming units (CFU), with a rough morphology and positive for biofilm formation. The *M. abscessus* subspecies abscessus OM194 strain is a rough

clinical isolate from Dr. Andres Floto, University of Cambridge, UK. The *M. abscessus* inoculum was prepared by thawing the bacterial vial. After that, we obtained the mycobacterial suspension from the vial with a 1-ml tuberculin syringe fitted with a 26.5-gauge needle and expelled back into the vial. We repeated this procedure back and forth into the vial 20 times without removing the needle to mix the suspension and break up small clumps of bacilli. All animals used were β-ENaC transgenic mice (Table 3).

### 2.2 Culturing of Nontuberculous Mycobacterial Strains

All strains of *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 2.5 x 10^8 CFU / mL. Afterward, the culture was centrifuged for 10 minutes at 23°C at 3,500 revolutions per minute (rpm). We resuspended the bacterial cell pellet in fresh 7H9 broth with glycerol, OADC, and Tween 80. After resuspending, the solution was centrifuged again for five minutes at 350 rpm at 23°C. The supernatant was saved, and the remaining pellet discarded. Lastly, 1.5 mL of bacterial culture was pipetted into glass vials, which were plugged and sealed. For each final culture, a titer was obtained, and labeled vials were stored in cryovial boxes at -80°C.

### 2.3 Animal Model βENaC-Tg

The cystic fibrosis mouse strain used in this study was the β-ENaC transgenic mice. These mice have an overexpression of the epithelial $Na^+$ channel (ENaC) which is responsible for regulation of airway surface liquid hydration. It is composed of three subunits: α, β, and γ, and those three subunits are over-expressed expressedin this

model. The over expression of the β subunit results in $Na^+$ hyperabsorption, which leads to dehydration of airway surface liquid, leading to thickened mucus accompanied by delayed bacterial clearance, and lung inflammation resulting in increased inflammation [65]

## 2.4 Bacterial Burden

On day 5, day 15, and day 30 post-infection with the *M. abscessus* OM194, four mice from each group were humanly euthanized by using 20 % dioxide ($CO_2$) euthanasia and their lungs, livers, and spleens were harvested to determine the bacterial burden baseline in each time point. Afterward, we homogenized organs in 5 mL of phosphate-buffered saline (PBS), and serial dilutions were plated on nutrient 7H11 agar and incubated for 1 week at 30°C when CFU was enumerated.

## 2.5 Flow Cytometry

Primary infected and re-infected βENaC-Tg mice lungs were evaluated by flow cytometric analyses. This analysis will allow us to get 24 parameters on each cell. We performed a lung cell digestion to provide single cell suspension of lung leukocytes for flow cytometry, in order to evaluate the pathogenesis by tracking the immune response after infection. We quantified the of CD4+ T cell expression of PD1+ expression by flow cytometry and the CD11b+ macrophages expression of CTLA4+ positive cells. This analysis will help to understand if the reinfection modulates the immune system to express markers of immune exhaustion (PD1+ and CTLA4+) causing cells to not function effectively to kill *M. abscessus*.

## 2.6 Statistical Analysis

Bacterial burdens in the *M. abscessus* infected animal organs are compared to naïve mice, animal infected once, twice, three times and four times 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 P-value of $p<0.050$.

# Chapter 3, Animal Model Development

## 3.1 Development of βENaC-Tg Mouse Model

There have been several mouse strains tested for *M. abscessus* infection [10]. It has been shown that bacterial infection is quickly being cleared by other immunocompetent mouse strains, which does not led to an acute infection. However, some

immunodeficient mouse models were discovered that demonstrate susceptibility to infection and maintained a high level of *M. abscessus*, including the CF mouse models with structural defects, βENaC-Tg transgenic mice [10].

Micro sprayer intratracheal instillation technique was used to deliver the bacteria to achieve pulmonary infection. By using this technique, we have been dosing the mice with moderate numbers $1x10^6$ CFU of *M. abscessus*, afterwards evaluating the disease progression at 10, 20, and 30 days after infection.

As it is shown in Figure 3 the mice have been exposed to isoflurane anesthesia and positioned on the intubation platform. In Figure 3C mice were microsprayed with 40 ul Cy5.5 dextran in a pulmonary aerosol demonstrating that the pulmonary aerosol goes to the pulmonary cavity and not the stomach. Figure 3D shows the *M. abscessus* bacterial burden infection, which has been delivered by using the pulmonary microsprayer technique. This procedure was used for our reinfection and after four reinfections we evaluated bacterial organ burden in the lungs and flow cytometic changes on days 10, 20, and 30 after the pulmonary re-infection procedure. Our results suggest that the βENaC mouse model after four re-infections demonstrates a productive disease, denoted by increased bacterial lung burden, airway inflammation, and can be used as a model to better understand what has been seen in human subjects with isolated CF *M. abscessus* lung disease.

Once we had established the primary infection βENaC mouse model we developed a reinfection βENaC mouse model [Figure 4 A-D]. We employed our experience and

knowledge gained with the βENaC-Tg microsprayer intratracheal instillation technique which was developed by exposing mice with low to high numbers ($1x10^4$- $1.0x10^{10}$CFU) of MABS and evaluated disease progression at 10, 15 and 30 days after infection. We optimized the reinfection process using suboptimal primary infection doses of MABS between $1x10^4$-$1x10^8$. Ultimately, the optimal amount of MABS to allow for βENaC mouse primary infection and initial immune clearance, reinfection and subsequent persistence and disease progression that did not result in mouse mortality was a dose of $1x10^6$ CFUs. [Figure 4] shows the reinfection process with MABS outbreak strain MABS OM194.

3.2 Immune Response to reinfection with *M. abscessus*

Dr. Ordway's laboratories previous research with reinfection models of *Mycobacterium tuberculosis* (MTB) [10] has shown that reinfection causes a substantial memory T cell response, which quickly collapses. This study gives us an initial idea of the reinfection process of *M. abscessus*. Verver et al. [66] showed that patients with active tuberculosis successfully treated with chemotherapy have a four times higher chance to present a new infection. In Dr. Ordway's laboratory, they modeled this in mice infecting them with the optimal dose of virulent *M. tuberculosis* W-Beijing strain HN878 and afterward treating these animals with chemotherapy to sterilize them. The protection and the memory response were lost with a concomitant increase in expression of the exhaustion marker PD-1 on these cells like pointing to the underlying cause.

A primary function of Tregs is to mitigate the potentially harmful effects of inflammation [45-47]. While Tregs may play a host-defense role in various mycobacteria, viral, parasitic, and fungal infections [48] as well as help limit inflammation and pathologic damage in disease, a reduced influx or exhaustion of Tregs to the lungs, particularly before MABS infection is under control, is known to result in increased inflammation and worsen mycobacterial disease in experimental animals and primary human cells [42, 67-70].

### 3.3 Reinfection Results in Immune Exhaustion

The understanding of the process of *M. abscessus* transmission, virulence and pathogenesis are limited. Of the few studies evaluating the microbial constituents of the home environment of individuals with CF and their association with lower airways infections, all showed CF patients acquire *M. abscessus* infections from contaminated nebulizers, and environmental sources [71, 72]. The limited studies from different groups [73, 74] and from our laboratory [75, 76] support the hypothesis that outbreak strains have a heightened capacity infection patterns, are more drug-resistant and have a worse clinical outcome.

We demonstrate in Figure 5 that after 4 suboptimal exposure of *M. abscessus* OM194 that using our flow cytometry gating strategy shown in Figure 5 that the CD4+ T cells show increased expression of PD-1 markers of immune exhausting after the fourth bacterial exposure. We predict that reinfection of cystic fibrosis mice will result in

increased bacterial burden, organ pathology, and immune exhaustion markers PD1+ on CD4+T-cells.

Increased percentages of CD4+PD1+T cells were present after a low dose reinfection of *M. abscessus* OM194 in cystic fibrosis mice analyzed by flow cytometry compared to naïve controls. T cells were gated with a primary gate on viable FSC vs. SSC lymphocytes compared to the naïve isotype controls and then on $CD4^+$ T cells, and analyzed for changes in the total mean percentages of CD4+ PD1+ cells over the course of infection Significantly increased percentages of migrating CD4+ PD1+ T cells between day 5 and 30 to the lungs of mice occurred after reinfection (Student T test*, p=0.001), compared to naïve mice. Results represent the mean percentage of cells of five mice for each condition from one experiment (±SEM).

We demonstrate in Table 4 the differences in the percentages of naïve and infected mice after 4 suboptimal exposure of *M. abscessus* OM194 using our flow cytometry gating strategy shown in Figure 5 that the CD4+ cells shows a significant increase in the percentage of PD1+ markers of immune exhausting after the four bacterial Intratracheal (IT) exposures and after day 5, 15 and 30 days.

We then went onto evaluate if then macrophage population of cells in the lungs of mice reinfected would also after four IT infections and on days 5, 15 and 30 demonstrate an gradual increase of then exhaustion maker CTLA4+ on CD11b+ macrophages. We demonstrate in Figure 6 that after 4 suboptimal exposure of *M. abscessus* OM194 that

using our flow cytometry gating strategy shown in Table 5 that the CD11b+ macrophages show significant increased expression of CTLA4+ markers of immune exhausting after the fourth bacterial exposures and 5, 15 and 30 days after infection.

Table C shows then percentage of postive CD11b+CTLA4+ naïve mice, after four pulmonary *M. abscessus* intratracheal (IT) reinfections, and after infection progressed on days 5, 15 and 30 in the Beta enacmice inducing significantly increased numbers of CD11b+CTLA4+ exhaustion markers compared to naïve mice. The changes of naïve and *M. abscessus* reinfected cells, were analyzed for changes in the total mean percentages of CD11b+CTLA4+ cells over the course of infection. Significantly increased percentages of CD11b+CTLA4+ between day 5, and 30 in the lungs of mice occurred after reinfection (Student T test*, p=0.001, compared to naïve mice). Results represent the mean percentage of cells of five mice for each condition from one experiment (±SEM).

The changes of naïve and *M. abscessus* reinfected cells, were analyzed for changes in the total mean percentages of CD11b+CTLA4+ cells over the course of infection. Significantly increased percentages of CD11b+CTLA4+ between day 5, and 30 in the lungs of mice occurred after reinfection (Student T test*, p=0.001, compared to naïve mice). Results represent the mean percentage of cells of five mice for each condition from one experiment (±SEM).

## Chapter 4, Discussion

To achieve progressive infection of *M. abscessus* after several reinfections in the cystic fibrosis mouse model, we developed this work to screen a cystic fibrosis mouse model to model *M. abscessus* reinfection with clinical endpoints with CF individuals.

Previously at Dr. Ordway's laboratory, we observed three cystic fibrosis mouse models infected with suboptimal *M. abscessus* sbsp. *massiliense* OM194 that results in

progressive pulmonary infection. This finding supports our original hypothesis that reinfection with *M. abscessus* from the environment is more likely to cause progressive infection in the CF mouse model.

In this work, we showed that after four times infection in β-ENaC transgenic mice there was a high level of infection and developed progressive disease. In β-ENaC transgenic mice, the high bacterial burden remained after four times of infection and maintained in the organs by until day 30. This data shows that this mouse model is unable to control an adaptive or innate immune response for clearing the mycobacterial infection. Thus, we concluded that this mouse model is the best mouse models for studying *M. abscessus* re-infection if we want to achieve some clinical endpoints with CF individuals. The disease progression and increase in bacterial burden in this mouse model show the potential to be used as a candidate mouse models for testing the novel anti-mycobacterial drug for CF patients.

Moreover, we found that β-ENaC transgenic mice developed lung pathology in the disease progressed on day 15 and day 30 post-reinfections. β-ENaC transgenic mice is a good model for histopathology since it mimics the same as in humans, as humans develop pulmonary infection both necrotizing and in-necrotizing granulomatous inflammations (data not included).

Our study also showed that in a β-ENaC transgenic mice mouse model after four times reinfection gradually resulted in increased markers of immune exhaustion. We

demonstrated that by analyzing the exhaustion markers PD-1 markers on CD4+ T cells were expressed in higher amounts after four *M. abscessus* exposure. Also, the β-ENaC transgenic mice mouse model after four times of *M. abscessus* exposure showed a gradual increase of CD11b+ macrophages expressed higher amounts of another immune exhaustion marker CTLA4. The expression of immune exhaustion markers results in the cell's being physically present in the mouse, but these cells have reduced antimicrobial functions to be able to kill *M. abscessus*. This means that after several times of infection the immune response is no longer able to fight against the pathogen, and that is what happens frequently in CF patients.

The need to understand the impact of the reinfection with *M. abscessus* in CF patients is an urgent matter in the NTM field. Our recent study on reinfection of our CF mouse models showed that the process of reinfection was able to inhibit immunity and allow *M. abscessus* to persist and cause pulmonary infection and progressive disease. The reinfection helps the development of increased bacterial burden and dissemination in the tissue and lung pathology.

We have two hypothesis that are plausible where reinfection could result in a progressive infection in a CF patient. The first hypothesis is that upon initial *M. abscessus* attachment to the upper airways and/or lungs *M. abscessus* could persist in these sites with the potential of the cough reflux to result in multiple pulmonary exposures. This initial endogenous reinfection process of *M. abscessus* persistence would allow *M. abscessus* to replicate to higher numbers over time and combined with the cough reflux instill *M.*

*abscessus* into mid to lower regions of the lung. Our second hypothesis proposes that an exogenous source of *M. abscessus* exposes the individual to *M. abscessus* and reinfection occurs directly in the upper airways persisting and/or directly infecting the lungs over time, increasing *M. abscessus* numbers causing progressive infection. Once we had established the primary infection βENaC mouse model we developed a reinfection βENaC mouse model. We employed our experience and knowledge gained with the βENaC-Tg microsprayer intratracheal instillation technique which was developed by exposing mice with low to high numbers ($1x10^4$- $1.0x10^{10}$CFU) of *M. abscessus* and evaluated disease progression at 10, 15 and 30 days after infection. We optimized the reinfection process using suboptimal primary infection doses of *M. abscessus* between $1x10^4$-$1x10^8$. Ultimately, the optimal amount of *M. abscessus* to allow for βENaC mouse primary infection and initial immune clearance, reinfection and subsequent persistence and disease progression that didn't result in mouse mortality was a dose of $1x10^6$ CFUs. shows the reinfection process with *M. abscessus* outbreak strain MABS OM194.

In conclusion, little is known about the early stages of *M. abscessus* infection or reinfection in the upper respiratory track of CF patients. However, additional studies are needed using *M. abscessus* strains expressing fluorescent markers using whole body mouse xenogen imaging (which allows for tracking the specific site of infection) after pulmonary infection shows to further understand the persistence of immune cells in the nasopharyngeal passageways prior to dissemination into the lower airways. In addition, understanding the true impact of immune exhaustion on other cellular phenotypes, such as granulocytes, dendritic cells and CD8+ cell's is warranted.

TABLES AND FIGURES

Table 1 describes the Cystic Fibrosis βENaC-Tg mouse model characteristics which has been shown to possess similar properties to human clinical disease, such as immunity supporting bacterial persistence, airway inflammation, and mucus plugging resulting in pulmonary pathology [1-3].

## Table 1. Mouse Models of Cystic Fibrosis

| Mutation | Genotype | Immune Effects in the Lung |
|---|---|---|
| βENaC over-expressing (βENaC-Tg) | Over expression of Epithelium Sodium Channel (ENaC) | • Macrophages and neutrophils accumulation in the airways<br>• Increased the neutrophil-attracting chemokines CXCL-1, CXCL-2 in BAL fluid<br>• Reduced/inactivated antimicrobial molecules; defensins, lysozyme, lactoferrin |

Table 2. Two major NTM complexes and the recommended treatment regimen for each.

| Species | Recommended Treatment Regimen |
|---|---|
| *Mycobacterium avium complex* | Standard regimen is a macrolide (azithromycin or clarithromycin), |
| ***Mycobacterium abscessus* complex** | For *M. abscessus* complex species with a functional erm41 gene, a recommended regimen includes daily clofazimine and cefoxitin (or imipenem) plus thrice weekly amikacin for the first 2-3 months. For species without the functional *erm41* gene, daily macrolide (azithromycin or clarithromycin) and cefoxitin (or imipenem) plus amikacin thrice weekly for the first 2-3 months. |

Table 3, above describes the Cystic Fibrosis βENaC-Tg mouse model characteristics which have been shown to possess similar properties to human clinical diseases, such as immunity supporting bacterial persistence, airway inflammation, and mucus plugging resulting in pulmonary pathology [1-3].

| Mutation | Genotype | Immune Effects in the Lung |
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Table 4.
Kinetic influx of CD4+PD1+ T cells in naïve and *M. abscessus* reinfected cystic fibrosis mice. Data from 1 experiment in which mice were infected daily for 4 days in a row with suboptimal doses of *M. abscessus* and then mice were assayed (that received the 4 suboptimal doses) on Day 5, Day 15, and Day 30. Kinetic influx of CD4+PD1+ T cell in *M. abscessus* naïve and reinfected cystic fibrosis mice. Increased percentages of CD4+PD1+T cells were present after a low dose reinfection of M. abscessus OM194 in cystic fibrosis mice analyzed by flow cytometry compared to naïve controls. T cells were gated with a primary gate on viable FSC vs. SSC lymphocytes compared to the naïve isotype controls and then on $CD4^+$ T cells and analyzed for

changes in the total mean percentages of CD4+ PD1+ cells over the course of infection. Increased percentages of migrating CD4+ PD1+ to the lungs of mice peaking after 30 days of reinfection. Results represent the mean percentage of cells of five mice for each condition from one experiment (±SEM).

**Table 4. Percentage PD1+ high CD4+ cells in the Lungs of Naïve and Reinfected mice**

| Percentage Positive cells ± SEM | Naïve (Day 0) | IT1 (Day 1) | IT2 (Day 2) | IT3 (Day 3) | IT4 (Day 4) | Day 5 | Day 15 | Day 30 |
|---|---|---|---|---|---|---|---|---|
| **Naive** | 0.18±0.12 | 0.16±0.10 | 0.17±0.08 | 0.15±0.05 | 0.18±0.09 | 0.18±0.06 | 0.17±0.19 | 0.16±0.18 |
| **Reinfected** | 0.18±0.12 | 0.16±0.10 | 0.17±0.08 | 0.15±0.05 | 0.18±0.09 | 19.90±0.09 | 23.30±0.1 | 25.50±0.1* |

**Table 5**. shows then percentage of postive CD11b+CTLA4+ naïve mice, after four pulmonary *M. abscessus* intratracheal (IT) reinfections, and after infection progressed on days 5, 15 and 30 in the Beta enacmice inducing significantly increased numbers of CD11b+CTLA4+ exhaustion markers compared to naïve mice. The changes of naïve and *M. abscessus* reinfected cells, were analyzed for changes in the total mean percentages of CD11b+CTLA4+ cells over the course of infection. Significantly increased percentages of CD11b+CTLA4+ between day 5, and 30 in the lungs of mice occurred after reinfection (Student T test*, p=0.001, compared to naïve mice). Results represent the mean percentage of cells of five mice for each condition from one experiment (±SEM).

## Table 5. Percentage CTLA4+ high CD11b+ macrophages in the Lungs of Naïve and Reinfected mice

| Percentage Positive cells ± SEM | Naïve (Day 0) | IT1 (Day 1) | IT2 (Day 2) | IT3 (Day 3) | IT4 (Day 4) | Day 5 | Day 15 | Day 30 |
|---|---|---|---|---|---|---|---|---|
| **Naive** | 0.12±0.15 | 0.14±0.10 | 0.13±0.05 | 0.17±0.11 | 0.19±0.06 | 0.10±0.15 | 0.09±0.15 | 0.14±0.15 |
| **Reinfected** | 0.12±0.15 | 0.14±0.10 | 0.13±0.05 | 0.17±0.11 | 0.19±0.06 | 15.0±0.08 | 17.0±0.11 | 22.00±0.0* |

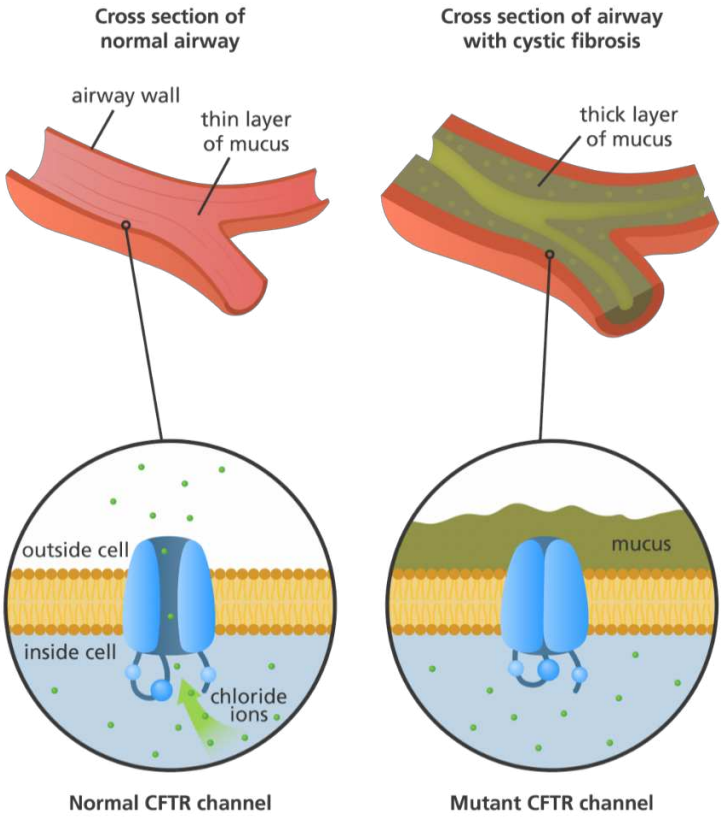


Figure 1. Comparison between healthy person's airway wall and Cystic Fibrosis patient airway wall. Normal CFTR channel compared no mutated CFTR channel [4].

| Discovery | Phase I/II | Phase III | Phase IV | Other |
|---|---|---|---|---|
| **LCB01-0371**<br>• Target 50S ribosome<br>• For *M. abs*<br>**PIPD1**<br>• Target MmpL3<br>• For *M. abs*<br>**Indole-2-carboxamides**<br>• Target MmpL3<br>• For *M. abs*<br>**Thiacetazone derivates**<br>• Target FAS-II dehydralase<br>• For *M. abs*<br>**CPZEN-45**<br>• Target decaprenyl-phosphate-GlcNAc-1-phosphate transferase<br>• For *M. abs and MAC*<br>**Clofazimine***<br>• Target NDH-2<br>• For *M. abs*<br>**Tedizolid**<br>• Target 50S ribosome<br>• For *NTM*<br>**Bedaquiline***<br>• Target ATP synthase<br>• For NTM<br>**β-lactams with avibactam***<br>• Target penicillin-binding protein<br>• For *M. abs* and *M.avium*<br>**Rifabutin***<br>• Target RNA polymerase<br>• For *M. abs* | **Clofazimine**<br>• Target NDH-2<br>• For *M. avium* PD<br>**Liposomal amikacin For inhalation (LAI)**<br>• Target 30S ribosome<br>• For *M. abs* PD<br>**Nitric Oxide**<br>• Enhance host defense<br>• Produce reactive nitrogen intermediates<br>• For CF patients with NTM (esp. *M. abs*)<br>• From AIT therapeutics<br>**Gaseous nitric oxide (gNO)[a]**<br>• Enhance host defense<br>• Produce reactive nitrogen intermediates<br>• For NTM<br>• Thiolanox from novoteris | **Liposomal amikacin for Inhalation (AI)**<br>• Target 30S ribosome<br>• For refactory MAC PD<br>**Clarithromycin vs azithromycin**<br>• Target 50S ribosome<br>• For MAC PD<br>**Clarithromycin vs moxifloxacin**<br>• Target DNA gyrase<br>• For *M. xenopi* PD | **Linezolid**<br>• Target 50S ribosome<br>• For NTM disease • | **Resiquimod**<br>• TLRm7/8 agonist<br>• For NTM disease<br>**Imidazoquinolines**<br>• TLRm7/8 agonist<br>• For NTM disease?<br>**Vaccines**<br>**BCG**<br>**Msmeg IKE**<br>**Msmeg IKEPLUS**<br>• IL-12<br>• For NTM disease? |

**Mechanism of Action**
- Inhibition of cell wall synthesis
- Inhibition of protein synthesis
- Inhibition of nucleic acid synthesis
- Other mechanisms
- Immunomodulatory

Figure 2. Discovery of the drugs and mechanism of action in different stages, showing the need of developing new immunomodulatory drugs[4].

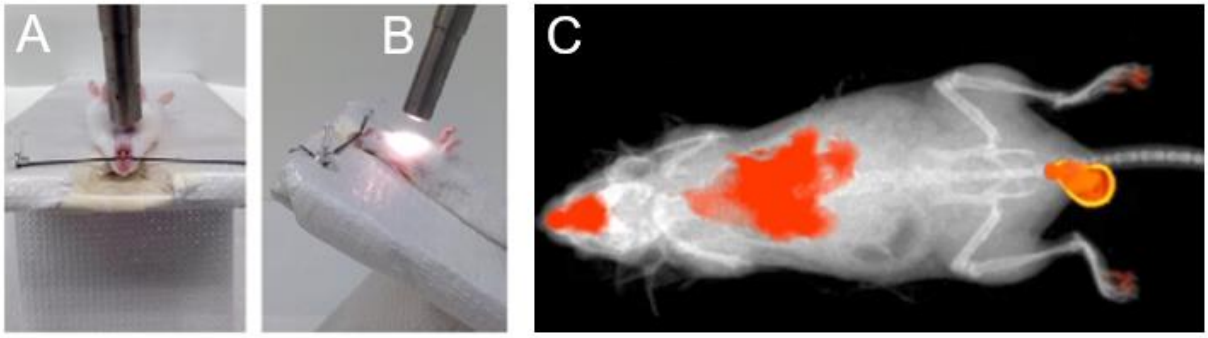


Figure 3. Non-invasive pulmonary intratracheal instillation infection in mice. The intratracheal instillation technique is used to deliver nontuberculosis mycobacteria

**Figure 1. A-D. Multiple MABS Reinfections Result in a Progressive Pulmonary Disease in the βENaC Mouse Model**

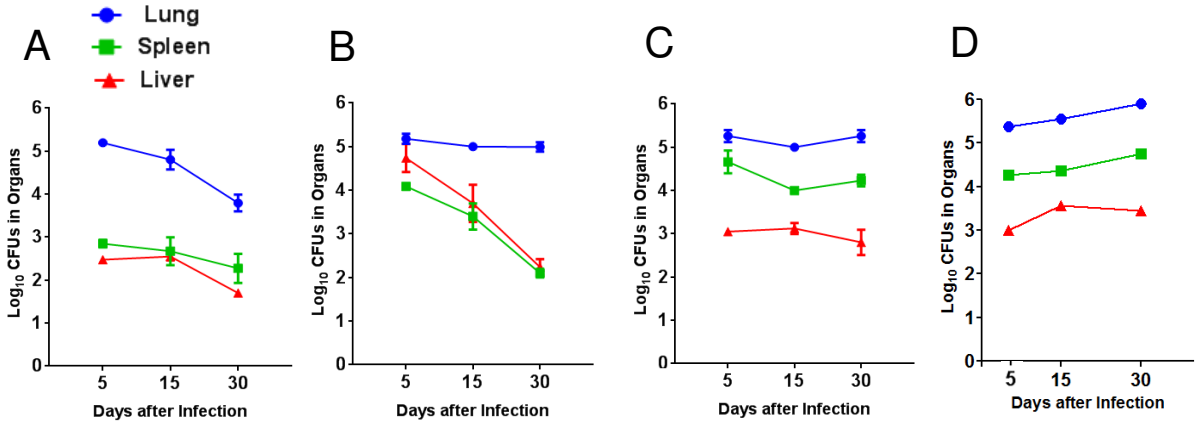


**Figure 3 A-D.** Pulmonary infection of βENaC overexpressing (βENaC-Tg) using an intratracheal instillation technique is used to deliver MABs 604 daily to mice. **A.** shows a primary infection. **B.** demonstrates two infections. **C.** demonstrates three infections and **D.** demonstrates four infections. The primary and subsequent reinfections were evaluated for bacterial burden in whole lung, spleen and livers on days 5, 15 and 30.

Figure 4. shows, the βENaC-Tg mouse model is showing progression of the disease in the lungs of βENaC-Tg following infection four times with a $1x10^6$ CFU/ml *M. abscessus* strains which were isolated from CF patient with NTM lung disease.

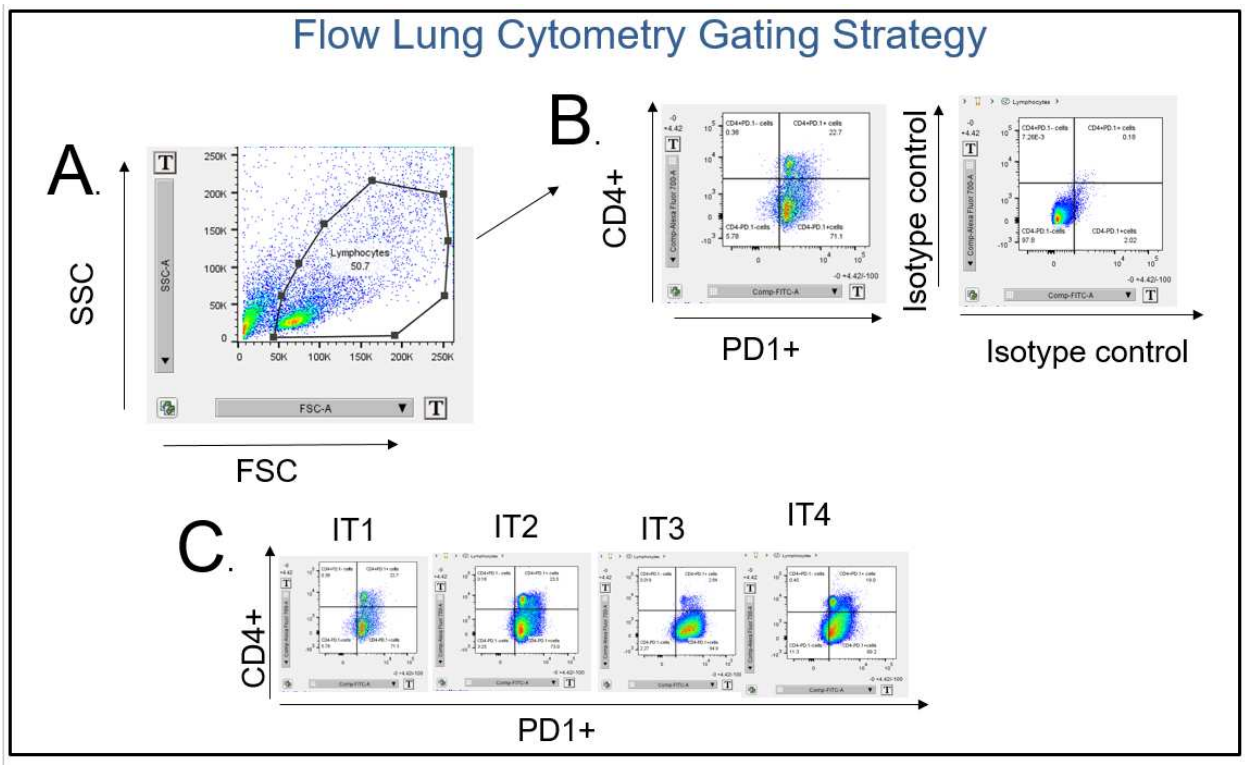


Figure 5. A. Demonstrates four pulmonary *M. abscessus* intratracheal (IT) reinfections given once per week with Beta enac inducing slowly increased numbers of CD4+PD1+ exhaustion markers over time compared to naïve mice

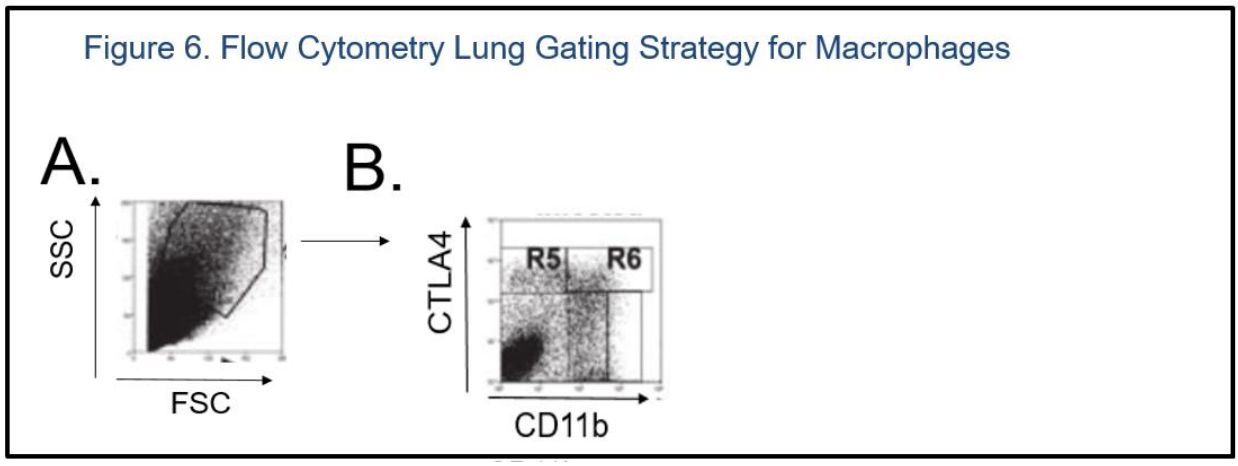


Figure 6. A. Shows CF mouse flow cytometry dot plots gated on lung macrophages and then B shows gated on CD11b and CD11c (not shown) then on alveolar macrophges CD11b+ /CD11c high, R5 CD11b+CTLA4+ were only were gated after each IT *M. abscessus* exposure.

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List of Abbreviations

| | |
|---|---|
| A700 | Alexa fluor 700 |
| AFB | Acid Fast Bacillus |
| ANOVA | Analysis of variance |

| | |
|---|---|
| APC | Allophycocyanin |
| APC | Antigen Presenting Cells |
| ATS | American Thoracic Society |
| bp | base pairs |
| BSL-2 | biosafety level 2 |
| BSL-3 | biosafety level 3 |
| C57bl/6 | C57 black 6 |
| CD11b | Cluster of differentiation 11b |
| CD11c | Cluster of differentiation 11c |
| CD4 | Cluster of differentiation 4 |
| CD8 | Cluster of differentiation 8 |
| C3HeB/FeJ | Kramnik |
| CFU | Colony forming units |
| $CO_2$ | Carbon dioxide |
| Csf2 | Colony stimulating factor 2 |
| Cybb | Cytochrome b β |
| DMEM | Dulbecco's Modified Eagle's Medium |
| ef450 | efluor 450 |
| erm(41) | Erythromycin Ribosome Methyltransferase (41) |
| FITC | Fluorescein isothiocyanate |
| FoxP3 | Forkhead box P3 |
| GKO | IFN-γ knockout |
| GPL | Glycopeptidolipid |

| | |
|---|---|
| HDA | High-dose aerosol |
| HPLC | High performance liquid chromatography |
| IDSA | Infectious Diseases Society of America |
| IFN-γ | Interferon-γ |
| IFNγR1 | Interferon-γ receptor 1 |
| IFNγR2 | Interferon-γ receptor 2 |
| IL-2 | Interleukin-2 |
| IL-3 | Interleukin-3 |
| IL-4 | Interleukin-4 |
| IL-10 | Interleukin-10 |
| IL-12 | Interleukin-12 |
| IL-12p40 | Interleukin-12 p40 |
| IL-12Rβ1 | Interleukin-12 receptor subunit β1 |
| IL-17 | Interleukin-17 |
| IL-32 | Interleukin-32 |
| iNOS | Inducible nitric oxide synthase |
| LAR | Lab Animal Resources |
| LDA | Low-dose aerosol |
| MAPK | Mitogen-activated protein kinase |
| MBC | Minimum bactericidal concentration |
| MFI | Mean Fluorescence Intensity |
| MHC II | Major histocompatibility complex class II |
| MIC | Minimum inhibitory concentration |

| | |
|---|---|
| MyD88 | Myeloid differentiation primary response gene 88 |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NK | Natural Killer |
| NTM | Non-tuberculous Mycobacteria |
| OADC | Dubos Oleic Albumin Complex |
| Ob/Ob | Leptin-deficient mice |
| OD | Optical Density |
| PBS | Phosphate Buffered Saline |
| PD1 | Programmed Cell Death Protein 1 |
| PE | Phycoerythrin |
| PE-Cy7 | Phycoerythrin-Cyanine7 |
| PerCP | Peridinin chlorophyll protein complex |
| PRA | PCR restriction endonuclease assay |
| Rag2-/- | Recombinant Activating Factor 2 knockout |
| RGM | Rapidly Growing Mycobacteria |
| rpm | Revolutions per minute |
| rRNA | Ribosomal RNA |
| SCID | Severe Combined Immunodeficiency |
| SEM | Standard Error of the Mean |
| $T_H1$ | T helper 1 |
| $T_H2$ | T helper 2 |
| TLR2 | Toll-Like Receptor 2 |
| TLRs | Toll-Like Receptors |

| | |
|---|---|
| TNFα | Tumor Necrosis Factor α |
| TNFαR | Tumor Necrosis Factor α Receptor |