THESIS

INHALATIONAL ANTIBIOTIC THERAPY FOR TREATMENT OF CHRONIC PULMONARY MYCOBACTERIUM ABSCESSUS DISEASE IN MICE

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2019

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ABSTRACT

INHALATIONAL ANTIBIOTIC THERAPY FOR TREATMENT OF CHRONIC PULMONARY MYCOBACTERIUM ABSCESSUS DISEASE IN MICE

Mycobacterium abscessus (*M. abscessus*) is a nontuberculous mycobacterium that causes chronic pulmonary infections. Due to *M. abscessus*'s intrinsic antibiotic resistance, treatment is often complex with low cure rates. Tigecycline, a glycylcycline class antibiotic, demonstrates bactericidal effects against *M. abscessus* without eliciting bacterial resistance mechanisms, however, this antibiotic requires intravenous administration and causes significant side effects that limit its use. Here, we tested the hypothesis that tigecycline administered via inhalation has the potential to maximize the bactericidal effect while reducing side effects. GM-CSF knockout mice with pulmonary *M. abscessus* infection were treated by intrapulmonary tigecycline aerosols in 0.25 mg, 1.25 mg, and 2.50 mg doses for 28 days. Assessment of pulmonary bacterial burden after full treatment duration shows that inhaled tigecycline is highly effective, dose-dependent, and well tolerated. We concluded that inhaled tigecycline represents a viable treatment option for *M. abscessus* pulmonary disease. Future studies should address the pharmacokinetics, and ultimately, translation into clinical trials.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my thesis advisor, Mercedes Gonzalez-Juarrero, for her guidance throughout these projects. Her willingness to share her expertise and challenge my ideas has shaped this thesis into a piece that I am proud to present.

I thank members and former members of the Gonzalez-Juarrero lab: Amanda Walz, Zohaib Ali, Marissa Quilici, and Andrea Sanchez-Hidalgo for their assistance and support over the course of the last couple years. I would also like to thank our collaborators, specifically, Dr Jakko van Ingen for his clinical expertise and generosity in supplying the tigecycline necessary for these studies.

A special thanks to my family and friends for their continuous support throughout my extended and long-distant studies at Colorado State University.

Lastly, I would like to thank the members of my graduate committee for their feedback and support throughout these projects.

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CHAPTER 1: MYCOBACTERIUM ABSCESSUS LITERATURE REVIEW

1.1 Introduction

Non-tuberculous mycobacteria (NTM) are ubiquitous in the environment and human exposure is frequent [1]. In certain populations, particularly in individuals with pre-existing inflammatory lung dysfunction, these microbes can cause serious chronic pulmonary infection. Falling into this group of NTM's is the *Mycobacterium abscessus* (*M. abscessus*) complex; a group of multidrug resistant and rapidly growing mycobacteria that, upon pulmonary infection, develops into a disease characterized by declining lung functionality and eventual respiratory failure.[1–3] Current antibiotics have a limited killing effect against *M. abscessus* because of a thick cell envelope, neutralizing enzymes, and complex drug efflux systems that enable persistent and constitutive or inducible antibiotic resistance.[4,5] The *in vitro* hollow fiber model demonstrates that current antibiotics lack the killing activity to selectively kill the intracellular bacillus before resistance is induced.[6,7] Altogether, treatment of pulmonary *M. abscessus* infections with antibiotic-based therapy is very complex with low cure rates.[8,9]

The diagnosis of pulmonary disease caused by *M. abscessus* infection is based on clinical, radiological, and microbiological culture isolation.[10] For diagnosis, the patient's samples (sputum, bronchoalveolar lavage fluid, or fecal samples) are collected and cultured on differing selective media to determine growth patterns, colony morphology, and ultimately, bacterial speciation.[11] Next, the bacteria are subjected to drug susceptibility testing against various front-line drugs to guide, but not dictate, the

treatment regimen.[11] Treatment regimens include intravenous (IV) administration of multiple drugs in conjunction with an oral or inhaled supplementary drug, which is often poorly tolerated.[12] Patient treatment outcome is highly variable, with one study citing 29% of patients remaining culture positive, and 23% of patients relapsing after only one year of ending treatment therapy.[1] Low rates of bacterial clearance and poor patient treatment outcome further confirm the immediate need for treatment improvement.

Tetracycline class antibiotics have been the focal point of multiple studies, due to their potent bacteriostatic effects.[13,14] Within this class of antibiotics is the drug tigecycline; the first tetracycline developed with a glycyl moiety that allows it to overcome bacterial resistance mechanisms.[6,15] Tigecycline was added to the antibiotic regimen to be employed during the initiation phase of pulmonary treatment because of its unusually effective bactericidal effect seen in minimum inhibitory concentration (MIC) assays.[11] The side effects of tigecycline, however, often limit the dose and duration of treatment. A recent *in vitro* study suggests that tigecycline produces dose-dependent results and that local pulmonary concentrations should be increased for optimal effectiveness.[6,8] With current administration guidelines, a dose increase would undoubtedly sacrifice the patients' health for optimal pulmonary antimicrobial activity.

Our studies focus on utilizing the murine model of pulmonary *M. abscessus* to develop an alternative delivery method of tigecycline that aims to overcome side-effects and increase treatment efficacy. A previous study demonstrates that granulocyte macrophage colony stimulating factor knockout (GM-CSF KO) mice can develop both acute and chronic stages of pulmonary infection, and be used as a preclinical model for testing drug efficacy.[16] Thus, we used the GM-CSF KO mouse model to test tolerability

and efficacy of inhaled therapy of tigecycline against pulmonary infection with *M. abscessus*.

1.2: *M. abscessus*: An Overview

1.2a History

In January of 1950, a woman was admitted to the hospital with complaints of knee stiffness and limited range of motion. During physical examination, doctors found a small abscess that stained positive for acid-fast bacilli, and a diagnosis of tuberculosis was made.[17] At the time, there were few reports of human infection caused by acid-fast bacteria besides *Mycobacterium tuberculosis and Mycobacterium leprae,* so when bacterial colonies grew rapidly and produced a unique colony morphology, doctors concluded that this was likely an undocumented mycobacterium subspecies. Further phenotyping led researchers to classify this bacillus under a new species: *Mycobacterium abscessus*. In 1972, a cooperative taxonomic analysis re-defined *M. abscessus* as a subspecies of *M. chelonea.*[18] *M. abscessus* was still characterized as having low virulence due to lack of lesion dissemination and the self-limiting nature of the infection.[17] In 1992 it was re-elevated to species status, and then after sequencing in 2012, *M. abscessus* was finally re-classified into what it is now, a complex with three subspecies: subsp. *abscessus,* subsp. *massiliense,* and subsp. *bolletii.*[19–21]

1.2b Types of infection

M. abscessus can cause a variety of infections types including: pulmonary, disseminated, and both cutaneous and subcutaneous infection.[22] Cutaneous and subcutaneous infections are categorized as soft skin and tissue infections (SSTI), which

are most commonly contracted through 1) contaminated water coming into contact with a surgical wound, trauma, or open skin wounds or 2) disseminated pulmonary or lymphatic disease.[23] Furthermore, multiple cases have been linked to the use of contaminated needles during body piercing, tattoo procedures, or any other surgical cosmetic procedure.[24,25] Localized SSTI's generally respond to antibiotics and are characterized by red skin lesions with a yellow discharge and unpleasant odor.[22]

Pulmonary *M. abscessus* disease is complicated and runs a progressive course of infection, often leading to a decline of pulmonary function and quality of life.[26] Those with pre-existing lung diseases or dysfunction are especially at risk for NTM infection. Patients with cystic fibrosis (CF), an autosomal disorder that results in the buildup of pulmonary mucous, are the leading population affected by *M. abscessus*.[27] It should be noted that pulmonary NTM disease, including *M. abscessus*, has increased prevalence in women and individuals with low body mass, with seemingly no predisposing factors.[28] Symptoms include dyspnea, fatigue, weight loss, and excessive coughing. Diagnosis of NTM disease is difficult, as most NTM disease associated symptoms are masked with symptoms related to the primary lung dysfunction.[29] Treatments generally include a macrolide-based therapy that has shown to have cytotoxic effects; only further deteriorating the patient's well-being.

1.2c Drug resistance

Infections due to *M. abscessus* are challenging to treat because of the bacteria's intrinsic or inducible resistance to most of the front-line drugs that are commercially available.[1] Furthermore, most drugs administered to patients cannot reach concentrations in tissue or serum above the minimum inhibitory concentration (MIC) necessary for *M.*

abscessus. Of the drugs with potential antimicrobial effects, there is high risk of host cytotoxicity and gene induced resistance.[5] *M. abscessus* has a thick and lipid rich cell membrane, and complex efflux pumps, amongst numerous other resistance mechanisms, that make it a "nightmare" to be eliminated with current drugs.[5] Different clinical isolates of *M. abscessus* may possess the inducible erythromycin resistance mechanism (*erm41*) gene or a constitutive macrolide resistance through a 23S rRNA point mutation.[30] The *erm41* gene confers delayed bacteria macrolide resistance *in vitro* and *in vivo*, and a 23S rRNA point mutation on an adenine of its single rRNA operon is responsible for constitutive macrolide resistance.[31] Clarithromycin and erythromycin (front-line macrolides) demonstrate reasonable inhibitory effects, however, after 10 days, growth is re-established, and MIC values increase dramatically.

1.3: *M. abscessus* Pulmonary Infection

1.3a Incidence

Until the early 1990's, *M. abscessus* was still considered a low-priority, low-virulence pathogen, with few disease implications beyond cutaneous infection. In 1992, a study of 154 patients with mycobacterium pulmonary infection, showed that over 80% of the isolates were *M.* abscessus.[2] Although the prevalence of *M. abscessus* pulmonary infection in this study are higher than the actual population mean, this was the first study to establish *M. abscessus* as a virulent and progressive disease that should be recognized as a serious public health concern. In the U.S., states along the southeastern coastline have reported the majority of cases, although pulmonary NTM disease has been reported from every geographical region within the U.S. [28]

One report shows that the prevalence of NTM infection in Northern Europe increased significantly between 1995 and 2006, with similar trends expected worldwide.[32] *M. abscessus* is broadly lumped into this category of NTM infection, and will typically comprise 5-20% of the total cases reported.[28] Rate of reports increased from 0.9 per 100,000 population to 2.9 per 100,000 population over a span of 11 years. A study in Denmark standardized the incidence rates of NTM disease to age groups and found that elderly over the age of 65 have a ten-fold increased risk of infection to that of people aged 10-19.[33] For every 10 years of age after 15, the odds of contracting NTM infection are estimated to be 17.5% higher.[34] Improvement in laboratory diagnosis has contributed to the increased rates in NTM pulmonary disease, however, factors such as increased life expectancy and increased proportion of the population experiencing immunosuppression may also be leading to an overall higher incidence rate.[27] Because *M. abscessus* are environmentally ubiquitous, and exposure is nearly impossible to avoid, disease prevention efforts have been limited to improving sterilization procedures that reduce and control health care-associated transmission.

1.3b *M. abscessus* in CF patients

Cystic fibrosis (CF) is a well described and characterized disorder primarily found in Caucasian populations of European ancestry. The mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene results in thick mucus build up and, subsequently, serious medical problems. Between 80-95% of patients with CF will die from respiratory failure caused by chronic infection, and not surprisingly, approximately 10-13% of CF sputum samples are NTM positive.[35,36] Large epidemiological analysis studies of NTM, especially in CF patients, are often limited by incomplete data on specific

NTM which prevents a more comprehensive analysis, and much of the data will vary based on region and time period.[27] A study by Esther *et al.* found that 55.6% of CF derived NTM positive cultures were *M. abscessus*, reinforcing that high infection rates with *M. abscessus* strongly influences the increased respiratory deterioration in those patients.[37]

Perhaps the most prominent phenotypic dysfunction correlated to a CFTR genetic mutation is the inability of the airway epithelium to undergo mucociliary action.[36] Normally, airway cilia perform synchronous movements that create a steady "current" and move the mucus layer up toward the nasopharynx. The effects of the CFTR mutation prevent the movement of the mucus layer because of its increased viscosity, thus resulting in stagnant mucus, often rich with foreign microorganisms and particulate.[36] Pulmonary disease caused by *M. abscessus* is extremely difficult to treat, and when added to primary complications associated with CF disease, therapy becomes even more challenging. It is recommended that all adult and adolescent patients with CF are screened yearly for the presence of NTM, and all patients, regardless of age, are evaluated for NTM during periods of declining health from separate infections.[38]

1.4: Diagnosis and Treatment

1.4a Diagnosis

As previously mentioned, *M. abscessus* is environmentally ubiquitous, therefore, diagnosis can be challenging because a positive culture from a respiratory sample does not always indicate pulmonary disease. Diagnosis typically requires radiological, clinical, and microbiological analyses.[11] An *M. abscessus*-positive chest radiograph shows patchy

or multilobed alveolar spaces; primarily visualized in the upper lung, supplemented with nodular lesions (abnormal lung growth) in the middle lobe that resemble pulmonary tuberculosis.[39] Symptoms are nonspecific, and difficult to distinguish from any underlying conditions (CF, bronchiectasis, COPD, etc.), but usually include: chronic coughing, excess sputum production, and fatigue.[39] Less frequently occurring symptoms are weight loss, fever, and night sweats, of which, often indicate an advanced stage disease.[29,40] NTM speciation is not possible from the radiograph or symptoms alone, hence multiple sputum samples are taken to confirm the presence of *M. abscessus* via microbiological characteristics.[28]

Following the American Thoracic Society (ATS) diagnostic guideline, at least three respiratory samples should be taken and promptly tested, and sampling intervals should be spaced out by several weeks.[39] An accurate diagnosis includes: careful monitoring of NTM colony growth for characteristics such as, growth rate (3-5 days or 3-4 weeks), color, and morphology, that further indicate mycobacterial infection. In difficult cases where sputum samples are difficult to obtain, or pathology is atypical, histological analysis of the sample can be useful.[39] When preparing samples for analysis, the sputum, bronchoalveolar lavage fluid (BAL), or fecal samples are emulsified and decontaminated using a 1% N-Acetyl-L-Cysteine (NaLC) mixed with NaOH (NaLC-NaOH solution).[41] After decontamination, samples are added to a mycobacteria-selective media (7H10/7H11) to select for any NTM's that may be present in the sample. The Mycobacteria Growth Indicator Tube (MGIT) is often employed as a quick, automated, method of ensuring the presence of NTM and obtaining valuable information of the specimen's growth patterns.[42,43] NTM speciation is very important in the diagnostic process, as differing

mycobacteria vary in their clinical relevance, disease severity, and therapeutic approach.[29] The most common identifying techniques include: biochemical testing, high-performance liquid chromatography, line probe assays, partial gene sequencing, and more recently, matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.[44–47] A thorough evaluation of these three different diagnostic techniques is necessary to make an accurate diagnosis. Despite the advances in radiography and laboratory diagnostic techniques; an *M. abscessus* diagnosis is complicated due to the limited sensitivity and the time consuming nature of culturing.[39]

1.4b Treatment

Therapy for *M. abscessus* infection consists of prolonged multi-drug treatments and are poorly tolerated by patients. Unfortunately, chemotherapy is often unsuccessful because of resistance to many of the most commonly used antibiotics.[48] The British Thoracic Society (BTS) outlines a suggested antibiotic regimen for adults with *M. abscessus* pulmonary disease for both macrolide sensitive and constitutively macrolide resistant isolates.[11] According to the BTS, patients should have two phases of treatment: the initial phase and a continuation phase. The initial phase consists of daily, or twice daily, IV administered: amikacin, tigecycline, and imipenem (or cefoxitin), supplemented with an oral clarithromycin (if isolate demonstrates macrolide susceptibility). The first month of the initial phase, is followed by a continuation phase for a minimum of 12 months. The continuation phase involves aerosolized amikacin, oral clarithromycin (if determined susceptible), and then 1-3 of the following oral antibiotics as guided by drug susceptibility results: clofazimine, linezolid, minocycline, moxifloxacin, and cotrimoxazole.[11] These methods of combining multidrug therapies with different

administration routes could control the symptoms and progression of pulmonary *M. abscessus* disease, however, the only "predictably curative" therapy involves a lung surgical resection combined with the multidrug therapy.[28]

1.4c Side effects

Current therapies against pulmonary *M. abscessus* infections are associated with a broad spectrum of side effects, obligating physicians to adjust or discontinue therapy.[48] In a recent U.S. study of 65 cases of pulmonary *M. abscessus*, 62% of the patients reported serious side effects, and nearly 50% of those were linked to administration of either amikacin or tigecycline.[48] Amikacin can cause renal failure, ototoxicity, and vestibular toxicity; which appears to occur correlative to higher concentrations of amikacin in the bloodstream.[49] Tigecycline, a relatively new glycylcycline often used as a secondary agent, is often associated with extreme vomiting/nausea. In one study with 52 patients receiving IV tigecycline as a part of a salvage regimen; 49 of the 52 patients experienced nausea and vomiting, 12 of which were directly associated with tigecycline.[50] Importantly, in this same study, 60% of the patients receiving tigecycline showed improvement and appeared to limit disease progression, however, side effects associated with the harsh drug therapy actually lowered the standard of living for patients.[50]

A similar study, involving 65 patients who received antibiotic therapy for more than 12 months, reveals that 60% of patients discontinue the use of cefoxitin after developing hepatotoxicity and/or a reduced white blood cell count (leukopenia).[51] An alternative to cefoxitin is imipenem, however, the side effects may prove to be just as severe as those induced by cefoxitin. Clarithromycin is the most common macrolide used in *M. abscessus* therapy, and even this is associated with hearing loss in up to 10% of patients.[52]

Combination antibiotic therapies tend to amplify the side effects, which in combination with the already resistant nature of *M. abscessus*, renders treatments relatively ineffective.

1.5: Preclinical animal models

1.5a Existing models

Preclinical animal models are a useful tool to study disease progression and evaluate drug efficacy against NTM infections. Various immunocompetent mouse strains have demonstrated their value as a disease model for similar pathogens with more virulence (*Mycobacterium avium* complex), however, the majority of these murine models demonstrate rapid clearance when infected with the less virulent *M. abscessus*.[53] Thus, developing an animal model against *M. abscessus* has been particularly challenging because the bacteria is relatively avirulent, and upon entrance in the airway, they are cleared by both innate and adaptive immune responses.[23,53]

One study by Ordway et al. shows that when C57BL/6 and leptin-deficient mice are infected with a low dose aerosol (~100 bacilli per mouse) of *M. abscessus*, the bacilli are rapidly cleared. With a high dose inoculum (~1000 bacilli per mouse), both strains of mice showed increased susceptibility to pulmonary infection. However, the animal's immune system responded to infection with an early influx of IFN-γ $CD4^+$ T cells that cleared the bacteria in both murine strains ($CD4^+$ T cells are the primary source of IFN-γ, which is important in controlling mycobacterial infections in both humans and animals).[23,54]

Other murine models, including: C3HeB/FeJ (Kramnik) mice, GKO (gamma interferon knockout) mice, MyD88$^{-/-}$ (myeloid differentiation primary response gene 88) KO mice [53], SCID (severe combined immune deficiency), nude, GM-CSF (granulocyte macrophage colony stimulating factor) KO mice, and most recently, CFTR KO and ENaC (epithelial sodium channel) KO mice, when infected with *M. abscessus*, allowed for the establishment and progression of acute infection.[55] Because *M. abscessus* in humans is a long-term chronic infection, testing drug efficacy in animal models is best if animal maintains infection well into a chronic phase. Some of these referenced murine models of *M. abscessus* infection can progress into a chronic pulmonary infection.[16,23,56]

1.5b GM-CSF KO mouse model

Host susceptibility to pulmonary *M. abscessus* infection relies on the defense provided by the pulmonary immune response.[16] Alveolar macrophages (AMs) are the first cells to encounter the bacillus in the lungs and are essential in pulmonary protection against infection.[57] In the lungs, the GM-CSF cytokine is essential for AM maturation, differentiation, as well as surfactant homeostasis; all of which directly affect the host's ability to fight an infection.[58] Without GM-CSF, lung AMs have defects in phagocytosis and lose surface expression of Toll-like receptors and signaling, which are essential for host-pathogen interaction.[59,60] When the GM-CSF gene is deleted from the mouse genome, the mice show pulmonary dysfunction and lack effective pulmonary immune responses, but demonstrate an otherwise normal hematopoietic system with normal spleen immunity.[61] Thus, given the importance of AM functionality in helping to control pulmonary bacterial infection, mice lacking functional pulmonary immunity are unable to clear *M. abscessus* bacilli after a primary aerosol challenge.[16] In a study done by De

Groote et al., GM-CSF KO mice were exposed to a high dose of aerosolized *M. abscessus* clinical isolate #21 (a virulent strain isolated from a patient with history of pulmonary tuberculosis), and maintained both acute and chronic infection.[16] Thereafter, the GM-CSF KO mouse model could be used as a reproducible animal model for the assessment of *M. abscessus* pulmonary infection. In our studies, we utilized the GM-CSF KO mouse model to monitor chronic pulmonary infection and test antimicrobial agents in a clinically relevant animal model.

1.5c *M. abscessus* isolates

Microbiologic isolation refers to the separation of a specific organism from a mixed population of microbes within the environment of interest.[62] Isolation of mycobacteria from a contaminated source requires digestion, decontamination, and concentration.[63] After the successful isolation, using the techniques previously outlined, the resulting *M. abscessus* sample can be tested against the desired antimicrobials. Different clinical isolates of *M. abscessus*, even within the same species, demonstrate varying susceptibility to front-line drugs and display either rough or smooth colony morphology.

The most commonly studied *M. abscessus* isolate is known as *M. abscessus* strain ATCC® 19977™, otherwise referred to as *M. abscessus* CIP 104536, amongst other less common names for the same organism. This strain was the first rapidly growing pathogenic mycobacteria to be sequenced and is most often used as a reference strain for comparison against newly isolated strains. The successful pulmonary infection in animal models has been published using *M. abscessus* strains CIP 104536, #103, and #21.[16,56] *M. abscessus* #21 was originally isolated from a patient with a history of pulmonary tuberculosis at the Colorado Hospital Clinical Microbiology Laboratory and

displays a predominantly smooth colony morphology. For our studies, all mice were challenged with *M. abscessus* strain #21, as previously published data suggests this bacterial strain can sustain both acute and chronic phases of pulmonary infection.[16]

1.5d Drug administration

Drug administration in preclinical murine studies can be performed using different routes of delivery, including but not limited to: oral, gastric, intravenous, epicutaneous/subcutaneous, intraperitoneal, intranasal, and intratracheal.[64] Each of these procedures require restraint or sedation of the animal and should be carefully selected to reduce invasiveness and optimize effects.[64]

As previously discussed, many antibiotics included in combined drug regimens against *M. abscessus* pulmonary infection are administered IV or orally, apart from nebulized amikacin in the continuation phase of treatment.[11] An alternative approach is to use an aerosolized method for a wider variety of drugs to deliver higher and more localized concentrations locally to the site of infection.[65] In the past, there have been difficulties in delivering aerosols to a mouse, stemming from both the formulation and the delivery method [66–70], however, a study by Gonzalez-Juarrero et al. demonstrates that the intrapulmonary aerosol delivery of front-line anti-TB drugs, using a high-pressure syringe device (MicroSprayer), results in a bacterial reduction similar to that of systemic administration.[65] By establishing the concept of testing antibiotic efficacy via aerosol in a mouse model, drugs with limited oral or IV efficacy may regain value using a novel delivery method. In our own studies, we utilize the MicroSprayer to assess the efficacy an aerosolized drug therapy against *M. abscessus* in the GM-CSF KO mouse model.

1.6: Tigecycline

1.6a Mechanism of action

In response to the increasing bacterial resistance, a semi-synthetic class of tetracyclines, called glycylcyclines, were introduced in the early 2000's as a "broad-spectrum" antibacterial treatment option.[71] In 2005, tigecycline was the first of its kind to be approved by the Food and Drug Administration (FDA) for injection via IV route. Tigecycline is approved in North America for treatment of skin and abdominal infections, pneumonia, and has been used to treat other infections, including sepsis, urinary tract infections, and ventilator-associated pneumonia.[72] Tigecycline has demonstrated activity against a wide variety of different bacteria, including many clinically relevant species of both gram-positive and gram-negative bacteria that demonstrate resistance against classic tetracycline derivatives.[71] Interestingly, the drug has also been identified as an agent with anticancer activity in a preclinical study of acute myeloid leukemia.[73] Tigecycline induces its bacteriostatic, sometimes bactericidal, effects through binding to the bacterial 30S ribosome and blocking the A-site with subsequent inhibition of protein translation.[74] Tetracyclines, in general, exhibit the same mechanism of action in ribosomal binding assays as in tigecycline, however, the addition of a glycyl- moiety in tigecycline allows the molecule to bind up to five times more effectively.[74] Tigecycline also has killing-effects in bacteria that display efflux-mediated resistance simply because many efflux pumps are unable to transport the molecule with an attached glycyl- moiety out of the cell.[75]

1.6b Efficacy against *M. abscessus*

M. abscessus, although notorious for its complex resistance mechanisms, has yet to demonstrate any resistance to tigecycline. A potent form of resistance in *M. abscessus* is the upregulation of a drug-modifying enzyme known as the WhiB7-independent tetracycline-inactivating monooxygenase, or MabTetx. One recent report shows that tigecycline has poor binding affinity to MabTetx, whereas tetracycline and tetracycline-like drugs are inactivated through MabTetx monooxygenation [14]. Early phase MIC testing of 72 different NTM isolates (*M. abscessus, M. chelonae, and M. fortuitum*) showed values ≤1 mg/l, which was 4- to 11- fold more susceptible than tetracycline, minocycline, and doxycycline.[76] This data confirmed that tigecycline should be explored as a potential candidate for therapy against *M. abscessus* infection. In 2013, Wallace et al. reported one of the first and largest clinical trials using tigecycline as a salvage treatment for patients with pulmonary *M. abscessus.*[50] They report that when tigecycline was administered for more than one month in a multidrug regimen, greater than 60% of patients showed improvement, despite all previous antibiotic therapies failing. Since this study, and with the influence of several other preclinical and clinical trials, the BTS suggests that tigecycline should be administered by IV in a 50 mg dose, twice daily.[11]

1.6c Therapeutic challenges

Administering tigecycline as an IV treatment twice-a-day is often poorly tolerated by patients. As previously mentioned, the side effects of tigecycline usually involve extreme flu-like symptoms, and frequently, the patient discontinues treatment before completing the recommended duration of therapy.[6,50] An additional limitation of tigecycline therapy is its poor stability in aqueous solution.[77,78] Prior to patient

administration, lyophilized tigecycline is reconstituted in 0.9% saline. Once reconstituted, the drug must be administered immediately as it degrades rapidly in solution.[77] A phenol group on the end of the tigecycline molecule leaves it susceptible to quick oxidation, especially at higher pH values. Similarly, at lower pH values, the molecule is prone to epimerization which causes significant instability in solution.[73]

Current tigecycline regimens suggest that patients receive 100 mg/day as an IV. Recent studies using the *in vitro* hollow fiber model (HFM) concluded that tigecycline doses at 200 mg/day would improve the drug efficacy.[6,79,80] Unfortunately, at this dose, patients would experience serious side effects, rendering this strategy unreasonable as an IV. In summary, tigecycline is a promising agent for improving treatment outcome in pulmonary *M. abscessus* disease, but treatment-ending side effects limit dosing to suboptimal concentrations. Tigecycline regimens against *M. abscessus* still have room for improvement, and future ventures might include combination therapies, or perhaps, novel and more efficient delivery methods.

Chapter 2: Inhaled Tigecycline as Therapy Against Pulmonary *M. abscessus* Infection

2.1 Introduction

Mycobacterium abscessus is a rapidly growing, non-tuberculous mycobacteria (NTM) that can survive in harsh environments where many other microorganisms would not.[33] *M. abscessus* is environmentally ubiquitous and can be responsible for both soft tissue infections as well as more serious pulmonary infection.[17] Diagnosis of pulmonary infection is difficult and often relies on culture of respiratory tract sample, symptoms, and a minimum of two positive sputum sample for NTM of the same species.[11] Patients with pre-existing lung disease or cytokine dysfunction have a predisposition to *M. abscessus* pulmonary infection, which can be particularly challenging to treat because this bacillus displays intrinsic antibiotic resistance to most front-line drugs.[4,81] The thick cell envelope, complex drug efflux systems, and wide variety of antibiotic neutralizing enzymes, all characterize its intrinsic antibiotic resistance.[5] Only few drugs have demonstrated *in vitro* activity against *M. abscessus*, and of those, there is often induced drug resistance resulting in minimum inhibitory concentrations (MICs) above the serum concentration achieved in patients during chemotherapy.[5] For these reasons, all existing drug-regimens against pulmonary infection with *M. abscessus* entails multidrug treatment, which are poorly tolerated by patients and often yields less than 60% cure rates.[12,16] The bacteriostatic effects of tetracyclines against *M. abscessus* have more recently become a focus point for developing new drug therapies, however, the intrinsic resistance

mechanisms developed by the bacillus result in these tetracycline therapies eventually failing.[13,14]

Tigecycline, the first tetracycline with a glycyl moiety, overcomes the common resistance mechanisms in rapidly growing mycobacteria (RGM) and exhibits potent bacterial inhibition *in vitro*.[6,82] It was shown to be effective *in vivo* against pulmonary *M. abscessus* infections, but adverse side-effects often limit the treatment duration and efficacy.[50] The recommended dosage includes an initial 100 mg dose, followed by a 30 – 60 minute intravenous infusion of 50 mg every 12 hours, and the side-effects commonly include extreme nausea/vomiting; with up to 94% of patients experiencing harsh flu-like symptoms.[50,72] Tigecycline demonstrates pulmonary improvement in many patients regardless of the side effects, yet, a recent study using the hollow fiber system (HFS) to mimic human intrapulmonary concentration of tigecycline, suggests that for optimal effectiveness, IV doses should be doubled to 200 mg/day.[6,8] This treatment approach would undoubtedly sacrifice patient well-being for drug efficacy.[6] Under the current administration guidelines, there is room for improvement; both in the delivery method and patient tolerability. Here, in preclinical studies using a murine *M. abscessus* model of pulmonary infection, we assessed the tolerability and efficacy of tigecycline delivered as an inhalational therapy.

2.2: Materials and Methods

2.2a Mice

Mice lacking the granulocyte-macrophage colony stimulating factor (GM-CSF KO) were generated by Dranoff et al.[61] The mice were bred at Colorado State University

animal housing facilities. Prior to infection, mice were verified as genetic knockout through PCR genotyping. Before and after infection with *M. abscessus,* mice were maintained in a separate Biosafety Level-2 room with restricted access and directional airflow. Animals had free access to water, standard mouse chow, and various cage enrichments. The pathogen-free nature of the breeder colonies was demonstrated by testing sentinel animals. University facilities cared for the animals in line with both Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and NIH guidelines. The Colorado State University Animal Users Committee (IACUC) approved all experimental protocols involving the animals. For these studies, animals had a minimum of 20 grams in weight and both male and female mice were used, however, the studies were not powered to study therapy differences between genders.

2.2b Bacteria

M. abscessus strain #21, originally isolated from an immigrant with a history of pulmonary tuberculosis, was generously provided by Dr. Mary Jackson of Colorado State University and Dr. Nancy Madinger from the University of Colorado Hospital Clinical Microbiology Laboratory. *M. abscessus* #21 is a virulent strain that, when cultured, primarily displays a smooth colony morphology. The bacilli were grown in Middlebrook 7H9 supplemented with OADC broth and 0.05% Tween 80 in a shaker incubator adjusted to 37°C. After ~24 hours of incubation, when the culture reached OD_{600} between 0.6-0.7 (CFU/ml about 1 x 10^7), culture was removed from incubation and immediately aliquoted. Aliquots of stock were serially diluted and plated on Middlebrook 7H11 agar supplemented with OADC. Agar plates were incubated during 3-4 days at 37°C until the

colony forming units (CFU) were visible to naked eye. Thereafter, the CFU were counted and expressed as $\log_{10}$ CFU/ml.

2.2c Antibiotics

Tigecycline (TGC) was obtained as the commercial drug TYGACIL® (Pfizer Inc.) in a single-dose 5 ml glass vial containing 50 mg of drug as lyophilized powder for reconstitution. Amikacin (AMK) was obtained from Gold Biotechnology as amikacin hydrate aminoglycoside. Both drugs were diluted in endotoxin free 0.9% saline (Teknova).

2.2d Minimum inhibitory concentration

For minimum inhibitory concentration (MIC) determination, the bacteria were grown on an 7H11 agar plate without antibiotics. After 4 days, 3-4 colonies were swabbed and added into distilled/deionized water and adjusted to a 0.5 McFarland Standard (visually). 50 µl of this solution was transferred into 10 ml of cation adjusted Mueller-Hinton II broth for a sample inoculum of about 5 x 10^5 CFU/ml. Next, 100µl of sample inoculum was added into each well of the 96-well RAPMYCO Sensititre® preloaded drug plate (TREK Diagnostic Systems Ltd). Thereafter, to avoid cross contamination of wells, the plates were covered with the supplied adhesive seal as per manufacturer's recommendation. Plates were incubated at 30°C for 72-96 hours during which time the plates were monitored for bacterial growth. After the primary incubation period, plates were removed and imaged for initial MIC determination. To ensure the detection of inducible macrolide resistance, the plates were then placed back in at 30°C for another 6-7 days. MIC throughout the study was determined as the lowest concentration of the drug lacking visible (viewed as transparent) growth of *M. abscessus* strain #21.

2.2e Mouse infection

GM-CSF KO mice were infected via intrapulmonary aerosol route with 1 x 10^6 CFU/50µL of *M. abscessus* #21. Bacteria was delivered using a FMJ-250 high-pressure syringe device (PennCentury, Philadelphia, PA, USA) with an attached MicroSprayer (MicroSprayer, model IA-C; PennCentury, Philadelphia, PA, USA) as previously described [83–86]. Briefly, the mice were anesthetized using a mixture of isoflurane and oxygen for 8-10 minutes. The mouse was quickly placed in a stand with its teeth suspended up at a 45° angle, and its tongue gently rolled out using a cotton tip. The MicroSprayer tip was then inserted into the trachea and bacteria inoculum sprayed out into the lungs. After administration, the mouse was placed back into the cage and monitored as it regained consciousness. To confirm bacterial uptake after infection, 3 mice were sacrificed within the first 24 hours of bacteria exposure and the lungs were removed for CFU enumeration as explained below. Mice were monitored daily; their weights were recorded, and upon showing signs of disease, or losing 20% of their original weight, were immediately euthanized. The remaining mice infected with *M. abscessus* #21 were rested for 10 days when animals were randomly assigned to groups (n=3 or n=5) and the drug (TGC, amikacin, or saline) aerosol treatments were initiated as explained below. One group of mice remained untreated and was housed in the same environment as the other mice in the study.

2.2f Drug treatment

All drug vials of TYGACIL® were wrapped in aluminum foil and stored in a temperature and humidity-controlled room to maintain drug stability. On the treatment day

and immediately before administration, each vial was reconstituted in 300 µl of 0.9% endotoxin-free saline solution (TekNova) and delivered via intrapulmonary aerosol delivery using the a MicroSprayer device, as outlined above. Similarly, amikacin (Gold Biotechnology) was delivered at 0.50 mg/dose also in endotoxin-free saline solution (TekNova). Treatments were repeated five days a week for four weeks. Toxicity and tolerability to treatments was monitored by recording the mice normal turgor, eating/drinking, posture, grooming and weights. Twenty-four hours after last treatment, all the mice were euthanized as indicated below. Two different studies are addressed in this chapter (study #1 and study #2), both of which utilize the same reagents and protocols.

2.2g Necropsy

One and ten days after infection, as well as four weeks after initiation of therapy, mice (including those not receiving treatment) were euthanized by CO_2 inhalation followed by cervical dislocation. During the necropsies, the lung and spleen were examined for gross pathology and recorded by taking pictures. Thereafter, lungs were prepared for enumeration of bacterial burden and histopathology.

2.2h Determination of pulmonary bacterial burden

Mouse lung and spleen tissues were homogenized using the Next Advance Bullet Blender (Averill Park, NY). Briefly, the left lobe of the lung or the entire spleen were placed in a 1.5 ml sterile, safe lock Eppendorf tubes containing 0.5 ml of sterile 1X PBS and 3 x 3.2 mm, sterile stainless-steel beads. Then the tubes were placed in the Bullet Blender and homogenized for 4 minutes at 8000 rpm. After homogenization, each sample was used for bacterial enumeration as follows. For bacterial load enumeration, serial dilutions

of homogenized organs were prepared and plated onto agar plates as explained above. Plates were incubated for 3-4 days at 37°C when the CFU in each plate were enumerated. To prevent drug carry-over effect onto agar plates, the organs were also plated on 7H11-OADC agar plates containing 0.4% activated charcoal. The data were expressed as the mean $\log_{10}$ CFU ± the standard error for each group.

2.2i Determination of drug carryover effect

To further determine if residual drug in tissue homogenate was inhibiting colony formation on agar plates, samples from each lung homogenate in study #1 were spiked with an equal number of bacteria (100 µl of 1 x 10^4 *M. abscessus* #21 culture in 7H9 was added to 100 µl of tissue homogenate). After mixing each sample four times, an aliquot of 200 µl was serially diluted and plated onto agar plates as above. Each sample was plated in duplicate across 2 different quad-plates consisting of 8 total one-to-five dilutions. Plates were placed in a 37°C standing incubator for 3 days before the CFU were visible to the naked eye and enumerated.

2.2j Histology

The right lung lobe was immediately placed into 4% paraformaldehyde diluted in PBS during the necropsy. Tissues were then paraffin embedded and sectioned onto charged slides (6-10 µm) to be stained by H&E or immunohistochemistry (IHC) using the Otsuka anti mycobacteria O-TB antibody.[87]

2.2k Statistical analysis

Graph Pad Prism version 8.1.1 was used for statistical analysis and data presentation. CFU data was presented as the mean number of CFU, and weight data represented as

a group average. A statistical analysis was performed using a Dunnett's multiple comparisons test as part of a one-way ANOVA test.

2.3: Results

2.3a *in vitro* activity of TGC against *M. abscessus* #21

In these studies, we tested the tolerability and efficacy of TGC when delivered as an aerosol to GM-CSF KO mice with *M. abscessus* isolate #21 pulmonary infection.[16] **Table 1** shows the in vitro MIC of *M. abscessus* strain #21 to the commercially available form of TGC as well as both amikacin and clarithromycin; two of the common front-line antibiotics currently used in patient therapy.[5] The MIC of TGC for *M. abscessus* strain #21 was found to be 2.5 x 10^{-4} μM, and the MIC for amikacin and clarithromycin resulted in 8.0 x 10^{-3} μM and 5 x 10^{-4} μM, respectively. MIC readings after 10 days of incubation to the macrolide, clarithromycin, increased from 5 x 10^{-4} μM to 4.0 x 10^{-3} μM, an 8-fold decrease in susceptibility. Similarly, TGC also had an 8-fold reduction in MIC. From these results we concluded that M. abscessus #21 is susceptible to TGC, and as expected, this isolate has inducible macrolide resistance. The increased MIC value for TGC is likely associated with the degradation of the drug in solution during incubation.

Next, we studied if TGC had *in vivo* efficacy against *M. abscessus* strain #21. GM-CSF knockout mice were infected with *M. abscessus* #21, as previously reported [16,65], and rested for 10 days prior to initiation of drug treatment. Shown in **Table 2**, mice were grouped based on their weight and sex (n=3 or n=5) and randomly assigned to treatment groups. The groups in study #1 were as follows: untreated, saline, amikacin (0.5 mg/dose), TGC intermediate dose (1.25 mg/dose), and TGC high dose (2.50 mg/dose).

Study #2 omitted both the amikacin and intermediate dose treatments from study #1, and instead tested a low dose of TGC (0.25 mg/dose) along with the untreated, saline, and a repeated TGC high dose (2.50 mg/dose) **(Table 1)**. The vehicle-saline and amikacin groups were both added as control groups; saline was used as a vehicle in all drug formulations, and the amikacin administered at approximately the MIC value that was previously tested in *M. abscessus* #21. An untreated group was maintained under the same conditions without receiving treatment. Study #2 was subsequently performed to determine a larger range of dose-dependent pulmonary and had a high dose of 2.50 mg/dose and a low dose of 0.25 mg/dose.

During the 40-day infection and drug treatments, mice were observed daily and given a behavior score. Additionally, the mice were individually weighed 3 times a week and averaged by treatment group, shown in **Figure 1**. Behavior scores range from A-D, with A representing a healthy rodent, and D indicating a moribund rodent. As per the approved IACUC protocol, animals dropping below a Score C had to be immediately euthanized. Fortunately, over the course of both 40-day studies, no animals scored below a B (data not included), and there were no significant decreases in weight. Importantly, there was no indication of poor treatment tolerability or markers of poor health.

Starting on day 10 of infection, and for 4 weeks thereafter, mice were treated with either saline, amikacin, TGC, or left untreated (control groups). As previously explained, mice groups (mixed sex and n=3 or n=5) were untreated (Unrx), vehicle (Saline), amikacin (AMK), or TGC low/med/high. Each group was treated for 5 days a week over 4 weeks, at which point, the mice were euthanized, the lungs removed, and further processed to determine the bacterial burden. In both studies, the pulmonary bacterial burden **(Figure**

2) at days 1, 10, and in the untreated group (4 weeks post treatment initiation) are all indicative of the natural progression of pulmonary *M. abscessus* infection with no drug treatment. In the study #1 **(Figure 2A)** there was a significant decrease of 3.3 and 4.0 $\log_{10}$ CFU/lung between the untreated mice and groups that received doses of TGC (1.25 mg/dose and 2.50 mg/dose respectively). Only 1 of the 5 mice that received 1.25 mg/dose (TGC Med. Dose) showed positive CFU numbers, resulting in an average reduction of ~$2x10^3$ bacteria/lung ($P<0.001$). The lung homogenates from mice receiving intrapulmonary aerosols of TGC at 2.50 mg/dose over 4 weeks of treatment showed no CFU ($P<0.00001$). Amikacin treated group (n=5) had no significant difference in bacterial load when compared to the untreated and saline treated groups.

In Study #2 **(Figure 2B)**, TGC was administered at high 2.50 mg/dose (as in study #1) and low 0.25 mg/dose (ten fold lower than high dose). Statistically, there was no difference between the TGC low dose and the untreated control group. The TGC high dose group, showed a CFU reduction in all five of the mice, however, one mouse out of five had a 2.0 $\log_{10}$CFU reduction.

Throughout these studies, it was a concern that the significant CFU reduction in drug treated groups could be TGC carry-over in lung tissue homogenate. To address this concern, duplicates of serial fold dilutions of each sample were plated on both standard 7H11 agar and 0.4% activated charcoal supplemented 7H11 agar. The plates were incubated and the CFU were determined. **Figure 3** shows the comparison of CFU results from the two different compositions of agar. To further determine if there was drug carryover in tissue homogenate, samples from each lung were spiked with 1 x 10^4 bacteria. By adding a known number of bacteria in each sample, any inhibiting factors in

the tissue homogenate should be detected as a decrease in CFU in comparison to the inoculum. **Figure 4** shows the resulting CFU counts after 72 hours of incubating each homogenate sample from every group with equal amounts of bacteria. There was no significant difference between any of the treatment groups and the inoculum. Additionally, a control group (n=3) using *M. abscessus* #21 spiked with 2.50 mg of TGC in 50 µl of saline was added to quantify how the addition of "free" TGC would affect the growth of the bacteria, resulting in 1 x 10^4 bacteria, which was not significantly different from any of the other samples. The lack of TGC killing is attributed to rapid degradation of the small amount of drug in the tissue homogenate.

Histopathological analysis of the lung sections obtained from *M. abscessus* infected GM-CSF KO mice **(Figure 6A, B, C)** when compared to similar samples from uninfected GM-CSF KO mice **(Figure 5)**, demonstrated appearance of granuloma or pre-granuloma formations **(Figure 7)** and influx of inflammatory cells, mainly plasma cells and neutrophils in parenchyma. Additionally, samples from infected animals demonstrated an influx of lymphocytes in perivascular zones and an increase of foamy cells in granulomatous like formations **(Figures 6-7)**. There was a noticeable difference in the histology between samples obtained from infected mice receiving aerosols of TGC when compared samples from mice not receiving treatment; TGC treated mice show increased necrotic debris in the alveolar lumen and proteinaceous edema residue **(Figure 6C)** and occasionally, alveoli were lined by hypertrophied cells with vesicular nucleus, suggesting Type II cell hyperplasia.

IHC using a well characterized anti-mycobacteria specific antibody (O-TB, Otsuka Pharmaceutical Co., Tokyo Japan) that recognizes the mannose 3 cap, in conjunction

with a blue-fluorescent DAPI DNA stain, revealed the presence of bacilli in all tissue samples obtained from infected animals **(Figures 8-9)**.[87] Small rod-like bacilli are observed in clusters in lung tissue collected from *M. abscessus* infected - untreated animals **(Figure 8)**, and similar, but at a lower frequency in lung tissue obtained from *M. abscessus* infected - TGC treated animals **(Figure 9)**. Importantly, staining with the O-TB antibody demonstrates that most bacilli are present intracellularly. The bacilli are more easily observed intracellularly under 40x magnification with the addition of transmitted light **(Figure 10)**.

Table 1 MICs of tigecycline, amikacin, and clarithromycin against *M. abscessus*

	Drug (μg/ml)					
	Tigecycline		Amikacin		Clarithromycin	
Incubation Time	72 hours	<10 days	72 hours	<10 days	72 hours	<10 days
Accepted	0.12	--	24	--	0.75	<16 [88]
Tested (#21)	0.25	2	8	16	0.50	4

-- Data not represented in literature

Table 2 Treatment dose for each treatment group of GM-CSF KO mice

	Dose	
Treatment Group	Study #1	Study #2
Untreated	No dose (n=5)	No dose
Vehicle Saline	0.9% saline (n=3)	0.9% saline (n=3)
AMK	0.50 mg/dose (n=5)	---
TGC Low Dose	---	0.25 mg/dose (n=5)
TGC Med. Dose	1.25 mg/dose (n=5)	---
TGC High Dose	2.50 mg/dose (n=5)	2.50 mg/dose (n=5)

[a] AMK, amikacin; TGC, tigecycline

Note: Each dose is delivered as a 50 μl aerosol, once a day.

--- Indicates treatment group was not included in the respective study

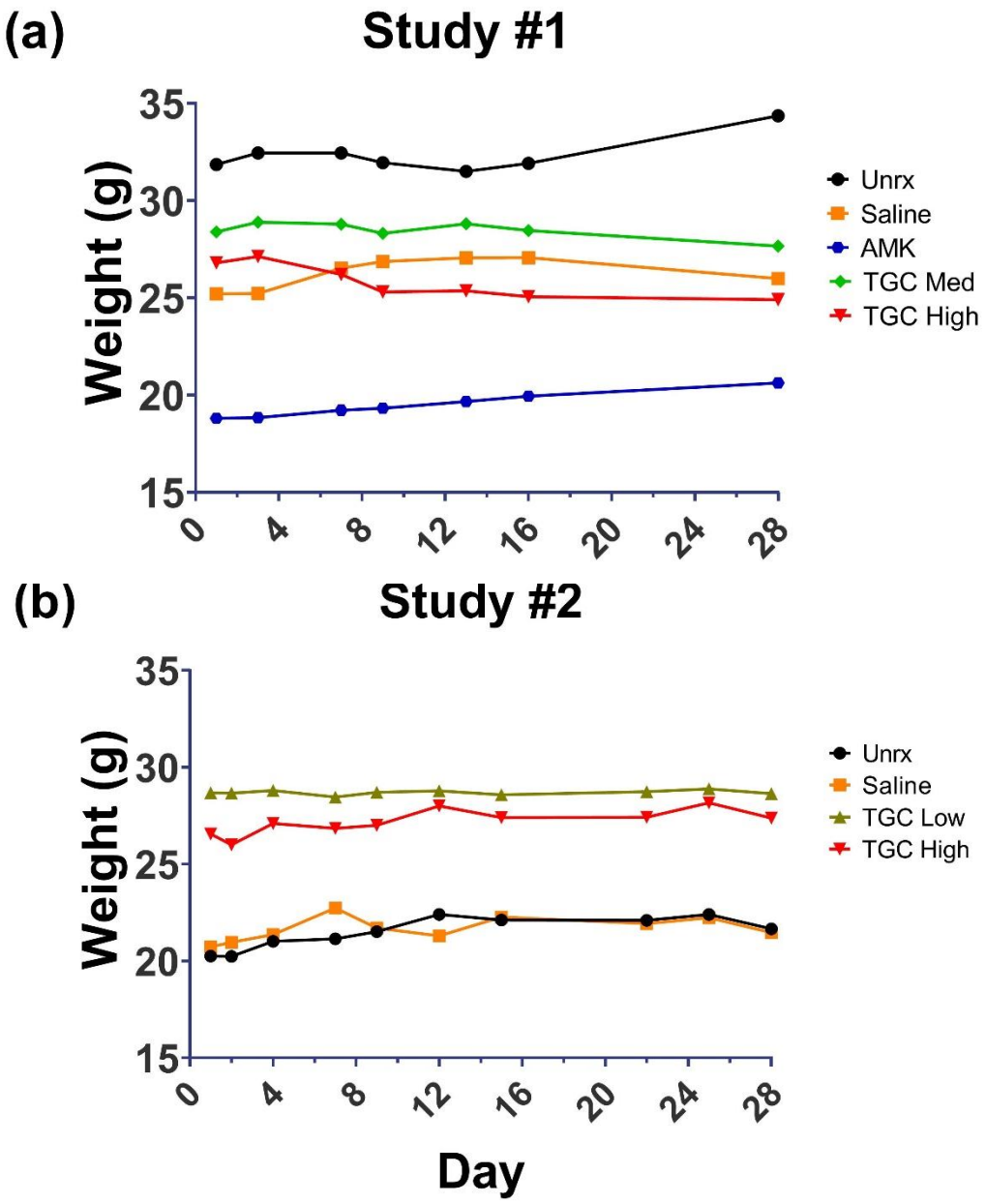
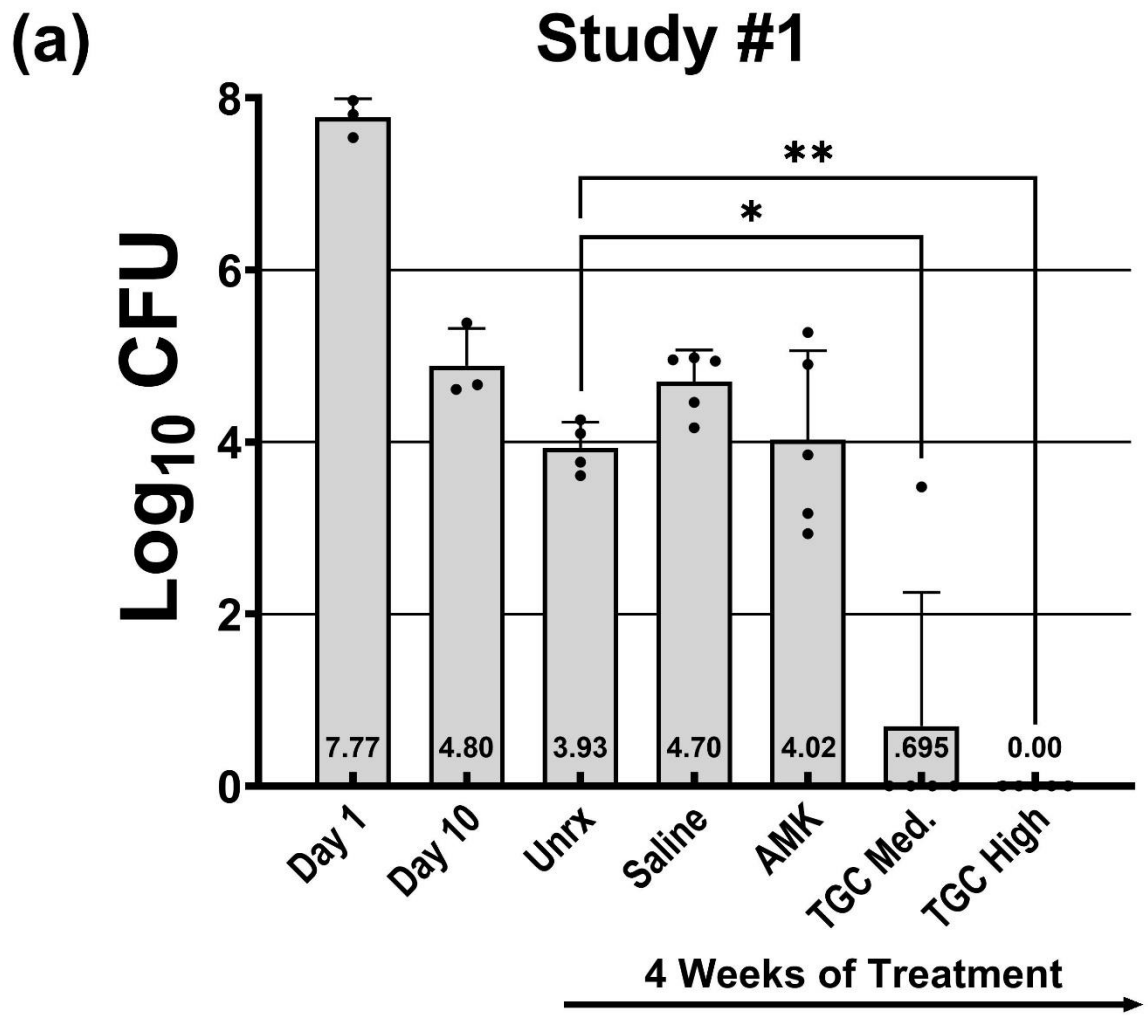


Figure 1. Graph of the mean weights from each treatment group across both study #1(a) and study #2 (b) at different times of infection and treatment.


(a)
Study #1
Log10 CFU
8
6
4
2
0
**
*
7.77
4.80
3.93
4.70
4.02
.695
0.00
Day 1
Day 10
Unrx
Saline
AMK
TGC Med.
TGC High
4 Weeks of Treatment

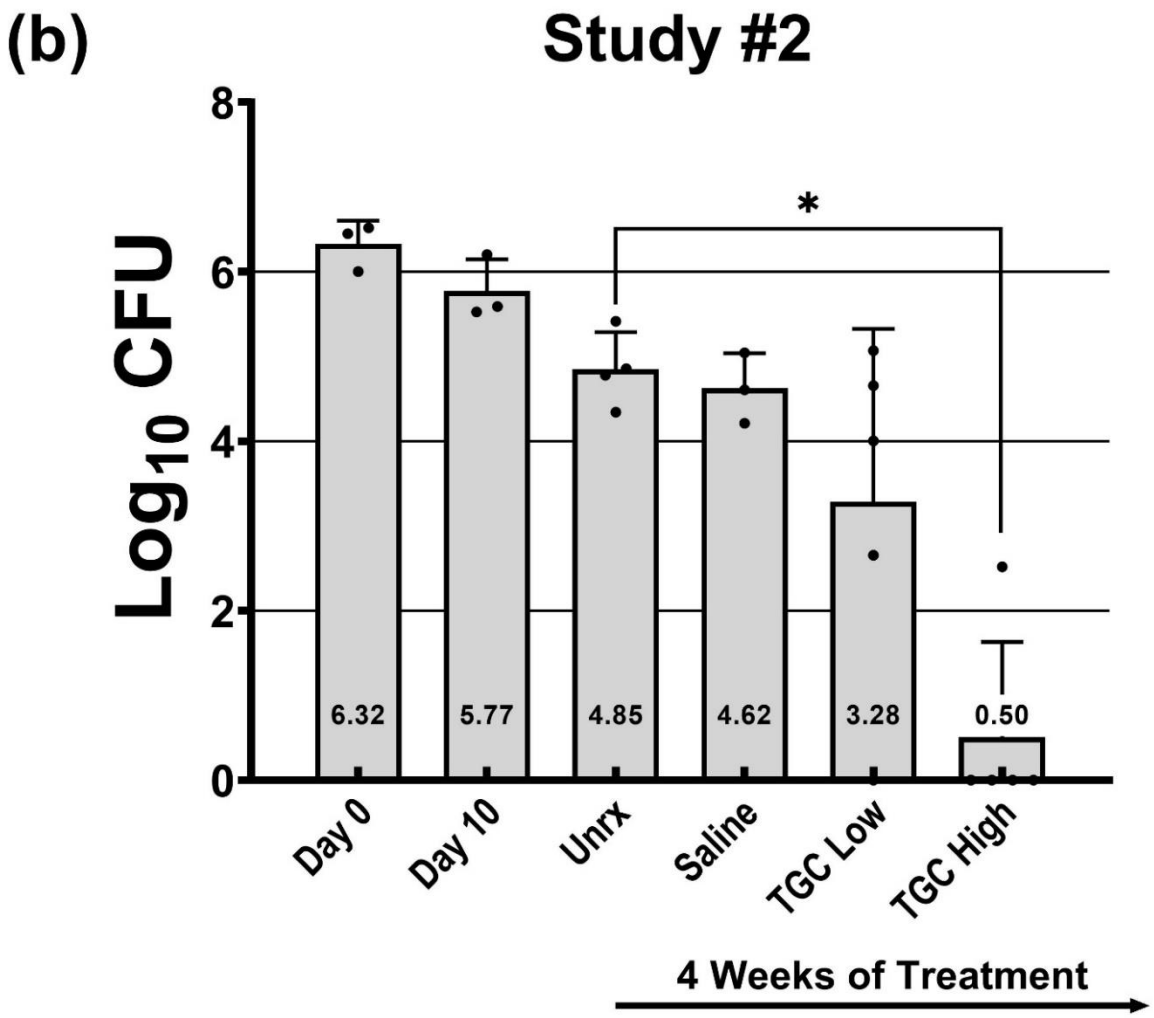


Figure 2. Bacterial load in the lungs of mice for study #1 (a) and #2 (b). Single data points in each group are represented by dots. The Y-axis represents the mean value and SEM of Log_{10}CFU in the lungs. The X-axis represents each group and time point or group treatment in the study. Day 0 and Day 10 are time-points included as infection control groups. All treatments were initiated on Day 10 and each group, excluding the untreated (Unrx) group, received an intrapulmonary aerosol dose of either saline, AMK (0.50mg/dose), or TGC at low/med/high concentrations (0.25/1.25/2.50 mg/50 μL dose respectively). The data represent the mean ± SD. Dunnett's multiple comparisons test; *, P<0.001, **, P<0.0001.

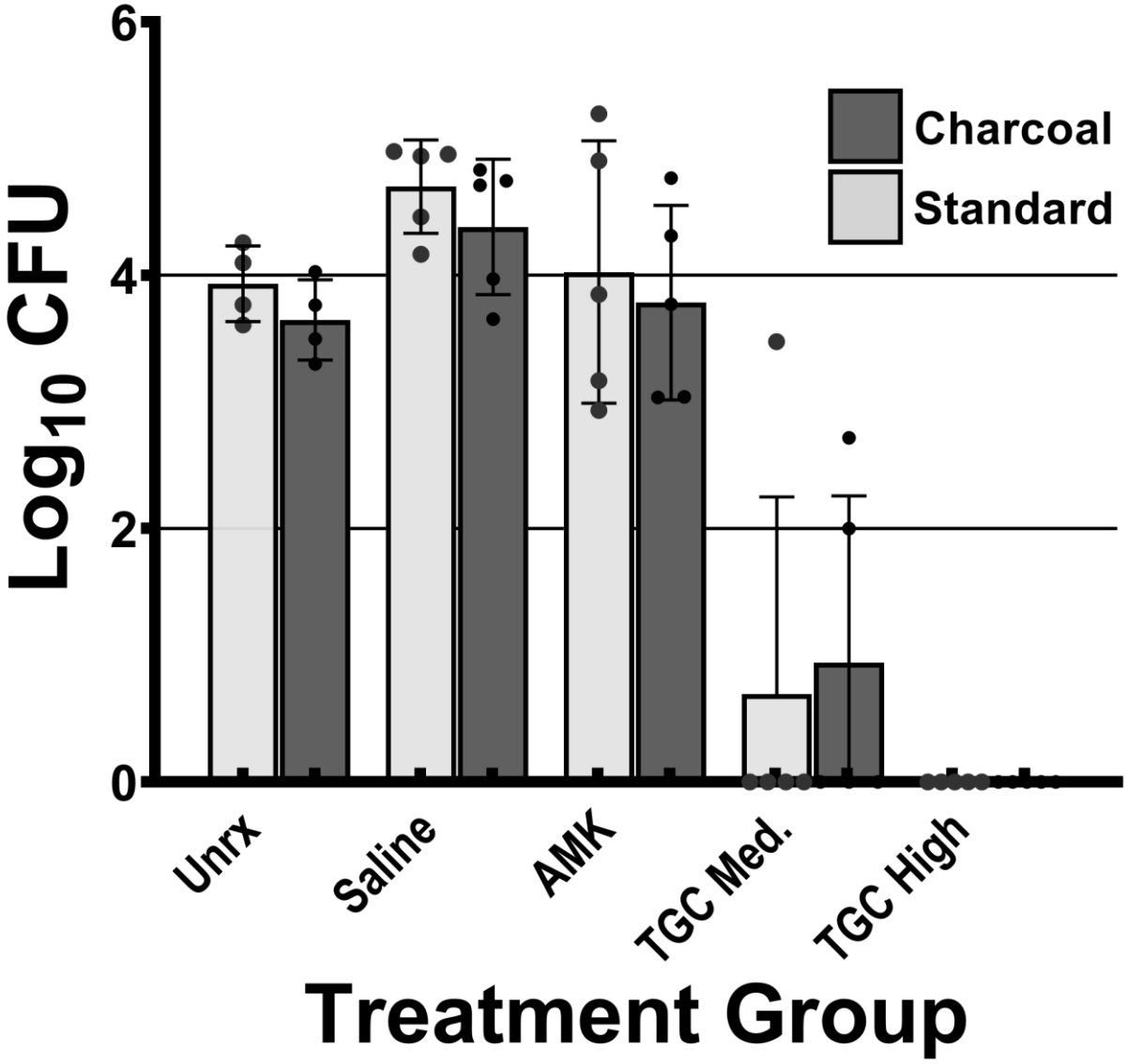


Figure 3. A comparison of study #1 CFU results between standard and activated charcoal agar plates after 4 weeks of treatment. There is no significant difference in CFU between the two types of agar. The x-axis represents the type of treatment, and the y-axis indicates log_{10}CFU after 72 hours of incubation. Each dot represents an individual mouse. Bars are shaded as white and gray to represent the use of standard and charcoal plates, respectively. The data represent the mean ± SD.

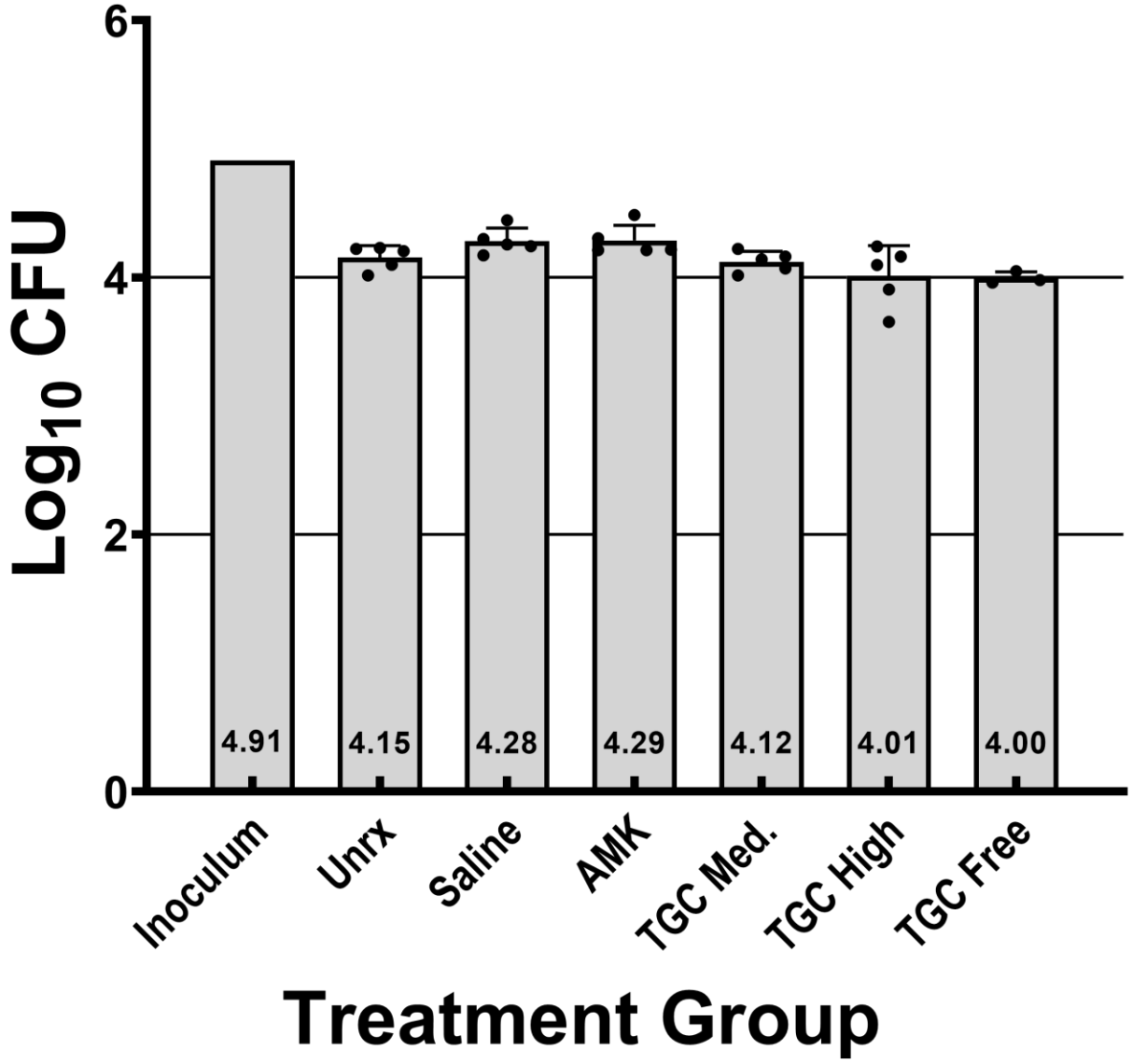


Figure 4. Lung homogenate samples from study #1 were spiked with 100µl of 1 x 10^4 *M. abscessus* #21. The x-axis represents the type of treatment, including: inoculum, used to inoculate the other 6 groups, the samples from the 5 treatment groups (Unrx, Saline, AMK, TGC Med., TGC High), and a control group with the addition of 2.50 mg of tigecycline (TGC Free). The data represents the mean ± SD. log_{10}CFU/ml.

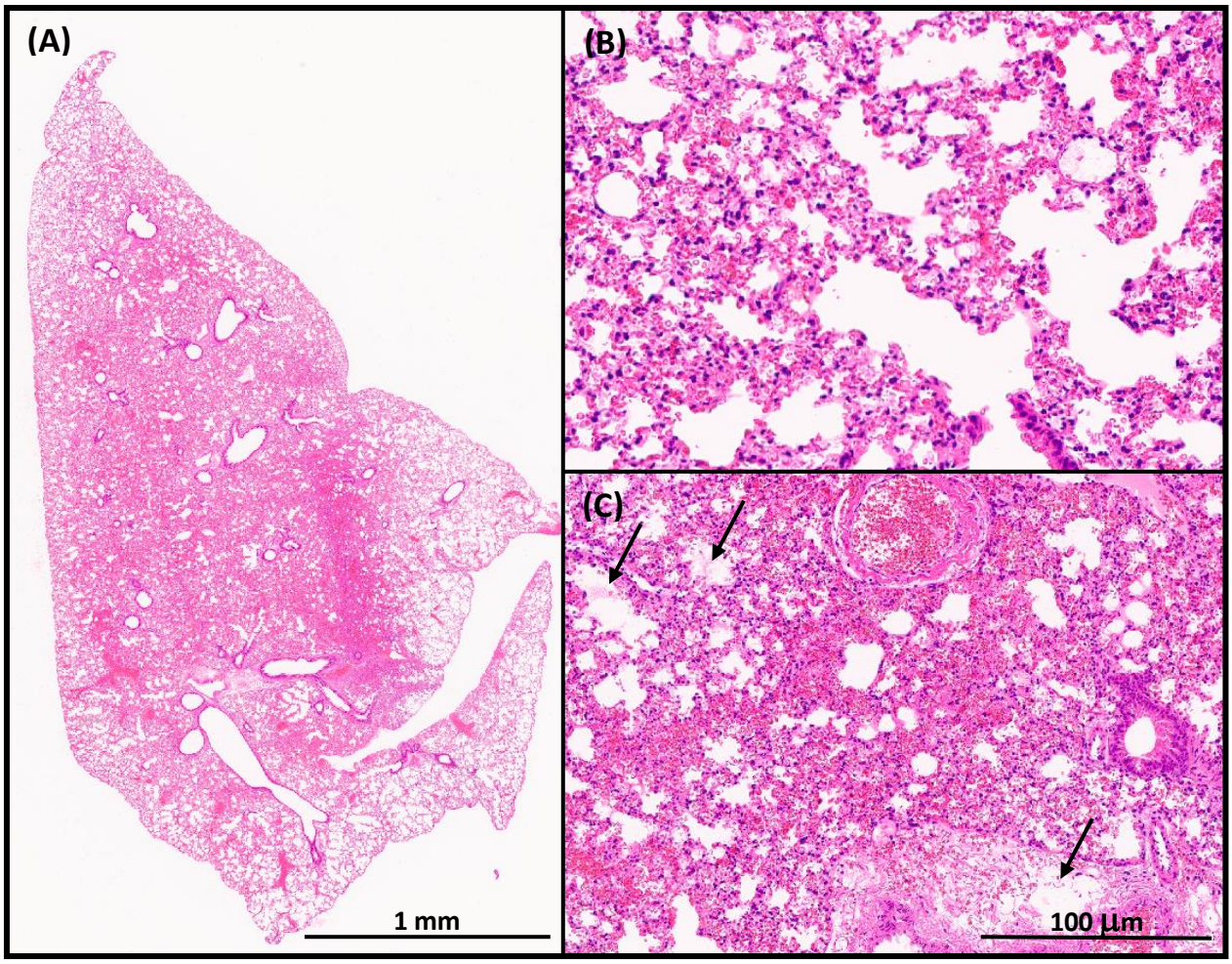


Figure 5. H&E stained lung tissue section of uninfected GM-CSF KO mice after fixation in 4% paraformaldehyde. (A) Left lung lobe. (B) 20x magnification showing alveolar space. (C) 20x magnification with black arrows depicting alveolar debris, typical for GM-CSF KO mice.

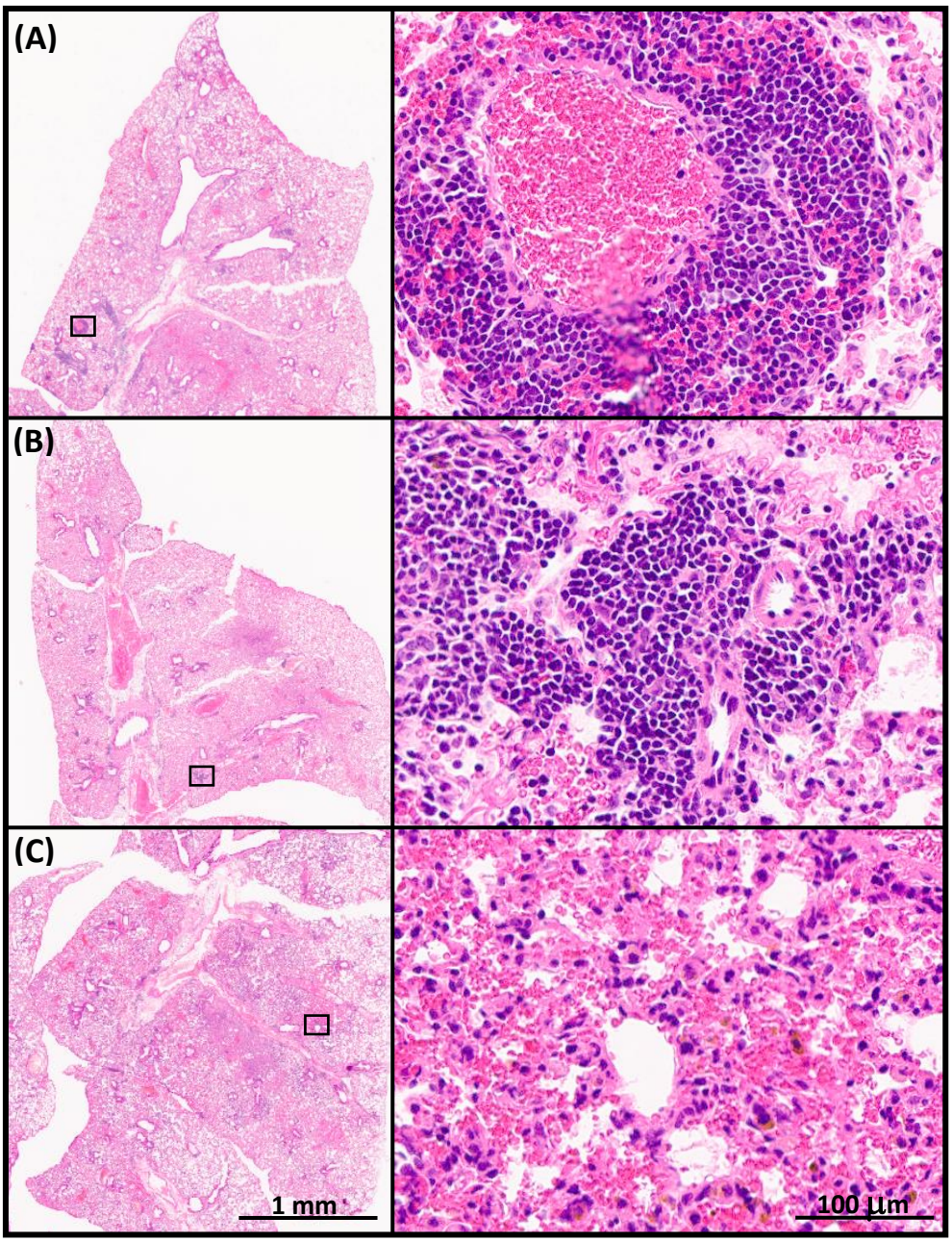


Figure 6. H&E stained tissue of mice 38 days post challenge of *M. abscessus* strain #21 from study #2. Left image panel is at 0.5x magnification and right image is at 20x magnification. (A) Untreated lung tissue with perivascular lymphocyte infiltration. (B) Representative 0.25 mg/dose TGC treated lung tissue. (C) Representative 2.50 mg/dose TGC treated lung tissue.

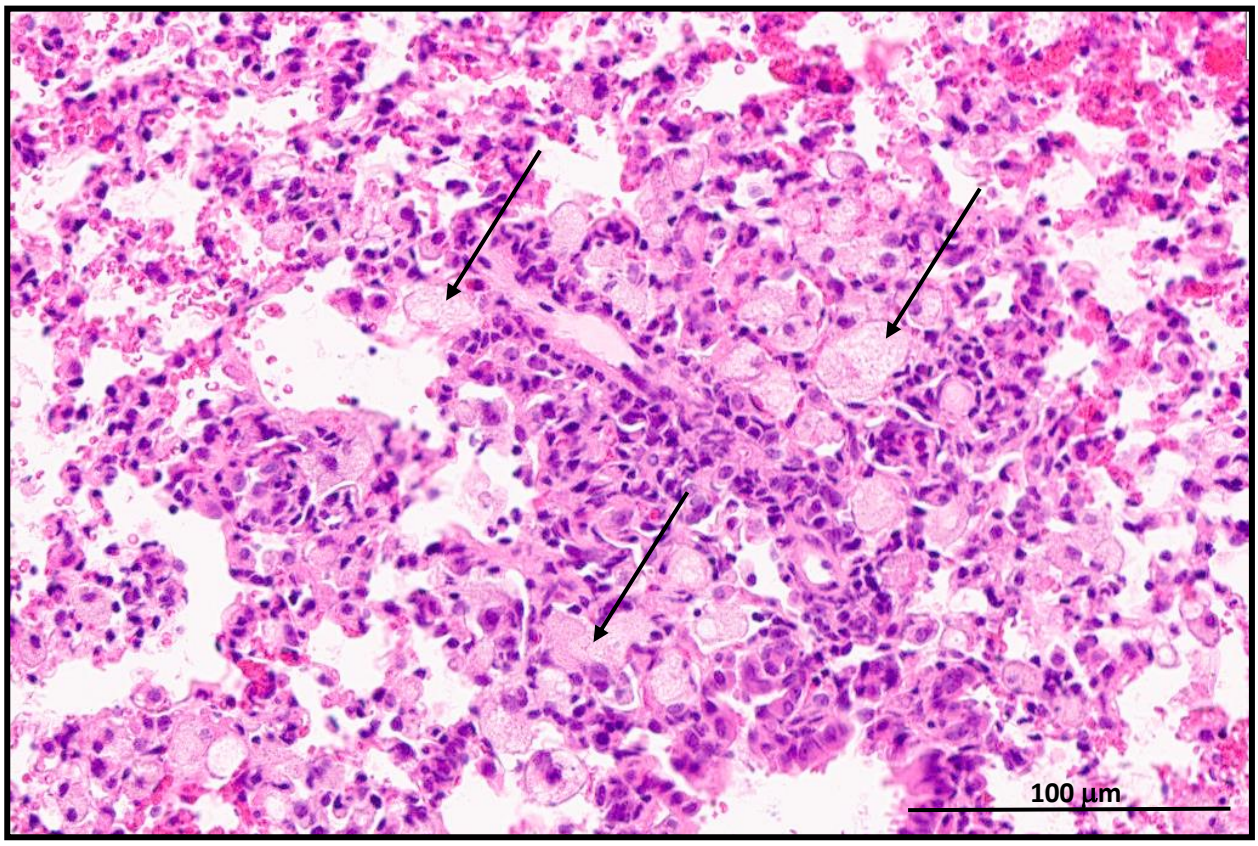


Figure 7. H&E stained lung tissue section showing foamy cells (black arrows) that are present in tissue samples from all *M. abscessus* infected groups and absent in uninfected tissue samples.

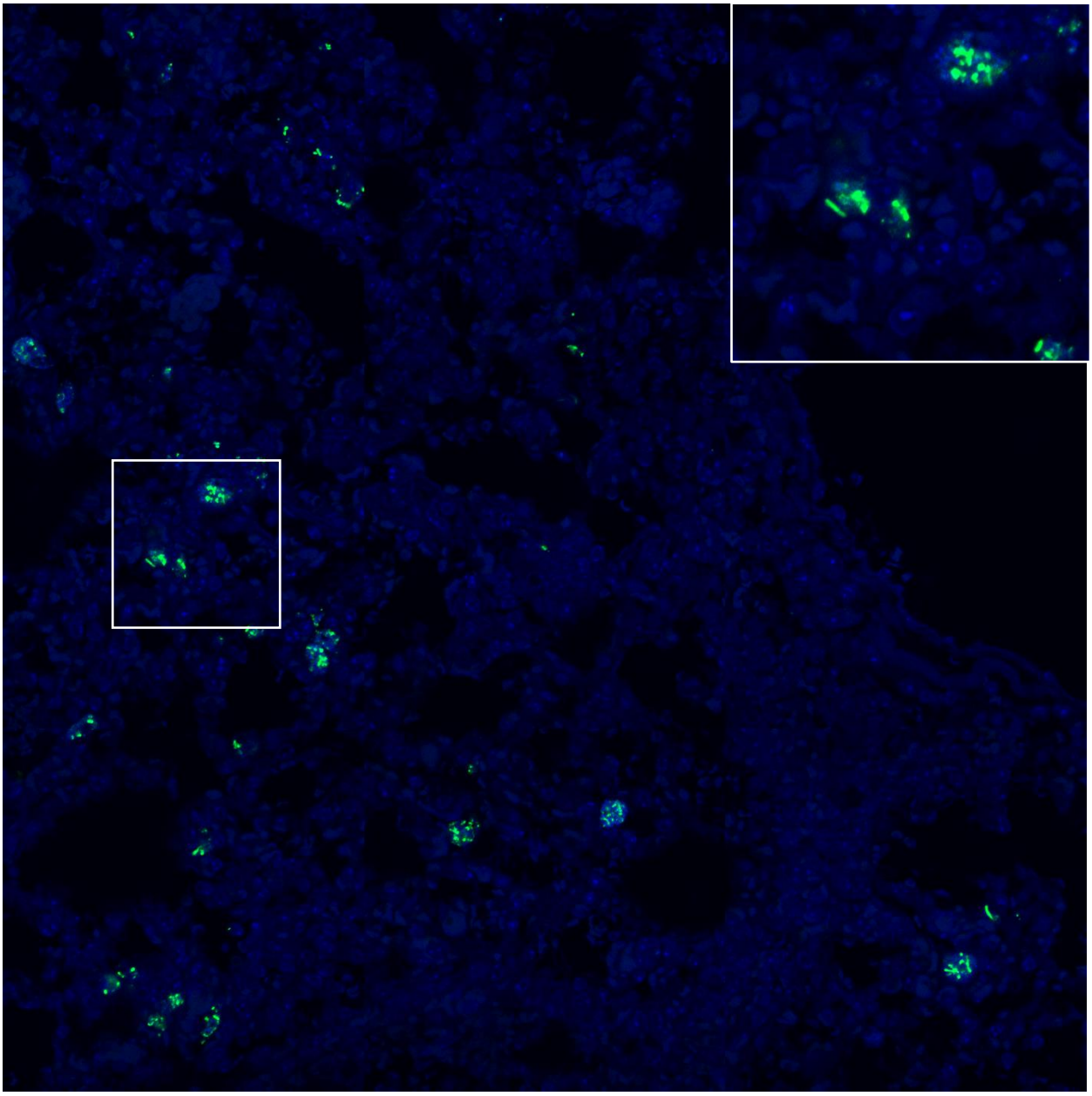


Figure 8. Confocal microscopy image of paraformaldehyde fixed lung tissue collected from an untreated and *M. abscessus* #21 infected mouse. *M. abscessus* #21 (green) is immune-stained with O-TB antibodies, and the surrounding nuclei are stained with the nucleic acid binding DAPI (blue).

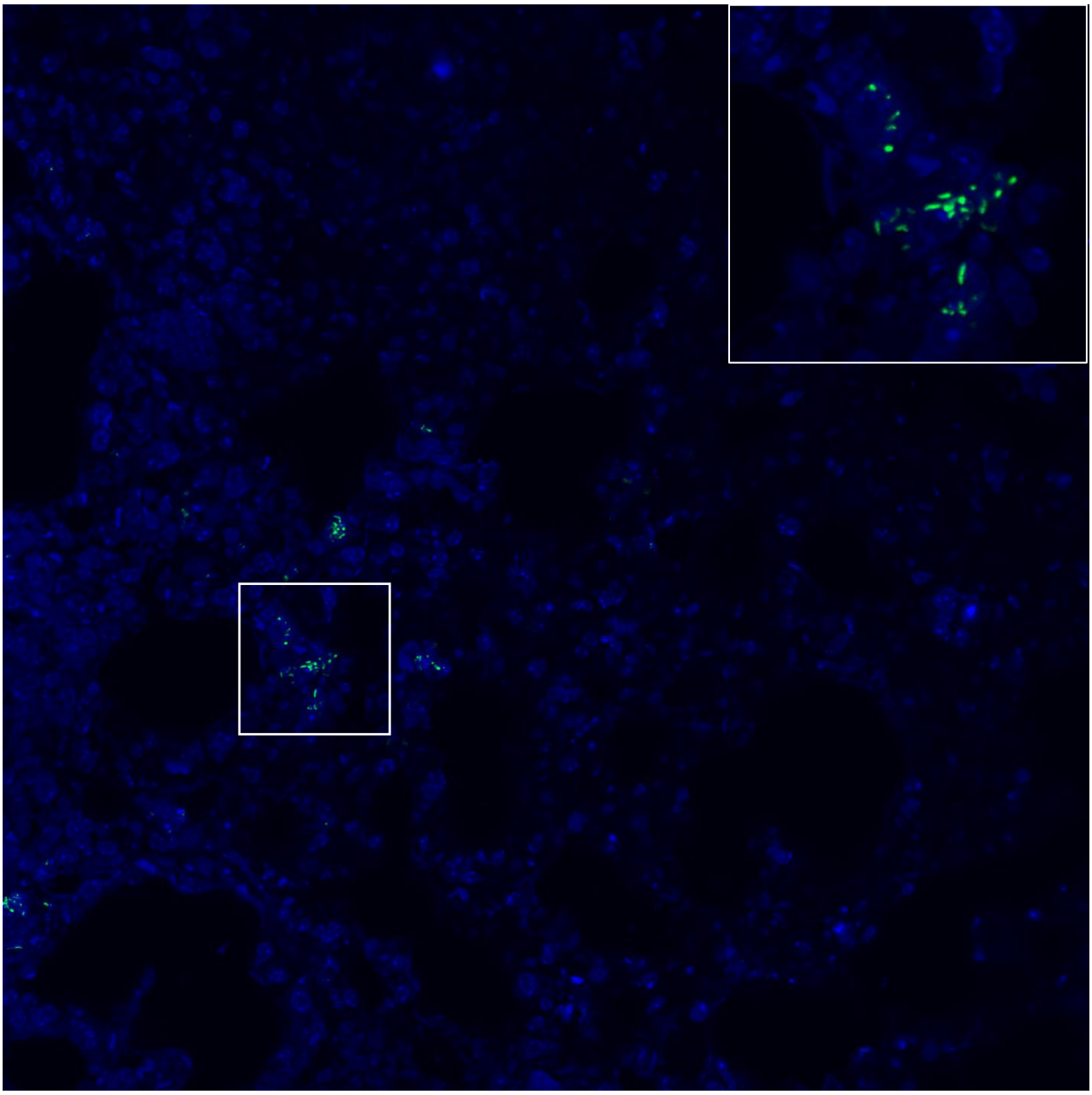


Figure 9. Confocal microscopy image of paraformaldehyde fixed lung tissue collected from an *M. abscessus* #21 infected mouse receiving TGC as a high dose aerosol (2.50 mg/dose). *M. abscessus* #21 (green) is immune-stained with O-TB antibodies, and the surrounding nuclei are stained with the nucleic acid binding DAPI (blue).

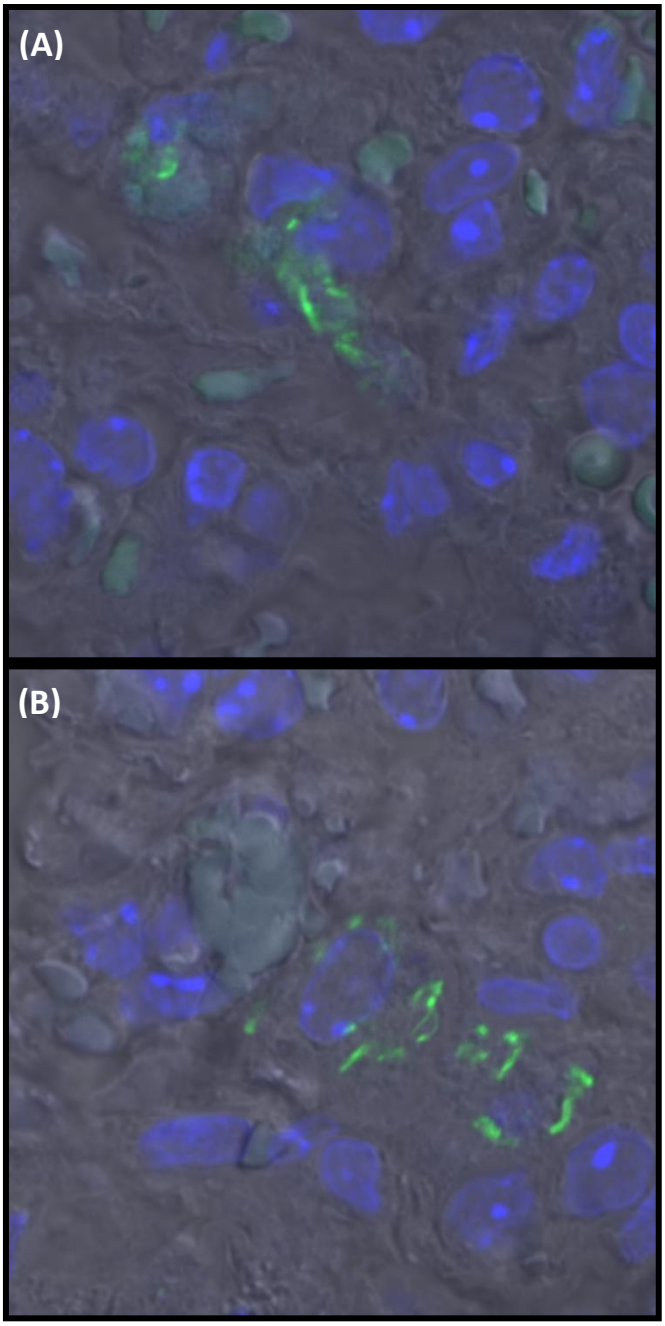


Figure 10. Confocal microscopy images of fixed lung tissue collected from an M. *abscessus* #21 infected mouse receiving: (A) no treatment, and (B) receiving TGC as a high dose aerosol (2.50 mg/dose). M. *abscessus* (green) is immune-stained with O-TB antibodies, and the surrounding nuclei are stained with the nucleic acid binding DAPI (blue). Cell morphology can be observed with the included transmitted light.

2.4: Discussion

Intravenous administration of tigecycline has shown to improve treatment outcome in patients that are able to overcome the side-effects, however, most patients discontinue IV administration of this antibiotic before the therapy regimen is completed.[14,48] In this study, we hypothesized that tigecycline is effective and well tolerated when administered via inhaled therapy. Indeed, using the preclinical GM-CSF KO mouse model, we demonstrated that inhaled tigecycline proved highly efficacious against pulmonary *M. abscessus* infection in mice, and furthermore, the daily therapy was well tolerated.

The data shows that the bacterial killing efficacy of inhaled tigecycline was dose-dependent. More specifically, the two studies were developed in GM-CSF KO mice infected with pulmonary *M. abscessus* that received a one month, 5 days-per-week, treatment of either 0.25, 1.25, or 2.50 mg/dose as an intrapulmonary aerosol. After the full duration of the treatment regimen, we determined pulmonary bacterial burden in each individual animal by CFU determination. Lung samples from animals treated with both 1.25 and 2.50 mg/dose, in all but one sample per group, lacked *M. abscessus* growth, whereas all samples from animals in the control groups (and 0.25 mg/dose tigecycline group) had positive *M. abscessus* bacterial recovery. To assess the potential of bacterial growth inhibition caused by carry-over tigecycline in the sample lung homogenate, each individual sample was plated on 0.4% activated charcoal-containing 7H11 agar to bind any remaining drug.[89] There were no significant differences between the resulting CFU counts from lung samples plated with and without the presence of charcoal, leading us to the conclusion that the bacterial growth was not a result of residual drug in sample homogenate. As a second method of verification all the remaining lung homogenates

were spiked with 1 x 10^4 CFU and cultured overnight. The results showed no inhibitory effect of bacterial growth in tissue samples, suggesting that the positive efficacy of inhaled tigecycline in these studies was not associated to an inhibitory effect in the sample, but rather to the administration of aerosolized tigecycline therapy.

The inhaled tigecycline tested in these studies was a liquid formulation in 0.9% saline. Tigecycline has been demonstrated to rapidly lose stability and degrade, both in aqueous solution and with light exposure.[73] Although all the formulations for these studies were administered immediately after suspension, and carefully shielded from the light, there was still potential for drug degradation during and after the administration procedure and it is possible that the delivered dose for each treatment was lower than the calculated dose. In humans, a liquid drug formulation is easily administered using a nebulizer, an approach currently used for the treatment of several other pulmonary diseases.[36] With regards to tigecycline, there is a need to study the side effects from exposure to a nebulized aerosol, as there is currently no literature that documents using tigecycline as an aerosolized inhalational therapy. Thus, future studies should develop new, more stable, inhaled formulations of tigecycline and test tolerability of inhaled tigecycline in humans. Furthermore, pharmacokinetic studies to determine pulmonary and systemic drug time - exposure of tigecycline after inhaled administration are still pending.

The dose dependent efficacy in these studies aligns with previously reported bactericidal effects of tigecycline that was seen in the hollow fiber model.[6] The current tigecycline regimens suggest that patients receive 2 x 50 mg doses per day through IV administration, but a recent study using the hollow fiber *in vitro* models by Ferro et al. suggests that tigecycline via IV would deliver optimal bactericidal effects at 200 mg/day,

approximately twice the current dosage. Clinical data, however, indicates that this high of an IV dose will be poorly tolerated by patients. Based on our results here, we reasoned that administration of tigecycline by inhalation at high doses (≤200mg/dose) will be well tolerated and that the rapid degradation of solubilized tigecycline could be beneficial in avoiding systemic exposure and decreasing side effects while subsequently increasing the local bactericidal effect in the lungs.

As previously discussed, TGC showed to be highly efficacious in pulmonary bacterial killing. Interestingly, IHC in combination with confocal microscopy analysis of lung sections from the mice in these studies clearly demonstrated the presence of numerous clustered and rod-shaped bacilli in every *M. abscessus* infected sample. The O-TB antibody used to visualize the bacilli binds to the surface exposed mannose-3 cap on the lipoarabinomannan (ManLAM), a lipoglycan that is specific to mycobacteria.[87,90] Our data highlights rod-shaped bacilli within lung cells (often within foamy macrophages) and indicate that lung samples from the untreated animals contain higher numbers of bacilli than the samples from TGC treated animals. Because we were unable to recover CFU out of the homogenate derived from TGC treated animals, we speculate that the bacilli visualized using the O-TB antibody were dead. It is well reported that lung macrophages in the GM-CSF KO mice have a deficiency in their ability to clear debris, therefore, the bacilli observed in TGC treated samples are likely dead and can still bind to the O-TB antibody.[91]

To summarize, we concluded that inhaled tigecycline represents a viable option for therapy against *M. abscessus* pulmonary infection that warrants further studies. In depth tolerability studies paired with pharmacokinetic exposure studies are necessary to

determine which concentrations of inhaled tigecycline may provide the maximum pulmonary bactericidal effects while minimizing side effects. The preclinical data presented here prompts further questioning of the possibility of delivering high concentrations of tigecycline to the lungs of human subjects.

Chapter 3: Limitations and Concluding Remarks

Historically, animals have played a critical role in the characterization of pathophysiology, antibiotic target identification, and evaluation of therapeutic agents and their respective pharmacokinetic relationships.[92] To our knowledge, the tolerability and pharmacokinetic relationships of tigecycline against mycobacterium has not been assessed in the mouse model with any form of delivery. A study in 2013 administered a low, subcutaneous, dose of tigecycline in a combination therapy against *M. abscessus* infection in Nude and GKO mice.[93] They state that tigecycline shows promising *in vivo* activity, however, due to the combinational therapy and lack of study longevity, it is difficult to evaluate the tolerability of tigecycline and further translate this to a human. Characterizing tigecycline's dose dependent effects on the infected mouse model is an important step in trying to understand the limitations that it may have in patients. The hollow fiber system provides us with a seemingly reliable model for determining the concentration-dependent effects that tigecycline may elicit but is currently limited to assessing MIC ratios against extracellular bacteria.

The first step in these studies was to characterize tigecycline's dose dependent effects in the GM-CSF knockout mice. Limited by the quantity of animals, determination of tigecycline tolerability in mice was limited to a high dose (nearly twice the daily recommended human dose) delivered as an intratracheal aerosol. The data from pilot studies indicate that the daily inhaled tigecycline treatment was well tolerated. This, however, has an unknown relevance in comparison to the tolerability of such high doses using IV administration techniques. The intravenous technique in murine preclinical

models, however, is challenging and poorly tolerated over repeated doses and repeated IV treatments.[64] In humans, IV is often a slow infusion on a continuous basis over several hours or days, and the alternative in mice is to use an electronic infusion pump that slow-injects through a catheter. This infusion pump alternative is costly and unreasonably time consuming for studies with large numbers of animals. Future studies should characterize the differences in tolerability, and eventually the pharmacokinetics (PK), of IV delivered tigecycline in mice relative to humans. Translating the *in vivo* mouse results into a human model of infection is meaningless until either the IV delivery of tigecycline is characterized in mice, or inversely, the inhaled tigecycline dose is characterized in humans. As of now, neither have been established, and translating this data into clinically relevant information is challenging.

The confocal microscope images collected for these studies clearly depict the presence of *M. abscessus* in the collected lung samples, however, additional supporting data is necessary for determination of the state of these bacilli. Future studies will address this issue using methods that include: RT-PCR for quantification of bacterial derived amplicons, mycobacterium growth indicator tubes (MGIT), and selective detection with microarray technology.

Firstly, using *M.* abscessus specific DNA primers, in combination with cDNA generated from a tissue sample RNA extraction, it should be possible to determine the relative quantity of bacterial RNA present in the lung tissue. A quantitative comparison of sample produced CT (cycle threshold) values is a good indicator of the relative amounts of live bacteria present in each individual sample. Next, the MGIT is a semi-automated detection machine that utilizes $^{14}CO_2$ to measure the decarboxylation that occurs in the

presence of metabolic processes.[43,94] This system is highly accurate in its time-to-detection, and will, throughout incubation, assign each sample a value that represents growth rate. The resulting growth value can be used to confirm the presence and determine relative quantity of *M. abscessus* in a given sample. By inoculating MGIT tubes with tissue homogenate from study #2, we will compare the relative rates of bacterial metabolism to confirm or deny the presence of *M. abscessus* in each sample. Lastly, DNA-intercalating dyes offer the possibility to selectively detect live bacteria. Dyes like ethidium monoazide and propidium monozide, paired with microarray signal detection, allow for accurate detection of live and dead bacilli concentrations through emission light intensities.[95] Ideally, the combined analysis of all three methods would provide enough evidence to determine, with certainty, the metabolic and genomic state of the *M. abscessus* bacilli in each lung sample.

M. abscessus is a pathogen that can survive long periods of time within the macrophage or dendritic cell. Optimizing *in vitro* cell infection assays is an indispensable tool that will be used to characterize the effects of tigecycline on intracellular *M. abscessus*. The CFU-based intracellular mycobacteria count technique is the most common method for quantifying the viability intracellular bacteria after antibiotic treatment.[96] This method involves introducing bacteria into a viable cell culture, incubating for a predetermined amount of time, eliminating extracellular bacteria, and finishing with cell lysis and CFU determination. In theory, the resulting CFU count represents the quantity of intracellular bacilli recovered from cell debris, however, few procedures consider confirming the removal of extracellular bacilli prior to assessing “intracellular” bacterial burden. Our current studies focus on the ensuring complete removal of

extracellular *M. abscessus* in macrophage cell culture by washing cells multiple times with antibiotic supplemented media, followed by supernatant CFU determination, and finally, sample fixation for confocal microscope image analysis. Through optimizing intracellular infection protocols, we ensure that the results are representative of the antibiotic effects that tigecycline has on intracellular mycobacterium, which should be addressed when understanding treatment of pulmonary *M. abscessus* infection.

The overall goal of these projects was to demonstrate that inhaled tigecycline could represent a viable option for therapy against *M. abscessus* pulmonary infection. The resulting data shows that tigecycline can be administered to mice as an aerosol and is well tolerated throughout the entire treatment duration. Although there are many limitations to these studies, our data suggests that inhaled tigecycline aerosols may increase the pulmonary bactericidal effects and decrease the severity of the side-effects. Studies addressing these limitations may provide the information necessary to translate this preclinical data into a clinically relevant inhaled tigecycline therapy for treatment of pulmonary *M. abscessus* infection in human patients.

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List of Abbreviations

AAALAC – Association for Assessment and Accreditation of Laboratory Animal Care

AM – Alveolar Macrophage

AMK – Amikacin

ATS – American Thoracic Society

BAL – Bronchoalveolar Lavage

BTS – British Thoracic Society

CF – Cystic Fibrosis

CFTR – Cystic Fibrosis Transmembrane Conductance Regulator

CFU – Colony Forming Units

COPD – Chronic Obstructive Pulmonary Disease

CT – Cycle Threshold

ENaC – Epithelial Sodium Channel

FDA – The Food and Drug Administration

GKO – Gamma Interferon Knockout

GM-CSF – Granulocyte Macrophage Colony Stimulating Factor

H&E – Hematoxylin and Eosin

HFM – Hollow Fiber Model

IACUC – Institutional Animal Care and Use Committee

IHC – Immunohistochemistry

IV – Intravenous

KO – Knockout

MALDI-TOF – Matrix-Assisted Laser Desorption/Ionization-Time of Flight

ManLAM – Mannosylated Lipoarabinomannan

MGIT – Mycobacterium Growth Indicator Tubes

MIC – Minimum Inhibitory Concentration

MyD88 – MyD88 Innate Immune Signal Transduction Adaptor

NaLC – N-Acetyl-L-Cysteine

NTM – Nontuberculous Mycobacteria

OADC – Oleic Acid, Albumin, Dextrose, Catalase

PK – Pharmacokinetics

RGM – Rapid Growing Mycobacteria

SCID – Severe Combined Immunodeficiency

SEM – Standard Error of the Mean