THESIS

UNDERSTANDING *MYCOBACTERIUM ABSCESSUS* PULMONARY AND DISSEMINATED DISEASE

Submitted by

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ABSTRACT

UNDERSTANDING *MYCOBACTERIUM ABSCESSUS* PULMONARY AND DISSEMINATED DISEASE

Mycobacterium abscessus is an emerging human pathogen which is difficult to treat and results in increased mortality. Moreover, the cause of increasing case rates and also the pathogenesis of *M. abscessus* are poorly understood. *M. abscessus* belongs to the family of nontuberculous mycobacteria (NTM) classified as members of the rapidly growing mycobacteria (RGM). These environmental pathogens are ubiquitous and found in shower heads, tap water, natural water sources, and soil. Humans contract pulmonary or disseminated infections with *M. abscessus* by breathing in the aerosolized bacteria or ingesting contaminated water. Immunocompromised individuals such as HIV or AIDS patients, are more susceptible to infection with *M. abscessus* as are those with cystic fibrosis, bronchiectasis, and individuals on tumor necrosis factor α (TNFα) inhibitors. Strangely, an increasing population of patients becoming infected with *M. abscessus* are immunocompetent, tall, slender, Caucasian, non-smoking women.

To expand our understanding of *M. abscessus* pathogenesis we developed mouse models that maintain high levels of bacterial infection to study immune responses induced by clinical strains of *M. abscessus*. Our results support the hypothesis that this bacteria can only persist in our animal models that possess a deficiency in macrophages and T cell function. Clustered bacterial strains obtained from

Cystic Fibrosis patients are more virulent than unclustered bacterial strains obtained from Cystic Fibrosis patients. Additionally, counts of viable mycobacterial colony forming units and histological analysis in (Severe Combined Immunodeficiency) SCID mice on a beige background infected with clustered versus unclustered *M. abscessus* strains which were isolated from Cystic Fibrosis patients also supported the increased virulence exhibited by the clustered strains. Lastly, we show that major human *M. abscessus* outbreak strains, when infecting IL-3, GM-CSF deficient mice on an IL-2, Rag2 deficient background (GM/Rag-dblKO mice), result in increased bacterial replication and organ pathology and impaired protective immunity against this pathogen.

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DEDICATION

I dedicate this work to all patients infected with nontuberculous mycobacteria, which is notoriously difficult to treat, and which is lethal all too often.

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1.1 Historical and Current Relevance of *M. abscessus*

M. abscessus is a ubiquitous, rapidly growing, acid fast bacillus (AFB) which can be found in soil, tap water, and shower heads [1, 2]. Ernest Runyon developed a system of identifying nontuberculous mycobacteria in the 1950s [3]. This included classification based on pigmentation and growth rates [3, 4]. This system of identification is discussed further in the next section. There are three distinct subspecies of *M. abscessus* including *M. abscessus* sbsp. *massiliense*, sbsp. *abscessus*, and sbsp. *bolletii*, which are discussed further below [5]. *M. abscessus* is an emerging human pathogen whose impact has grown considerably in the past decade [6, 7]. Mycobacteria have a unique cell wall made up of mycolic acid, peptidoglycan, and arabinogalactan, which likely contributes to their drug resistance and virulence [8, 9]. Clinical strains of *M. abscessus* can have smooth, rough, or mixed morphology [10, 11]. The association of the bacterial morphology and its implication on disease outcome are poorly understood. However, generally a rough morphology predicts a worse clinical outcome due to the cords which interfere with macrophage phagocytosis [10, 11, 12]. The smooth morphology associated with glycopeptidolipid (GPL) production confers the ability of these organisms to form biofilms [13, 14]. Biofilm formation allows smooth variants of *M. abscessus* to colonize the environment [13, 14].

Pulmonary or disseminated disease is caused by inhalation of aerosolized *M. abscessus* or even by person to person transmission [15, 16]. Additionally, cutaneous infections can result from exposure to *M. abscessus* by non-sterile tattooing practices,

unclean needles used for acupuncture treatments, or use of contaminated surgical equipment, often for cosmetic surgery [17, 18, 19]. It has long been thought that only immunocompromised humans were susceptible to infection with *M. abscessus*. However, recent evidence points to the possibility that immunocompetent individuals are becoming infected with *M. abscessus* as well [15, 20]. Even so, immunocompromised individuals such as HIV or AIDS patients and lung transplant patients are more susceptible to infection with *M. abscessus* than are members of the general population [15, 21]. Cystic fibrosis patients are also at a higher risk for contracting *M. abscessus* infection [14, 22]. Structural abnormalities of the lungs, such as bronchiectasis, contribute to host susceptibility as well [16, 21]. Additionally, immunocompetent, post-menopausal Caucasian, non-smoking, tall, slender females tend to be at higher risk for *M. abscessus* infection [23]. This may be caused by the elongation of the chest cavity in tall, slender women [23]. Further, it has been suggested that because slender patients have less leptin and more adiponectin, they are more susceptible to infection with *M. abscessus* [14]. This hypothesis is supported by a study in which leptin deficient mice were found to be more susceptible to *M. abscessus* infection [14]. Moreover, lower levels of leptin lead to decreased expression of T helper 1 (Th1) cells, which kill intracellular pathogens such as *M. abscessus* [14]. Increased levels of adiponectin upregulate the expression of interleukin 10 (IL-10) and the expression of IL-1R antagonist, which prevents the expression of TNFα [14]. Since moderate expression of TNFα is protective against mycobacterial infection, this leads to more severe disseminated disease [14]. Additionally, it is thought that perhaps women cough less effectively than men for social reasons, otherwise known as “Lady

Windermere syndrome" [3, 14]. This may create difficulty in clearing the bacteria from the lungs. A lack of estrogen in post-menopausal women may be responsible for the increased susceptibility of this particular demographic [14]. The binding of estrogen to estrogen receptors on macrophages induces phagocytosis [14]. Thus, without estrogen, macrophages may be less likely to phagocytose *M. abscessus*. The growing list of effects of *M. abscessus* upon public health points to the great need for further study of pathogenesis and effective drug therapies against this pathogen.

As stated previously, three different subspecies of *M.* abscessus including *M. abscessus* sbsp. *abscessus*, sbsp. *massiliense*, and sbsp. *bolletii* have been isolated from infected patients [5]. The traditional drug regimen for treatment of *M. abscessus* infection includes a macrolide such as clarithromycin or azithromycin, amikacin, which is an aminoglycoside, and either a fluoroquinolone such as levofloxacin or ciprofloxacin, or else a cephalosporin such as cefoxitin [26, 27]. Macrolides and aminoglycosides bind to the 50S and 30S ribosomal subunits, respectively, to inhibit protein synthesis by *M. abscessus* [28]. Macrolides also function by binding to the 23S ribosomal RNA (rRNA) to inhibit protein synthesis [29]. Aminoglycoside resistance is conferred upon *M. abscessus* strains by a p55 efflux pump [3]. Fluoroquinolones interfere with nucleic acid synthesis [28]. Cephalosporins are β-lactams, which interfere with cell wall synthesis [28, 30]. Many isolates of *M. abscessus* sbsp. *abscessus*, as well as some isolates of *M. abscessus* sbsp. *massiliense*, have demonstrated expression of erythromycin ribosome methyltransferase (41) genes (*erm*(41) genes) [24]. These *erm*(41) genes confer inducible macrolide resistance in these pathogens by methylation of the 23S rRNA [3, 24, 25]. Because of the inducible macrolide resistance expressed by many

clinical isolates of *M. abscessus*, this pathogen is incredibly difficult to treat [31]. Often patients are on this course of antibiotics for months at a time [32]. This may also be due to the fact that *M. abscessus* is an intracellular pathogen, which is able to survive within macrophages [33, 34]. This protects the bacteria from host defenses as well as exposure to drug compounds, and contributes to the chronic nature of infection [34]. Many individuals develop liver toxicity from prolonged exposure to antibiotics [26]. It is common for patients to have pieces of their lungs resected in order to eradicate the *M. abscessus* [35, 36, 37]. Even after successful treatment, many patients will experience relapse of the disease [35]. In one case, a lung transplant patient was diagnosed with subsequent pulmonary *M. abscessus* infection, and was treated successfully. However, one month later this patient developed deep gluteal abscesses caused by relapse of the *M. abscessus* infection, and eventually succumbed to bronchiolitis obliterans [35]. Thus, it is important to find more efficacious drug treatment regimens to prevent relapse. We will expand further on the drug resistance of *M. abscessus* in Section 1.3.

There are known differences between the human immune profile, and the murine immune response [38]. For example, humans produce interleukin-32 (IL-32), a pro-inflammatory cytokine which is not expressed in mice [39, 40]. The immune response to pulmonary or disseminated *M. abscessus* infection in humans has been well characterized [23]. Additionally, there is a growing body of knowledge regarding the pathogenesis of *M. abscessus* in humans. For example, we have recently learned that unlike slow-growing mycobacteria, *M. abscessus* is less likely to survive over time inside of macrophages, and more commonly destroys macrophages leading to the damaging release of reactive oxygen species [20]. The persistence of *M. abscessus*

within phagosomes of human macrophages may depend on whether the strain has rough or smooth morphology [10]. Upon infection of alveolar cells with *M. abscessus*, an adaptor protein found on toll-like receptors (TLRs) called MyD88 (myeloid differentiation primary response gene 88) activates the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, which produces cytokines that mediate inflammation [6].

We hypothesize that GM/Rag-dblKO mice (Table 1) and SCID mice on a beige background will allow us to understand the host immune response to infection with NTM and pathogenesis of *M. abscessus*. We suspect that these mice will demonstrate high bacterial loads into chronic infection, much like human patients, and unlike most other mouse strains which do not obtain high bacterial burdens [41]. GM/Rag-dblKO mice express human colony stimulating factor 2 (Csf2) and human interleukin-3 (IL-3) [42]. Thus, they promote the development of human alveolar macrophages, and demonstrate pulmonary disease processes that are commonly observed in human patients [41, 42]. The development of human alveolar macrophages within the GM/Rag-dblKO mice allowed Willinger, *et al*. to extract the macrophages from sickened mice, and use them for in vitro studies [42]. For our purposes, the human alveolar macrophages are non-functional in vivo. Thus, the knock-in of the human Csf2 is effectively equivalent to a knockout of Csf2 in our study. The GM/Rag-dblKO mice are on a recombination activating gene 2 knockout (Rag2-/-) interleukin-2 (IL-2) -/- background [42]. Thus, the GM/Rag-dblKO mice produce neither functional T cells nor functional B cells [42]. In order to understand the pathogenesis of *M. abscessus*, we carried out experiments

comparing the survival rates of GM/Rag-dblKO mice to those of SCID mice on a beige background upon infection with *M. abscessus*.

The GM/Rag-dblKO mice were developed by Flavell, Willinger, and their associates as described by Willinger, *et al* [42]. IL-3 stimulates the proliferation of myeloid cells, specifically, pro-inflammatory granulocytes and monocytes [43]. *Csf2* promotes the proliferation of pro-inflammatory granulocytes and monocytes, which differentiate into macrophages and dendritic cells [42]. Together, IL-3 and *Csf2* promote the development of alveolar macrophages [42]. Rag2 is responsible for the production of lymphocytes including T cells and B cells [44]. It is important to note that the absence of Rag2 does not inhibit the production of natural killer (NK) cells [45]. IL2 regulates inflammatory response by promoting the expansion of regulatory T cells which suppress inflammation [46]. Thus, these mice have non-functional granulocytes, macrophages, T cells, and B cells. Clearly, these mice have an extremely depleted immune system.

While the immune response to influenza viral infection in GM/Rag-dblKO mice has been characterized, the immune response to *M. abscessus* infection in GM/Rag-dblKO mice has not yet been characterized in the literature [42]. The GM/Rag-dblKO mice lack functional lymphocytes because they are on a Rag2 -/- background [44]. Furthermore, it has been observed that cystic fibrosis patients infected with *M. abscessus* have a higher level of CD40L+ (Cluster of Differentiation marker 40 Ligand positive) IL2- (Interleukin 2 negative) T cells [47]. This indicates that IL-2 contributes to protective immunity against *M. abscessus* infection [45]. Thus, the GM/Rag-dblKO mice will be more susceptible to infection with *M. abscessus* because they do not produce IL-2. Therefore, these mice will demonstrate high bacterial loads into chronic infection, much

like human patients, and unlike most other mouse strains which do not obtain high bacterial burdens [41]. Although it has been proposed by De Groote, *et al.* that the GMCSF-/- mouse is a good model for infection and preclinical testing of antimycobacterial compounds, this mouse does not attain high bacterial burdens in the spleen over time [48]. This implies that the mouse is not experiencing disseminated infection, unlike humans who often do develop disseminated disease over the course of chronic infection [35]. We will use flow cytometry to determine whether GM/Rag-dblKO mice are immunologically similar to humans upon infection with *M. abscessus* and to characterize the pathogenesis of *M. abscessus* within this mouse model.

SCID mice on a beige background are Rag2-/- mice on a beige background [49]. Again, we reiterate that the Rag2-/- indicates that these mice have no functional T cells or B cells [49]. The beige background renders the natural killer (NK) cells of these mice non-functional [49]. NK cells are cytotoxic cells which produce interferon-γ (IFN-γ) in response to infection [50]. IFN-γ protects against infection with Mycobacteria [51]. We will elaborate on this mechanism later in this discussion. NK cells also produce cytokines which enhance the T cell response to invading pathogens [50]. NK cells are known to protect against bacterial respiratory infections [50]. Dysfunction of microtubules in NK cells result from the beige mutation in beige mice [52]. This leads to the presence of giant granules in the cytoplasm of the NK cells, because they are unable to effectively degranulate [52]. Thus, the granzymes and perforins which would normally be released by NK cells in response to infection are not released [50, 52]. Hence, these NK cells are non-functional. In humans, such NK cell dysfunction resulting from analogous genetic mutation causes Chediak-Higashi syndrome [52]. Lysosomal

trafficking is disrupted in humans with Chediak-Higashi syndrome [53]. When this occurs in childhood, it is often lethal unless transplantation with hematopoietic stem cells can be performed before the disease begins to progress [53]. Symptoms include albinism, increased incidences of purulent bacterial infections, excessive bleeding, and neurological dysfunction [53].

Minimum inhibitory concentrations (MIC) of standard and novel anti-mycobacterial compounds against *M. abscessus* can easily be determined *in vitro* using broth microdilution assays [54]. However, *in vitro* assays are poor models for infection and immune responses in humans. Furthermore, there is no definitive correlation between drug susceptibility of *M. abscessus in vitro* with drug efficacy in human patients [55]. Thus, it is important to develop a good *in vivo* model of pulmonary or disseminated *M. abscessus* infection in order to study pathogenesis and screen novel anti-mycobacterial compounds in an animal before going into clinical trials. Without such a model, the public would not be safe from potentially hazardous or lethal drug compounds. Previous studies have found that most mouse strains do not maintain a high level of infection over time, and do not become clinically ill. Thus, it is of the utmost importance to find and implement a mouse strain which remains ill with a high bacterial load in its organs into chronic infection in order to model the disease process in humans. The GM/Rag-dblKO mice and the SCID mice on a beige background seem to fulfill this requirement, and hence, may be good models of *M. abscessus* infection and immune response in human patients.

Previous studies established that most immunocompetent mouse strains exhibit clearance of *M. abscessus* in the first 8 weeks post-infection with the less virulent *M.*

abscessus isolates, making model development and selection difficult [6, 51]. These earlier studies infected C57BL/6 and leptin-deficient (Ob/Ob) mice with a low-dose aerosol (LDA, ~100 bacilli per mouse) which did not develop a progressive infection [6, 51]. Conversely, when infected with a high-dose aerosol (HDA, ~1,000 bacilli per mouse), C57BL/6 and Ob/Ob mice developed an established infection and an early influx of IFN-γ+ cluster of differentiation 4 (CD4)+ T cells in the lungs [51]. This was a primary immune response which preempted the clearance of *M. abscessus* in both C57BL/6 and Ob/Ob mice [51]. On the other hand, IFN-γ knockout (GKO) mice infected with a LDA or HDA of *M. abscessus* yielded a progressive pulmonary infection related to the influx of T cells, macrophages, and dendritic cells leading to granuloma development [51]. Remarkably, a HDA *M. abscessus* infection of the GKO mice promoted the expansion of CD4+ and CD8+ T-cells able to produce IL-4 and IL-10 in the pulmonary cavity [51]. This was interesting because these are T helper 2 (T_H2) polarizing cytokines, and are produced by T_H2 cells [51]. One would expect to see a greater T helper 1 T_H1 polarization in mice infected with an intracellular bacteria. However, the absence of IFN-γ likely allowed for the shift to the T_H2 cytokines IL-4 and IL-10, which are immunosuppressive [51]. Thus, *M. abscessus* was capable of inflicting much more damage on the lungs of the GKO mice with HDA infection [51].

Notwithstanding the aforesaid obstacles associated with establishing a progressive model of infection, further studies using mouse models with significant insufficiencies in innate or acquired immunity have given insight into immune clearance mechanisms and novel models of infection [6]. Initial studies established mice with specific deficits in innate or acquired immunity infected with $1x10^6$ *M. abscessus*

intravenously were able to control the infection [6]. Mouse strains capable of clearing *M. abscessus* include beige (dominant TH_2 immunity), inducible nitric oxide synthase knockout (iNOS-/-), cytochrome b β (Cybb)-/- (devoid of super-oxide generating enzyme), TNFαR-/-, C3HeB/FeJ, GKO, and MyD88-/- mice [6]. Throughout a 40 day chronic infection, *M. abscessus* could still be detected at low levels in the lungs of the C3HeB/FeJ, GKO, and MyD88-/- mice [6]. Moreover, the GKO and MyD88-/- mice exhibited a decrease in the amount of *M. abscessus* in the spleen and liver after 40 days [6].

Conversely, SCID, nude, and GM/Rag-dblKO mice infected intravenously with *M. abscessus* exhibited persistent or progressive *M. abscessus* burden, illustrating the critical importance of T and B cell immunity and GM-CSF reliant cell phenotypes for establishment of protective immunity against *M. abscessus* [6]. An advantage of using severely immunocompromised mice (SCID, nude, and GM/Rag-dblKO) for modelling *M. abscessus* infection was the occurrence of foamy cells, necrotizing, and non-necrotizing granulomas in the lungs 40 days post-infection, a cellular phenotype ordinarily observed in the histopathologic samples of human NTM lung disease [6].

1.2 Classification of Nontuberculous Mycobacterial Strains

The development and acceptance of 16S rRNA gene sequencing has resulted in the identification of several new species of NTM [3, 4]. The mycobacterial 16S rRNA gene is highly conserved [4]. Thus, differences in the sequence of 1% or greater indicate a new species [4]. However, 16S rRNA gene sequencing is not a reliable

method for differentiating *M. abscessus* from *M. chelonae* because the two species only differ by 4 base pairs (bp) within the 16S rRNA genes [4].

It is important to obtain species-level identification of NTM clinical isolates because differences in antimicrobial susceptibility determine treatment options [4]. Phenotypic testing of NTM species is comprised of two classification criteria, including growth rate and pigmentation [3, 4]. To identify NTM species by growth rate, the bacteria are classified as either rapidly growing mycobacteria (RGM), or slowly growing mycobacteria [4]. RGM are defined as clinical isolates of NTM which form colonies upon subculture in at most seven days [4]. Examples of RGM include *M. abscessus*, *M. chelonae*, and *M. fortuitum*. Slowly growing mycobacteria require more than 7 days to form isolated colonies upon subculture [4]. Examples of slowly growing mycobacteria include *M. avium* and *M. intracellulare*. Pigmentation is not used as frequently to identify Mycobacterial species, but it is clear that strains of *M. tuberculosis* lack pigmentation, and have rough colony morphology [4]. Thus, the presence of pigmentation or smooth colony morphology is indicative of a strain of NTM [4]. High performance liquid chromatography (HPLC) is a reliable method of species-level identification for slowly growing Mycobacteria, but not for RGM [4].

The American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA) recommend that *Mycobacterium avium* complex and *Mycobacterium intracellulare* do not need to be differentiated from each other because they are currently clinically identical, and treatment would not change based on species-level identification [4]. Additionally, ATS and IDSA suggest that PCR restriction endonuclease assay (PRA) be used to differentiate between *M. abscessus*, *M. chelonae*, and *M.*

fortuitum [4]. Also, the ATS and IDSA advise that susceptibility of RGM to amikacin, cefoxitin, clarithromycin, ciprofloxacin, doxycycline, linezolid, sulfamethoxazole, and tobramycin be used to obtain species-level identification of *M. chelonae*, *M. abscessus*, and *M. fortuitum* [4].

Aside from ATS recommendations, it has been suggested in more recent literature that *rpoB* gene sequencing will allow identification of *M. abscessus* even to the subspecies level [56]. In other words, sequencing the *rpoB* gene will allow one to determine whether the isolate in question is *M. abscessus* sbsp. *abscessus*, *M. abscessus* sbsp. *massiliense*, or *M. abscessus* sbsp. *bolletii*.

1.3 Drug Resistance

As previously described, drug resistance is a major obstacle to treating *M. abscessus* infections. In this section, we will elaborate on the information provided in the Introduction regarding this drug resistance. All clinical isolates of *M. abscessus* are resistant to antituberculous drug therapies [4]. *M. abscessus* is multi-drug resistant [56]. Mutations in the 23S rRNA gene of *M. abscessus* can cause resistance to clarithromycin [4]. Additionally, mutations in the 16S rRNA gene of *M. abscessus* can lead to amikacin resistance [4]. *M. abscessus* tends to be susceptible *in vitro* to clarithromycin, amikacin, cefoxitin, imipenem, and clofazamine [4]. However, when treating pulmonary *M. abscessus* infections, there are no drug regimens that reliably result in sputum conversion from culture positive to culture negative over any considerable length of time [4]. Currently, the best course of action to treat pulmonary *M. abscessus* infections is to incorporate surgical resection of the affected lung tissue

as well as at least 12 months of rigorous combination antibiotic therapy using clarithromycin, amikacin, and cefoxitin [4]. In most cases, *M. abscessus* pulmonary infection is chronic and incurable [4].

NTM create many obstacles to efficacious antibiotic therapy [3, 24, 25]. Mycobacteria have a hydrophobic cell envelope that provides significant protection against lipophilic antimycobacterials [8, 57, 58]. In addition, antibiotic inactivation plays a significant role in NTM drug resistance, with resistance mechanisms including β-lactamases conferring resistance to β-lactams, which disrupt cell wall synthesis [28, 30, 59]. Aminoglycoside phosphotransferases and aminoglycoside acetyltransferases, which have been produced by Mycobacteria are known to be responsible for bacterial resistance to aminoglycosides, such as amikacin [60]. Mycobacteria also display natural resistance to isoniazid, rifamycins, pyrazinamide ethambutol [61-63]. Rapidly growing mycobacteria are generally quite resistant to ethambutol [64]. As previously mentioned, the p55 efflux pump gives mycobacteria resistance to aminoglycosides as well as tetracyclines [3]. We reiterate here that any isolates of *M. abscessus* sbsp. *abscessus*, some isolates of *M. abscessus* sbsp. *massiliense*, and isolates of *M. fortuitum* have demonstrated expression of erythromycin ribosome methyltransferase (41) genes (*erm*(41) genes) [3, 24]. These *erm*(41) genes confer inducible macrolide resistance in these pathogens by methylation of the 23S rRNA [3, 24, 25]. These *erm*(41) genes are not found in *M. chelonae* [3]. The stimulation of *erm*(41), and subsequent macrolide resistance, is not detected by traditional susceptibility testing, because this requires prolonged incubation observation [65]. Azithromycin contributes less to inducible macrolide resistance than clarithromycin [66].

Compounding the difficulty of drug development is the fact that in vitro susceptibility broth assays seldom correlate significantly with in vivo clinical efficacy [55, 67]. Another obstacle to accurate in vitro testing of antimycobacterial compounds is that NTM form biofilm, which causes them to enter stationary phase, and leads to imprecise measurements of drug susceptibility [55].

Moreover, there is sparse knowledge regarding pharmacokinetics of antimycobacterial compounds at the site of infection [67, 68]. There is a high degree of variability in the rates of loading of drug into the liposomes of macrophages from one compound to another, and so the pharmacokinetics and pharmacodynamics must be assessed for each individual compound tested, creating increased burden and expense for drug development [69]. As a result of these multiple resistance mechanisms paired with incorrect MIC and minimum bactericidal concentration (MBC) measurements, very few antibiotics have been identified through in vitro screening that have good in vivo activity against NTM [61-67]. The inadequacy of available chemotherapies against NTM poses serious challenges to effectively treating *M. abscessus* infections [70]. Often, surgical resection of infected lung tissue is required in order to eradicate the infection [70]. Yet patients whose lung tissue has been excised still experience relapse of NTM infection [70].

1.4 Pathogenesis

The epidemiology, virulence and disease processes associated with NTM are poorly understood [71]. It has been challenging to demonstrate a causative relationship between NTM in the environment and transmission resulting in pulmonary infection,

disseminated disease and lymphadenitis [72]. Whole genome sequencing has shown evidence supporting person to person transmission of *M. abscessus* between patients with cystic fibrosis [16]. A continuing study by Dr. Jackson and Dr. Ordway on the virulence of *M. abscessus* sbsp *massiliense* has shown the extremely pathogenic nature of BRA100 outbreak isolates [73]. The BRA100 strain of *M. abscessus* is particularly virulent in part because of remodeling of the cell envelope leading to resistance to glutaraldehyde, a common disinfectant [73]. Nevertheless, this is only one study and future studies are mandatory to assess high and low transmission strains to shed light on the properties of virulence in our animal models. Given the chance, NTM result in worsening pulmonary infection, superficial lymphadenitis, disseminated disease, or skin and soft tissue infections [4].

While HIV infected patients are more susceptible to infection with *M. abscessus*, usually disseminated infection will not begin until after $CD4^+$ T cell numbers have been depleted beneath a certain threshold [4]. Thus, T cells provide some protective immunity against *M. abscessus* [4].

Although cell-mediated, adaptive immunity is important and protective against *M. abscessus* infection, innate immunity is arguably more significant and crucial to fighting off infection with *M. abscessus*. NTM are phagocytosed by macrophages, which then produce interleukin-12 (IL-12) [4]. IL-12 production triggers expression of interferon-γ (IFN-γ) [74]. In turn, IFN-γ activates neutrophils and macrophages to kill mycobacteria and other intracellular pathogens [74]. IL-12 is produced principally by antigen presenting cells [74]. IL-12 receptors are found on T cells and NK cells [74]. IFN-γ is produced by T cells and NK cells [74]. Dysfunction in IFN-γ receptors 1 and 2 (IFNγR1

and IFNγR2) or in IL-12 receptor subunit IL-12Rβ1 and IL-12p40 have been shown to increase patient susceptibility to NTM infection because such mutations disrupt the polarization of T_H1 cells [4, 74]. IL-12p40 is a bioactive form of IL-12, and functions as a chemoattractant which beckons macrophages and dendritic cells to the site of infection [75].

Rough morphology of some *M. abscessus* strains contributes to hyper-virulence caused by cording, which allows for evasion of phagocytosis by macrophages [12]. Abscesses are formed by extensive cording of rough morphotypes of *M. abscessus* [12]. While moderate TNFα expression has been shown to be protective against mycobacterial infections, some studies show that rough variants of *M. abscessus* are easily recognized by Toll-like receptor 2 (TLR2) on macrophages because of their lack of GPL production [14]. This causes overexpression of TNFα, leading to pathologic inflammatory response [14]. These hyper-virulent rough variants of *M. abscessus* are subsequently more capable of invading lung tissue [14].

TLR2 signaling, mitogen-activated protein kinase (MAPK) signaling, and activation of the NF-κB pathway elicit TNF-α production by human monocytes in response to *M. abscessus* infection [14, 76]. Quickly following infection with *M. abscessus*, dendritic cells are activated by IL-12 p40 [14]. These activated dendritic cells then migrate to the mediastinal lymph nodes, where mycobacterial antigen is presented to naïve T cells [14]. This causes the differentiation and activation of effector T cells [14]. Further dissemination of *M. abscessus* is then prevented by the arrival of several monocytes, macrophages, neutrophils, and adaptive immune cells to the site of mycobacterial replication [14]. Thus, a granuloma is formed encapsulating the *M.*

abscessus [14]. Finally, in immunocompetent hosts, CD4+ and CD8+ T cells secrete granulysin and perforin, which kill intracellular pathogens such as mycobacteria [14]. Of course, if an individual is infected with a variant of *M. abscessus* which is resistant to phagocytosis by macrophages, as is the case with rough variants of *M. abscessus*, then much of the necessary antigen presentation and cytotoxic killing of intracellular pathogens is futile, and the host immune response is bypassed resulting in severe and lethal pathology.

1.5 Aims

The objective of this project is to identify small animal models which demonstrate a high bacterial burden in the lungs, spleens, and livers upon infection with *M. abscessus*, and which exhibit necrotic and non-necrotic granuloma formation in the lungs upon infection with *M. abscessus*. Additionally, we hope to find an animal model which has a similar immune response to that of the human upon infection with *M. abscessus*. Accordingly, our aims are as follows:

Aim 1: We will conduct a survival study and characterize the bacterial burden, organ pathology, and immune responses to M. abscessus in infected GM/Rag-dblKO and SCID mice on a beige background.

Aim 2: We will characterize the bacterial burden, organ pathology, and immune responses induced by human M. abscessus outbreak strains.

Chapter 2, Materials and Methods

2.1 Animal Infection

Mice were infected with a tail vein injection of 100 µL containing 1×10^6 colony forming units (CFU) of *M. abscessus* 103 with a rough colony morphology and positive for biofilm formation (a gift from Dr. Mary Jackson, Colorado State University). This 103 strain is a clinical isolate of the subspecies *bolletii*. The *M. abscessus* inoculum was prepared by thawing the bacterial vial. Thereafter, the mycobacterial suspension was obtained from the vial with a 1-ml tuberculin syringe fitted with a 26.5-gauge needle and expelled back into the vial. This procedure was repeated back and forth into the vial 20 times without removing the needle to mix the suspension and break up any small clumps of bacilli. All animals used were either SCID mice on a beige background (mouse strain: C.B-Igh-1b/GbmsTac-Prkdcscid-Lystbg N7) or GM/Rag-dbIKO mice (mouse strain: C;129S4-Rag2tm1.1Flv Csf2/Il3tm1.1(CSF2,IL3)Flv Il2rgtm1.1Flv/J).

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. The culture was then centrifuged at 3,500 revolutions per minute (rpm) for 10 minutes at 23°C. The bacterial cell pellet was resuspended in fresh 7H9 broth with glycerol, OADC, and Tween 80. This solution was again centrifuged for five minutes at 350 rpm at 23°C. The supernatant was saved, and the remaining pellet discarded. Finally, 1.5 mL of bacterial culture was pipetted into

glass vials, which were plugged and sealed. A titer was obtained for each final culture, and labelled vials were stored in cryovial boxes at -80°C.

2.3 Clinical Strains

All strains of *M. abscessus* used in this study were clinical strains obtained from Dr. Steve Holland, National Institute of Allergy and Infectious Disease. The outbreak strains were obtained from Dr. Andres Floto (Cambridge, UK) (*M. abscessus* sbsp. *massiliense* OM194), Brazilian outbreak strain (*M. abscessus* sbsp. *massiliense* CRM-0019) and the Seattle outbreak strain (*M. abscessus* sbsp. *massiliense* MC2638).

2.4 Animal Model

The two mouse strains used in this study were GM/Rag-dblKO mice (mouse strain: C;129S4-Rag2tm1.1Flv Csf2/Il3tm1.1(CSF2,IL3)Flv Il2rgtm1.1Flv/J), and SCID mice on a beige background (mouse strain: CB17.Cg-Prkdc^{scid}Lyst^{bg-j}/Crl). The GM/Rag-dblKO mice were obtained from Jackson Laboratories, and have strain code 014595. Six- to eight-week-old, specific-pathogen-free female SCID mice on a beige background were obtained from Charles River (Wilmington, MA), with stock number 250. As stated in Chapter 1, the GM/Rag-dblKO mice have undergone a knock-out of the murine IL-3 and Csf2, and a subsequent knock-in of human IL-3 and human Csf2 [41]. These GM/Rag-dblKO mice are on a Rag2, IL-2 knockout background [41]. The SCID mice on a beige background have non-functional T cells, B cells, and NK cells [49].

2.5 Bacterial Burden

On day one post-infection, three mice were euthanized and their lungs, spleens, and livers were harvested to determine the bacterial burden baseline. Organs were homogenized in phosphate-buffered saline (PBS), and serial dilutions were plated on nutrient 7H11 agar for 1 week at 30°C, when CFU were enumerated.

2.6 Organ Histology

The whole lungs, livers, and spleens from *M. abscessus* 103 infected mice were fixed in 10% formalin, and left inside of a biosafety level 3 (BSL-3) barrier at CSU for two weeks at 4°C. The organs were then transported to a biosafety level 2 (BSL-2) laboratory at CSU, and the 10% formalin was discarded from the organs. Then the organs were placed in 70% ethanol at 4°C for at least two days.

Next, the 70% ethanol was discarded from the organs, and the organs were immersed in 1XPBS at 4° C and sent to Premier Laboratories for histopathological analysis.

2.7 Immune Responses

GM/Rag-dblKO mice were infected with 10^6 CFU of *M. abscessus*. On day 30 and day 50, we humanely euthanized 5 infected mice from each group by carbon dioxide (CO_2) euthanasia.

Immune Cell Isolation from Lungs and Spleens of Infected Mice

Lungs, spleens, and small intestines of infected mice were placed in small petri dishes. A razor blade was dipped in 70% ethanol, and then dipped in incomplete Dulbecco's Modified Eagle's Medium (DMEM). Then the organs were chopped to disrupt the tissue in the petri dish using the razor blade, but still keeping the organs intact. Next each organ was returned to a 15 mL conical vial. At that point 1 mL of a collagenase / DNase mixture in DMEM was added to each conical vial containing an organ. The collagenase / DNase mixture was prepared by preparing a 1:5 dilution of our collagenase / DNase diluted with DMEM. After adding the collagenase / DNase to each conical vial containing an organ, the vials were incubated in a rocking water bath at 37°C for 30 minutes. Following this incubation, the conical tubes were placed on ice to neutralize the collagenase / DNase.

Next, sterile cell strainers were placed in small petri dishes. The contents of the 15 mL conical vials containing the organs were emptied into the cell strainers, and sterile plungers from a 5 mL syringe were used to push the organs through the cell strainers. We then pipetted 6 mL of incomplete DMEM into the cell strainer to rinse the cells into the petri dish. Next, the contents of each petri dish were pipetted back into each 15 mL conical vial. These vials were then centrifuged at 1300 rpm for 10 minutes at 4°C. Then the supernatant was discarded, and the cell pellet in each vial was disrupted by scraping the vial along the inside surface of the biosafety cabinet.

In order to lyse the red blood cells isolated from each organ, 1 mL of Gey's solution was added to each vial. The organs were incubated in the Gey's solution for five minutes at room temperature. After incubation, 5 mL of complete DMEM were

added to each conical vial in order to neutralize the Gey's solution. Again, the tubes were centrifuged at 1300 rpm for 10 minutes at 4°C. Then the supernatant was discarded, and the cell pellet was resuspended in complete DMEM. The organs were then incubated in the 37°C CO_2 incubator for three hours.

Cell Staining

We stained the isolated cells from the lungs, spleens, and small intestines of *M. abscessus* infected mice for markers of M cells, dendritic cells, macrophages, and neutrophils, including CD11b, CD11c, IL-4, IL-12, major histocompatibility complex class II (MHC II), IL-10, and Gr-1. We also stained for diverse T-cell populations including CD4+, CD8+, IL-4+, Programmed cell death protein 1 (PD1)+, forkhead box P3 (FoxP3)+, IL-17+, and IFN-γ+ T-cells (Table 1). Fluorophores used for staining were fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein complex (PerCP), Allophycocyanin (APC), efluor 450 (ef450), Alexa fluor 700 (A700), and phycoerythrin-cyanine7 (Pe-Cy7) (Table 1). We repeated this process again on Day 51. We used Fluorescence Minus One controls for compensation. We analyzed our data in Excel by comparing the percentage of the diverse cell populations to the percentage of the parent cell population.

2.8 Survival Study

Six SCID mice on a beige background and five GM/Rag-dblKO mice were infected with 10^6 CFU of *M. abscessus* 103 by intravenous tail injection. A group size of n=5 was sufficient given the power analysis which we share in section 2.9. These mice

remained untreated throughout the duration of the study. We, and staff at Colorado State University's Lab Animal Resources (LAR), monitored the mice daily for signs of distress indicating sickness from infection. If mice became distressed, they were humanely euthanized by CO_2 inhalation, and their whole lungs, livers, and spleens were harvested. Every other mouse which succumbed to disease was selected to have their organs sent out for histology, and the other mouse organs were used to assess bacterial burden in the organs.

A Kaplan-Meier survival plot of the survival rates over time post-infection of the SCID mice on a beige background and GM/Rag-dblKO mice was graphed using GraphPad Prism.

2.9 Statistical Analysis

Bacterial burdens in the *M. abscessus* infected animal organs were analyzed with GraphPad Prism version 4 (GraphPad Software, San Diego, CA), using analysis of variance (ANOVA) and Dunnett and Tukey multiple comparison tests. Data are presented using the mean values (n = 5) plus or minus the standard error of the mean (SEM). Significance was considered below a P value of $p<0.050$.

A Kaplan-Meier analysis was carried out on the survival data obtained from *M. abscessus* infected SCID mice on a beige background and GM/Rag-dblKO mice in the survival study outlined in section 2.8 using GraphPad Prism.

Chapter 3, Animal Model Development

3.1 Development of Mouse Models

Several mouse strains have been screened for susceptibility to infection with *M. abscessus* [6]. Recent research has found that many mouse strains clear bacterial infection quickly, and do not achieve acute infection (Table 2) [6]. However, in previous studies, it was discovered that among mice with immune defects, Nude mice, SCID mice, and GM/Rag-dblKO mice demonstrate a high level of infection (Figure 1) [6]. Of these three immune defective mouse strains, SCID mice and GM/Rag-dblKO mice exhibited the highest levels of infection upon intravenous inoculation with *M. abscessus* 103 [6]. Additionally, SCID mice were the only mouse strain of these three immune defective mice which produced both necrotic and non-necrotic granulomas in the lungs [6] (Figure 2). The GM/Rag-dblKO mice and Nude mice only exhibited non-necrotic granulomas in the lungs [6]. Thus, a survival study was carried out to determine the effects of chronic *M. abscessus* infection in SCID mice on a beige background and GM/Rag-dblKO mice (Figure 3).

Upon infection with *M. abscessus* 103, GM/Rag-dblKO mice demonstrate much lower survival rates than those of SCID mice on a beige background. In fact, some SCID mice on a beige background survived longer than their expected lifespan post-infection.

3.2 Pathogenesis of M. abscessus

M. abscessus infected GM/Rag-dblKO mice exhibited severe lung consolidation upon examination of lung histology due to coalescence of granulomas in the lungs (Figure 4). While AFB were detected in the pulmonary tissue of both GM/Rag-dblKO mice and SCID mice on a beige background, bacillary load was much more extensive in the GM/Rag-dblKO mice than in the SCID mice on a beige background (Figure 5).

Alveolar macrophages are observed in the lung histology of both the GM/Rag-dblKO and SCID mice on a beige background, but the excess of AFB seen in the lung histology of GM/Rag-dblKO mice compared to SCID mice on a beige background revealed that only the macrophages in the SCID on a beige background mouse lungs are effectively clearing AFB from the tissue. It is apparent from acid fast staining that many more AFB are surviving within the alveolar macrophages of the GM/Rag-dblKO mice than in those of the SCID mice on a beige background.

Chapter 4, Global Emergence of Human-Transmissible *M. abscessus*

4.1 Global Circulating Clones

For decades, it has been generally accepted that NTM infection was acquired through environmental contamination, by contact with NTM strains in tap water and soil [2, 77-79]. In a global collaboration, we found strong evidence of indirect human to human transmission of *M. abscessus* [80]. While previous studies have shown that individual patients are infected with genetically disparate strains of *M. abscessus*, this study has shown via whole genome sequencing that individual cystic fibrosis patients have been infected with genetically identical, globally circulating strains of *M. abscessus* [80-83]. Patients in a cystic fibrosis center in the UK were grouped into two clusters [80]. Each cluster consisted of patients with genetically indistinguishable isolates of *M. abscessus* [80]. Moreover, patients at a cystic fibrosis treatment facility in Seattle, Washington were found to have the same isolates of *M. abscessus* as those in the UK treatment facility [80]. We determined that these *M. abscessus* strains were transmitted via indirect human-to-human transmission within the treatment facilities, using social network analysis [80]. Further similar analysis was conducted using 1,080 *M. abscessus* isolates from 517 infected individuals being treated at various cystic fibrosis clinics in the UK, the United States of America, Ireland, Denmark, Sweden, the Netherlands, and Australia [80]. The majority, 730 of the NTM strains isolated, were identified as *M. abscessus* sbsp. *abscessus* [80]. Next, there were 256 of the 1,080 *M. abscessus* isolates which were identified as *M. abscessus massiliense* [80]. Finally, the least prevalent of the isolates was *M. abscessus bolletii*, with only 91 of the isolates being so

identified [80]. Phylogenetic analysis revealed that while environmental acquisition of unclustered *M. abscessus* was still occurring, there were also groups of nearly genetically identical, clustered isolates of *M. abscessus* from different regions [80]. It was concluded that human-transmissible, globally circulating clones of *M. abscessus* are emerging among cystic fibrosis patients and becoming clinically significant [80].

In a separate study, Tettelin, *et al.* used whole genome sequencing to identify nearly identical outbreak strains in cystic fibrosis patients in the UK, the United States, and Brazil [84]. Our collaborators shared these outbreak strain isolates with us, and we infected GM/Rag-dblKO mice with them. Then we performed flow cytometry, counted viable CFU, and carried out histological analyses of the lungs of these infected GM/Rag-dblKO mice. The strains used were *M. massiliense* outbreak strain CRM-0019 (Brazil), *M. massiliense* outbreak strain MC2638 (Seattle, USA), and *M. massiliense* outbreak strain OM194 (Cambridge, UK) [84].

4.2 *M. abscessus* Virulence

In order to determine whether there are disparities in the virulence of clustered versus unclustered isolates of *M. abscessus*, we carried out various phenotypic classifications of the clinically isolated clustered and unclustered strains [80]. No differences were detected between clustered and unclustered strains regarding colony morphology, biofilm formation, or capability to induce the expression of cytokines by macrophages [80]. However, there were significant differences in the rate of phagocytosis of clustered versus unclustered *M. abscessus* isolates by macrophages and the ability of clustered versus unclustered *M. abscessus* isolates to survive within

macrophages (Figure 6) [80]. Additionally, there were significant increases in the number of viable colony forming units of clustered *M. abscessus* strains when compared with unclustered *M. abscessus* strains in the lungs of SCID mice (Figure 6) [80].

The outbreak strains first classified by Tettelin, *et al*. were used to infect GM/Rag-dblKO mice. The viable colony forming units in their lungs, spleens, and livers were counted (Figure 7). The lungs, spleens, and livers of the GM/Rag-dblKO mice maintained a high bacterial burden when infected with these outbreak strains. Thus, the strains were found to be quite virulent in GM/Rag-dblKO mice.

4.3 Immune Responses to *M. abscessus*

In this study, we established that there was significantly increased phagocytosis of clustered *M. abscessus* strains by macrophages than that of unclustered *M. abscessus* strains [80]. Also, clustered strains of *M. abscessus* demonstrated greater intracellular survival than unclustered strains of *M. abscessus* (Figure 8) [80]. However, the cytokine profiles of macrophages responding to clustered versus unclustered *M. abscessus* strains were not significantly different [80]. Additionally, there was no significant difference in the biofilm formation observed in clustered *M. abscessus* strains when compared with unclustered *M. abscessus* strains [80].

Flow cytometry was performed on cells isolated from the lungs of GM/Rag-dblKO mice infected with the outbreak strains first described by Tettelin, *et al* [84]. It was observed that there were more polymorphonuclear cells (stained for Gr-1 with PeCy7), macrophages (stained for CD11c and MHC II using PE and ef450, respectively), and

dendritic cells (stained for CD11b and MHC II using FITC and ef450, respectively) in the lungs of GM/Rag-dblKO mice infected with *M. abscessus* sbsp. *massiliense* strains OM194 (Cambridge) and CRM-0019 (Brazil) (Figure 9). However, the outbreak strain *M. abscessus* sbsp. *massiliense* MC2638 (Seattle) infected GM/Rag-dblKO mouse lungs had fewer polymorphonuclear cells in their lungs, an increase in macrophages in the lungs, and only a slight increase in dendritic cells in the lungs between day 30 and day 50 post-infection (Figure 9).

Furthermore, double staining with PE and ef450 revealed that major histocompatibility complex class II (MHC II) was upregulated on macrophages from day 30 to day 50 post-infection of GM/Rag-dblKO mice infected with the outbreak strains *M. abscessus* sbsp. *massiliense* OM194 and MC2638 (Figure 10). Conversely, MHC II was down-regulated on macrophages in the lungs of GM/Rag-dblKO mice infected with the outbreak strain *M. abscessus* sbsp. *massiliense* CRM-0019 from day 30 to day 50 post-infection. No significant change was observed in the level of expression of MHC II on dendritic cells in the lungs of GM/Rag-dblKO mice infected with the outbreak strains *M. abscessus* sbsp. *massiliense* OM194 and CRM-0019. There was mild down-regulation of MHC II on dendritic cells in the lungs of GM/Rag-dblKO mice infected with the outbreak strain *M. abscessus* sbsp. *massiliense* MC2638.

Moreover, we observed decreased IL-12 production (by staining with APC for IL-12) by macrophages and dendritic cells in the lungs of GM/Rag-dblKO mice infected with outbreak strains *M. abscessus* sbsp. *massiliense* OM194 and CRM-0019, when compared with those mice infected with the outbreak strain *M. abscessus* sbsp. *massiliense* MC2638 (Figure 11).

Finally, there was much more production of IL-10 by macrophages and dendritic cells in the lungs of GM/Rag-dblKO mice infected with the outbreak strains OM194 and CRM-0019 than in the lungs of GM/Rag-dblKO mice infected with the outbreak strain MC2638 (Figure 12).

4.4 Pathogenesis of Major Clones

There was a significant increase in granulomatous inflammation in the lungs of SCID mice in response to infection with clustered *M. abscessus* strains versus unclustered *M. abscessus* strains (Figure 13) [80].

In this study, Bryant, *et. al* have established that the clustered globally circulating clones of *M. abscessus* are being transmitted by fomites [80]. Individual cystic fibrosis patients may be contaminating the surfaces in the rooms of their treatment centers [80]. In addition, it is surmised that *M. abscessus* may be spread by aerosols which linger for quite some time, and which are generated when infected patients cough [80]. Clustered strains of *M. abscessus* obtained mutations in their 16S ribosomal RNA which provided resistance to amikacin, contributing to increases in chronic infection in affected cystic fibrosis patients [80]. Similarly, clustered *M. abscessus* clones acquired mutations in their 23S ribosomal RNA which conferred resistance to macrolides, making chronic infection incredibly difficult to treat [80].

In a separate study, histological analysis was performed on the lungs of GM/Rag-dblKO mice infected with one of the outbreak strains *M. abscessus* sbsp. *massiliense* OM194, CRM-0019, or MC2638 (Figure 14).

Chapter 5, Discussion

We carried out a survival study in GM/Rag-dblKO mice and SCID mice on a beige background infected with *M. abscessus* 103, in order to see how these mice responded during chronic infection. We observed a much shorter survival time in GM/Rag-dblKO mice compared with SCID mice on a beige background. Since GM/Rag-dblKO mice have no functional neutrophils, macrophages, or lymphocytes, they are even less capable of mounting an effective immune response against *M. abscessus* than the lymphocyte-depleted SCID mice on a beige background. We will be more likely to see a significant difference in GM/Rag-dblKO mice in the survival times of drug-treated, infected mice compared with those of untreated, infected mice.

In our survival study, we prepared the lungs of the *M. abscessus* 103 infected GM/Rag-dblKO mice and SCID mice on a beige background for histological analysis. H&E staining of the lungs of *M. abscessus* infected GM/Rag-dblKO mice exhibited severe lung consolidation upon examination of lung histology due to coalescence of granulomas in the lungs. While acid fast staining revealed that AFB were detected in the pulmonary tissue of both GM/Rag-dblKO mice and SCID mice on a beige background, bacillary load was much more extensive in the GM/Rag-dblKO mice than in the SCID mice on a beige background.

Alveolar macrophages are detected in the lung histology of both the GM/Rag-dblKO and SCID mice on a beige background, but the plethora of AFB observed in the lungs of GM/Rag-dblKO mice compared to SCID mice on a beige background revealed that only the macrophages in the SCID on a beige background mouse lungs are

effectually clearing AFB from the tissue. It is clear from acid fast staining that additional AFB are persisting in the alveolar macrophages of the GM/Rag-dblKO mice than in those of the SCID mice on a beige background.

To discover whether or not there are differences in the virulence of clustered versus unclustered isolates of *M. abscessus*, we performed many phenotypic classifications of the clinically isolated clustered and unclustered strains [80]. There were significant differences in the rate of phagocytosis of clustered versus unclustered *M. abscessus* strains by macrophages and the capability of clustered versus unclustered *M. abscessus* strains to survive within macrophages [80]. In addition, there were significant increases in the quantity of viable colony forming units of clustered *M. abscessus* isolates when compared with unclustered *M. abscessus* isolates in the lungs of SCID mice [80]. Thus, the emergence of global circulating clones of *M. abscessus* may be due to increased transmissibility and worsening virulence [80]. Because there was no evidence of contact between cystic fibrosis patients in disparate geographical regions across the globe, it can be assumed that zoonotic transmission and environmental vectors are responsible for the spread of nearly identical strains of *M. abscessus* [80]. Since clustered isolates of *M. abscessus* are more virulent than unclustered strains, it is clear that global circulating clones have altered phenotypes that are allowing increased transmission and worsening infections [80].

The outbreak strains first classified by Tettelin, *et al*. were used to infect GM/Rag-dblKO mice. The viable colony forming units in lungs, spleens, and livers of infected GM/Rag-dblKO mice were quantified. We observed increased bacillary load in the lungs, spleens, and livers of GM/Rag-dblKO mice infected with the outbreak strains

M. abscessus sbsp. *massiliense* OM194, CRM-0019, and MC2638. Additionally, histological analysis revealed that there was worse lung pathology observed in GM/Rag-dblKO mice infected with these outbreak strains. The increased virulence demonstrated by these outbreak strains may account for their persistence and person-to-person transmission in cystic fibrosis patients.

We reiterate here that GM/Rag-dblKO mice develop non-functional polymorphonuclear cells and alveolar macrophages [42]. While flow cytometry of cells isolated from GM/Rag-dblKO mice infected with the outbreak strains *M. abscessus* sbsp. *massiliense* OM194, CRM-0019, and MC2638 revealed that there were increased numbers of granulocytes and macrophages produced by these infected mice, they were not functional granulocytes or macrophages, which accounts for the decreased production of IL-12 and MHC II by macrophages and dendritic cells. Furthermore, it seems that these outbreak strains are managing to survive intracellularly, and this may be due in part to the stimulation of IL-10 production, since IL-10 decreases the inflammatory response. On the other hand, this cytokine milieu may only be a result of the use of the GM/Rag-dblKO mouse which has no functional macrophages or granulocytes, and which has no functional T cells. Yet, the GM/Rag-dblKO mouse is a good model of cystic fibrosis, and so we can see a snapshot of what may be occurring in these susceptible patients.

When screening candidate mouse strains to model human NTM infection, Obregón-Henao, *et al.* found that SCID, GM/Rag-dblKO, and Nude mice achieved a high level of infection [6]. This high bacterial burden in SCID mice is not surprising. Since the SCID mice have a Rag2 knockout, they have no T cells or B cells. Thus, they

cannot mount a T_H1 response to promote the production of IFN-γ. Hence, SCID mice are unable to clear the mycobacterial infection. This implies that SCID mice model human NTM infection, and are a useful candidate for novel antimycobacterial drug screening because a high level of infection ensures that if drug treatments are effective, then there will be a significant difference in the number of *M. abscessus* CFU found in the organs of drug-treated, infected mice compared with untreated, infected mice.

The lack of lymphocytes, neutrophils, and macrophages in the GM/Rag-dblKO mice explains the high bacterial load maintained in the organs of these mice. These mice are unable to mount any adaptive or innate immune response to infection. Therefore, the GM/Rag-dblKO mouse model is an appropriate candidate for screening novel antimycobacterial drugs. The SCID mice were the only mice in which both necrotizing and non-necrotizing granulomas were observed [6]. Since humans develop both necrotizing and non-necrotizing granulomas, the SCID mouse model is a useful model of the histopathology observed in humans [6].

Table Legend

Table 1. Mouse Strains. This is a guide to the specific mutations of the mouse strains used in this study.

Mouse Strain	KO's	KI's	Background
GM/Rag-dblKO	Murine IL-3, Murine Csf2	Human IL-3, Human Csf2	IL-2 KO, Rag2 KO
SCID / beige	Rag2	None	beige (Non-functional NK cells)

Table 2. Screening of Animal Models. Animal models were screened for susceptibility to *M. abscessus* infection [6]. Animal models which maintained a high level of infection are in blue, animal models which cleared the bacteria are in red, and those animal models which have not been tested yet are in black [6].

Structural Defects	Immune Defects	Immunocompetent
B6CFTR^{tm1UNC}/CFTR^{tm1UNC}	GKO-/-	C57/BL6
ßENaC transgenic	MYD88-/-	C3HeB/FeJ
	Nude/Beige	Lep^{ob}/Lep^{ob}
	SCID	
	NOS2-/-	
	SOD-/-	
	TLR2/4 -/-	
	TLR2/4/9 -/-	
	GM/Rag-dblKO	**Cutaneous**
Other Models	TNFR-1-/-	Nude/Beige
Guinea Pigs	IL-1-/-	CCL17 transgenic
Rabbits	Beige (M. avium)	Beige

Figure Legend

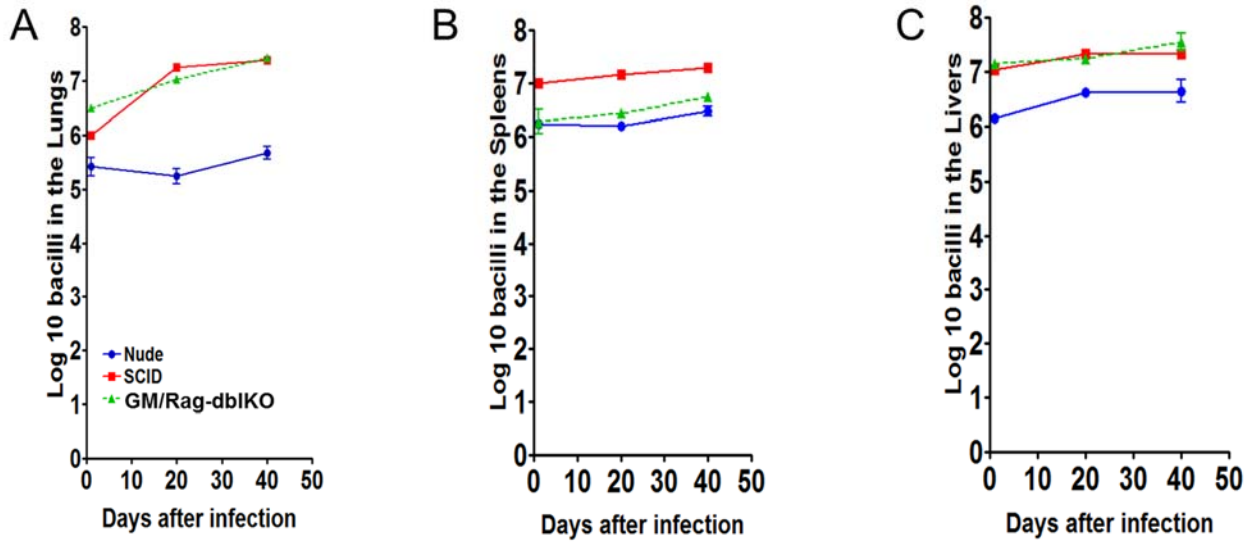

Figure 1. Bacterial Burden in Lungs, Spleens, and Livers of Nude, SCID, and GM/Rag-dblKO Mice. A high mean bacterial load is maintained in Nude, SCID, and GM/Rag-dblKO mice upon intravenous infection with *M. abscessus* 103 [6]. Please note that this figure was originally published by Obregón-Henao, *et al*. and has been slightly modified [6]. This research was conducted by Obregón-Henao, *et al*. in 2015.

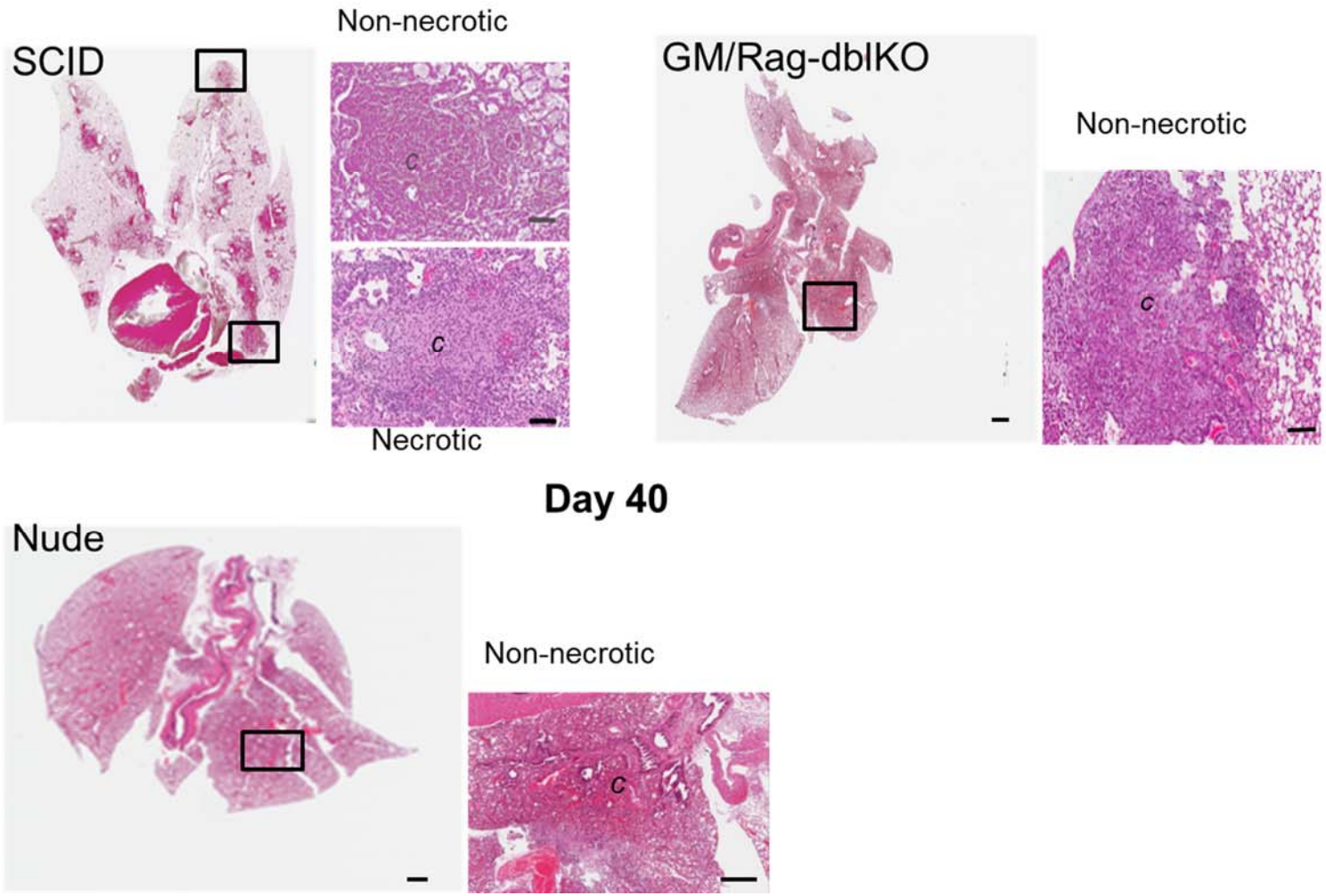

Figure 2. Lung Histopathology of *M. abscessus* Infected SCID, GM/Rag-dblKO, and Nude Mice. Necrotic and non-necrotic granulomas formed in SCID mice, while only non-necrotic granulomas formed in GM/Rag-dblKO and Nude mice upon infection with *M. abscessus* 103 [6]. Please note that this figure was originally published by Obregón-Henao, *et al*. and has been slightly modified [6]. This research was conducted by Obregón-Henao, *et al*. in 2015.

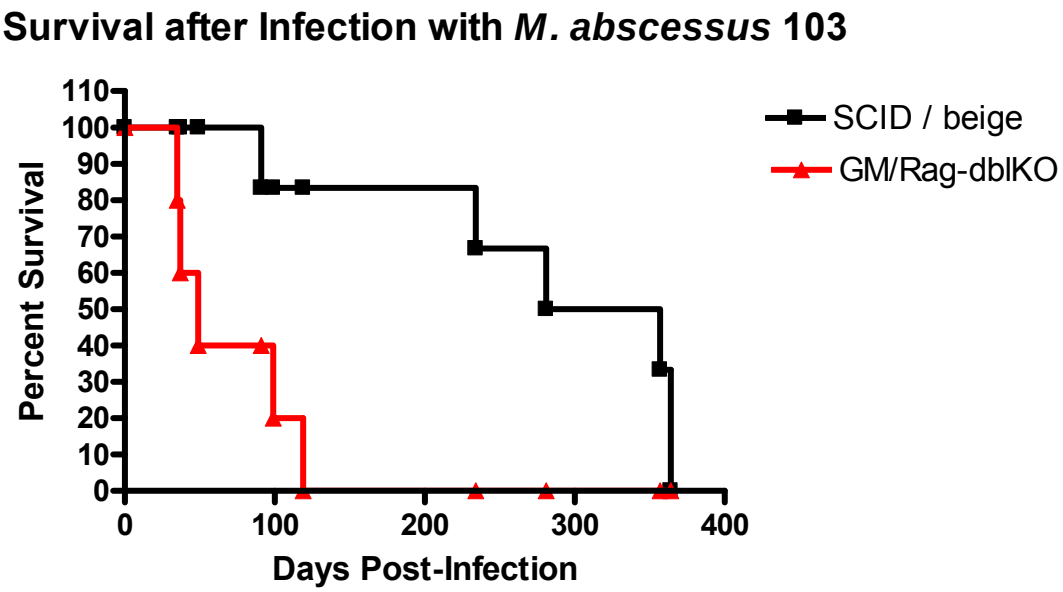

Figure 3. Kaplan-Meier survival plot of SCID mice on a beige background and GM/Rag-dblKO mice post-infection with *M. abscessus* 103. There were 6 SCID mice on a beige background in this study, and there were 5 GM/Rag-dblKO mice in this study.

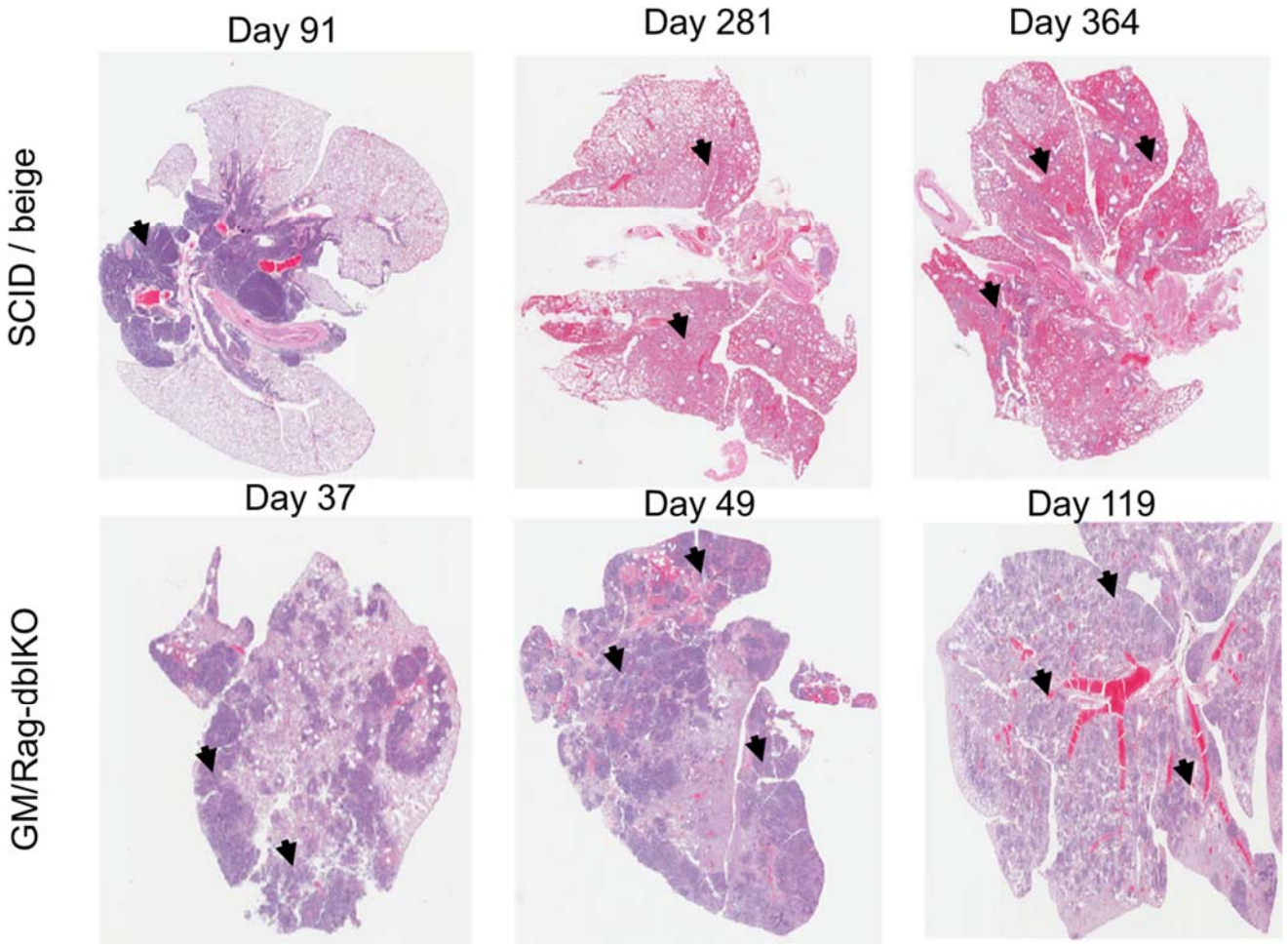

Figure 4. Lung Histopathology of *M. abscessus* infected GM/Rag-dblKO Mice and SCID mice on a beige background. Lung histopathology of *M. abscessus* infected GM/Rag-dblKO (n=3) and SCID mice on a beige background (n=3) from our survival study revealed quicker development of more extensive lung consolidation in GM/Rag-dblKO mice than in SCID mice on a beige background.

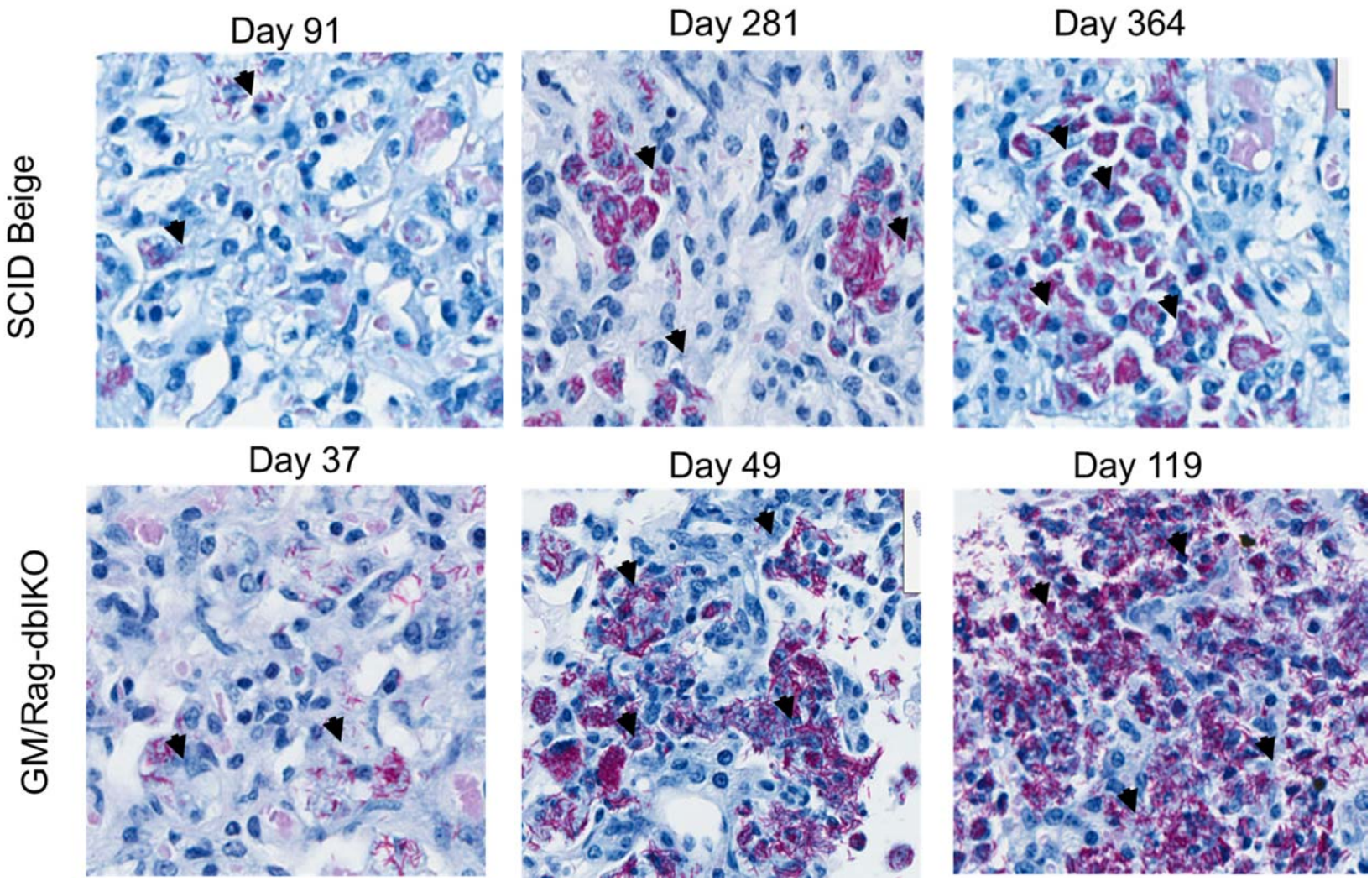

Figure 5. Acid Fast Staining of *M. abscessus* 103 Infected SCID Mice on a Beige Background and GM/Rag-dblKO Mice. Acid fast staining revealed that GM/Rag-dblKO mice (n=3) develop higher bacillary load much more rapidly than SCID mice on a beige background (n=3), as seen in histological analysis of pulmonary tissue taken from *M. abscessus* infected mice in our survival study.

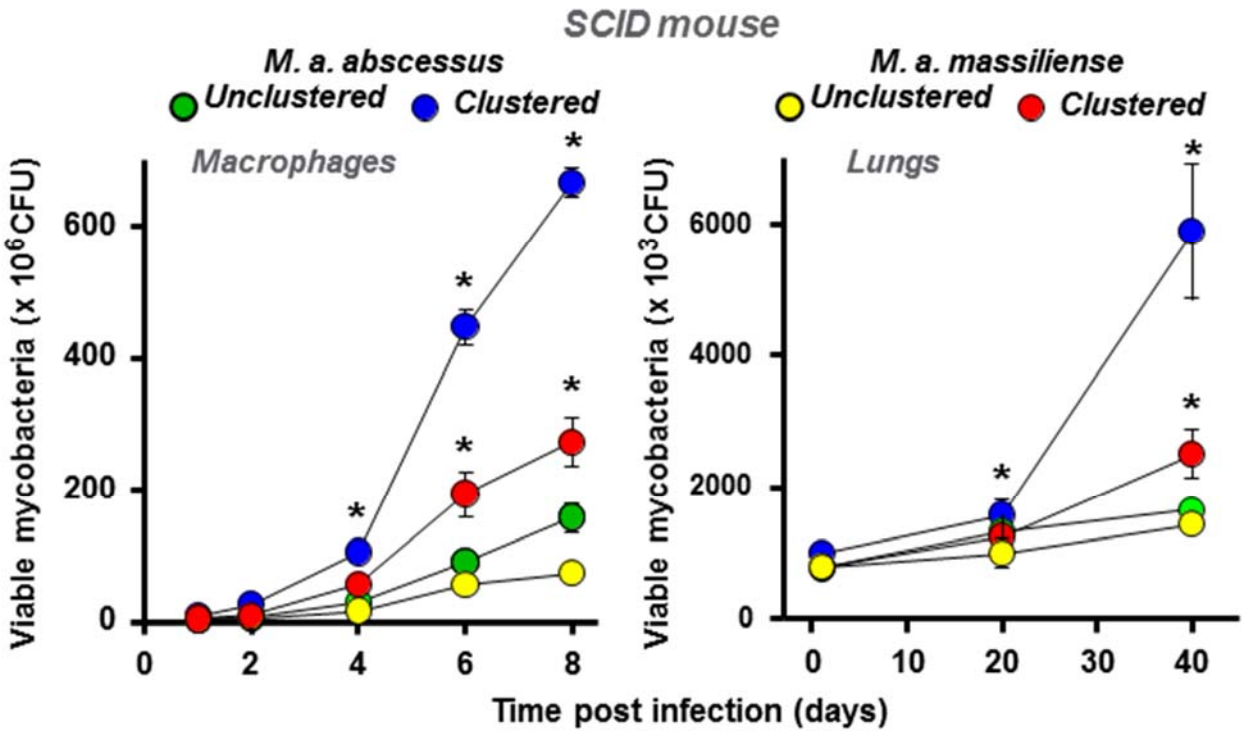

Figure 6. Phenotypic Characterization of Clustered versus Unclustered Strains of *M. abscessus*. Infection of SCID mice with clustered *M. a. abscessus* (blue) and *M. a. massiliense* (red) resulted in increased intracellular survival within bone marrow–derived macrophages, and higher bacterial loads in the lung after inoculation with 1×10^7 bacilli per animal [80]. Colony-forming units data are shown as mean ± SEM; *P < 0.05; **P < 0.005, n=5 (two-tailed unpaired Student's t test) [80].

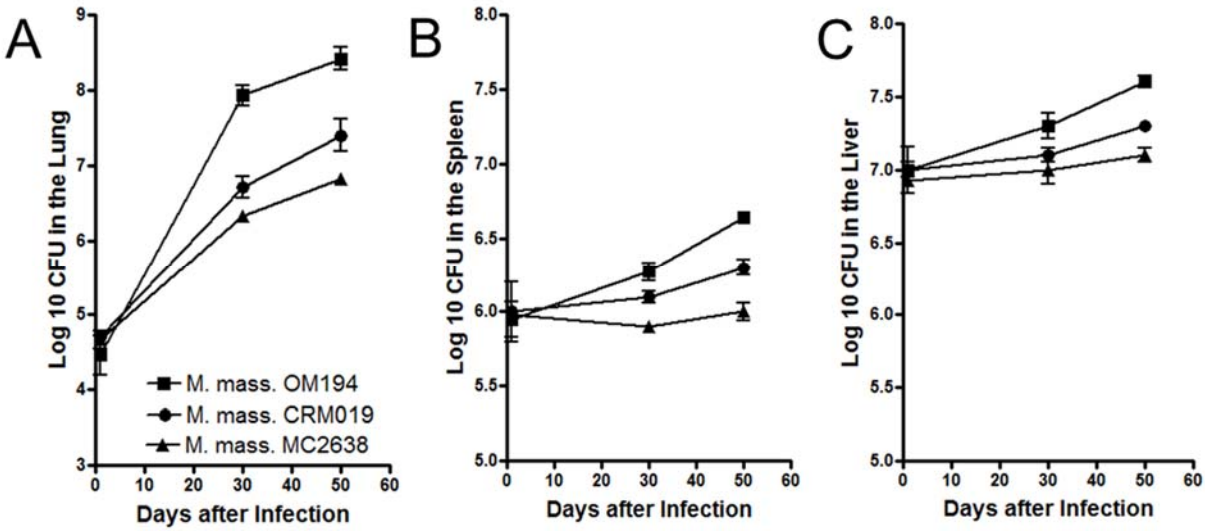

Figure 7. Increased bacteria in the lungs, spleens and livers of GM/Rag-dblKO mice infected with *M. massiliense* outbreak strain OM194. Bacterial counts in the lungs (A) and spleens (B), and livers (C) on days 1, 30 and 50 from GM/Rag-dblKO mice infected with an intravenous infection of 10^6 CFU mouse the *M. massiliense* outbreak strain CRM-0019 (Brazil), *M. massiliense* outbreak strain MC2638 (Seattle, USA) and *M. massiliense* outbreak strain OM194 (Cambridge , UK) were compared. Results are expressed as the mean (n=5) of the bacterial load in each group expressed as Log_{10} CFU, ± standard error mean (SEM). * $p<0.050$, by ANOVA and Tukey post-test.

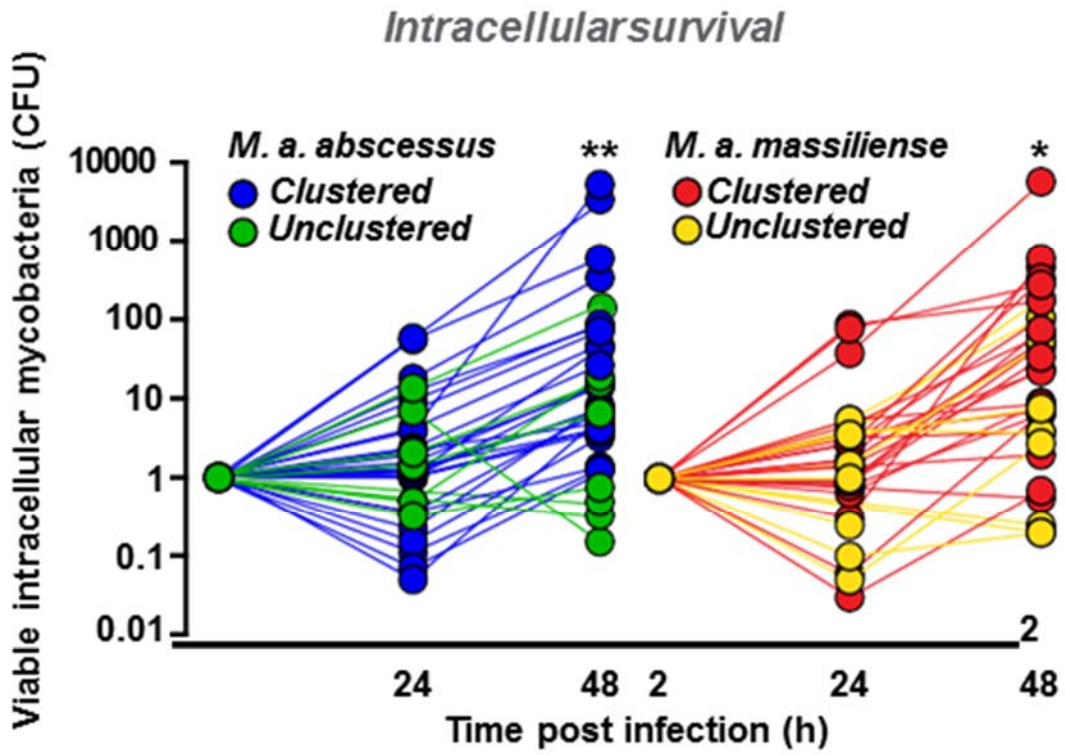

Figure 8. Intracellular Survival of Clustered versus Unclustered Isolates of *M. abscessus*. In vitro phenotyping of representative isolates of clustered (blue) and unclustered (green) *M. a. abscessus* and clustered (red) and unclustered (yellow) *M. a. massiliense* comparing intracellular survival within differentiated THP1 cells. Data points represent averages of at least three independent replicates. *P < 0.05; **P < 0.005 (two-tailed unpaired Student's t test) [80].

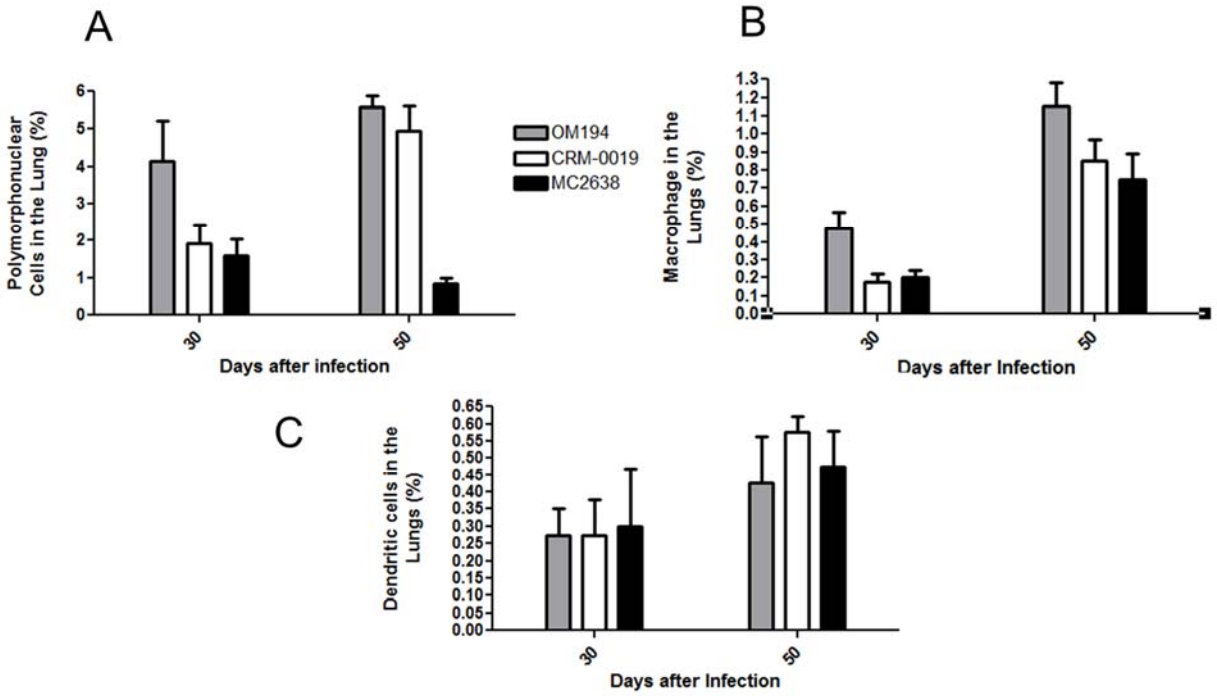

Figure 9. Immune Response of GM/Rag-dblKO Mice upon Infection with Outbreak Strains of *M. abscessus*. A) Increases were observed in the number of polymorphonuclear cells in the lungs of *M. abscessus* sbsp. *massiliense* outbreak strain OM194 (Cambridge) and CRM-0019 (Brazil) infected GM/Rag-dblKO mice. A decrease in the number of polymorphonuclear cells was observed in the lungs of GM/Rag-dblKO mice infected with *M. abscessus* sbsp. *massiliense* strain MC2638 (Seattle). **B and C)** Increases were observed in the number of macrophages and dendritic cells in the lungs of GM/Rag-dblKO mice infected with each of the *M. abscessus* sbsp. *massiliense* outbreak strains. Error bars represent ±SEM; n=5.

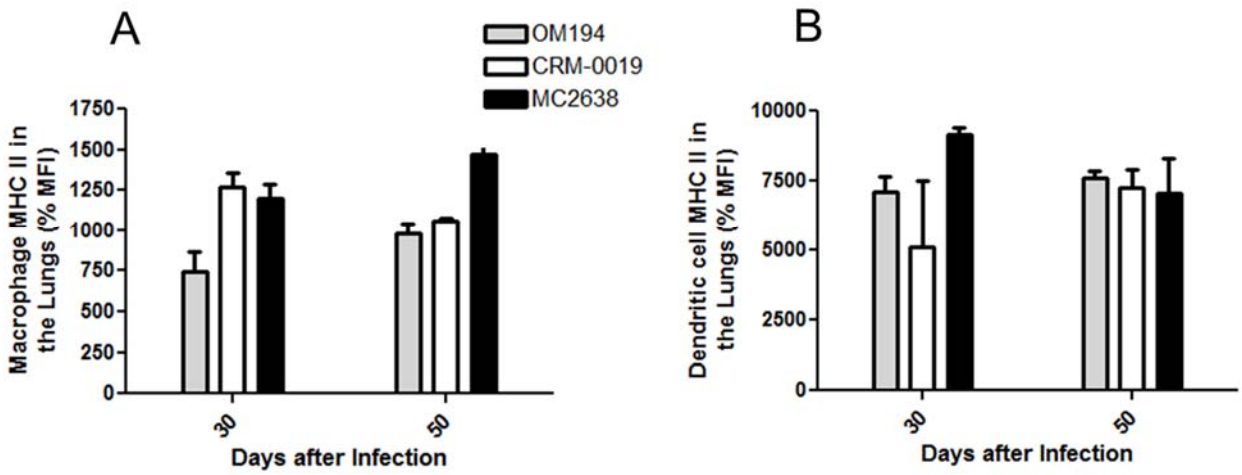

Figure 10. MHC Class II Expression in Antigen Presenting Cells of GM/Rag-dblKO Mice upon Infection with *M. abscessus* Outbreak Strains. A) MHC II was upregulated on macrophages in the lungs of GM/Rag-dblKO mice infected with the outbreak strains *M. abscessus* sbsp. *massiliense* OM194 and MC2638 between day 30 and day 50 post-infection. There was a significant decrease in the level of expression from day 30 to day 50 post-infection of MHC II on macrophages in the lungs of GM/Rag-dblKO mice infected with *M. abscessus* sbsp. *massiliense* CRM-0019. **B)** No change was observed in the level of expression of MHC II on the dendritic cells in the lungs of GM/Rag-dblKO mice infected with outbreak strain *M. abscessus* sbsp. *massiliense* OM194 or CRM-0019. There was a slight decrease in the level of expression of MHC II on dendritic cells in the lungs of GM/Rag-dblKO mice infected with the outbreak strain *M. abscessus* sbsp. *massiliense* MC2638. Error bars represent $\pm$SEM; n=5.

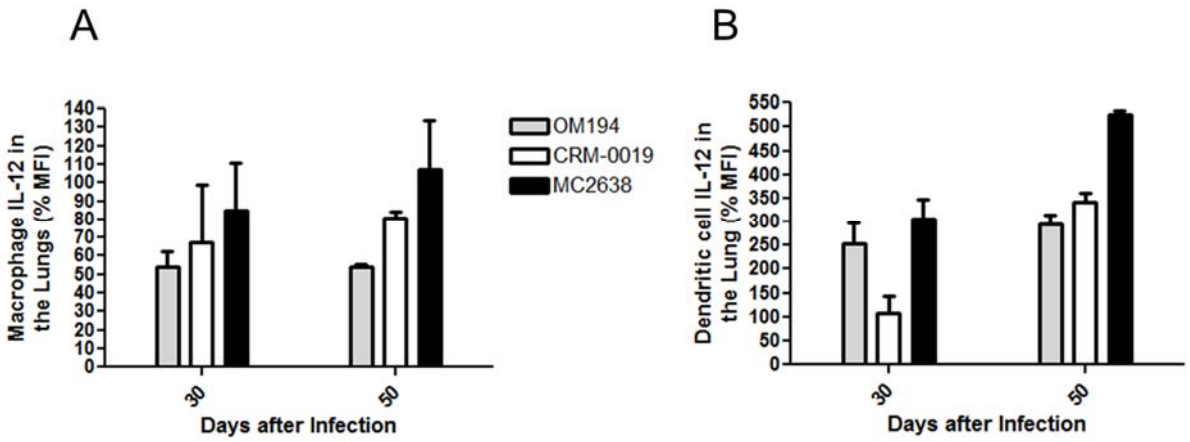

Figure 11. IL-12 Expression by Antigen Presenting Cells in the Lungs of GM/Rag-dblKO Mice upon Infection with *M. abscessus* Outbreak Strains. There was less expression of IL-12 by macrophages (A) and dendritic cells (B) in the lungs of GM/Rag-dblKO mice infected with outbreak strains *M. abscessus* sbsp. *massiliense* OM194 (Cambridge) and CRM-0019 (Brazil) when compared with GM/Rag-dblKO mice infected with *M. abscessus* sbsp. *massiliense* MC2638 (Seattle). Error bars represent ±SEM; n=5.

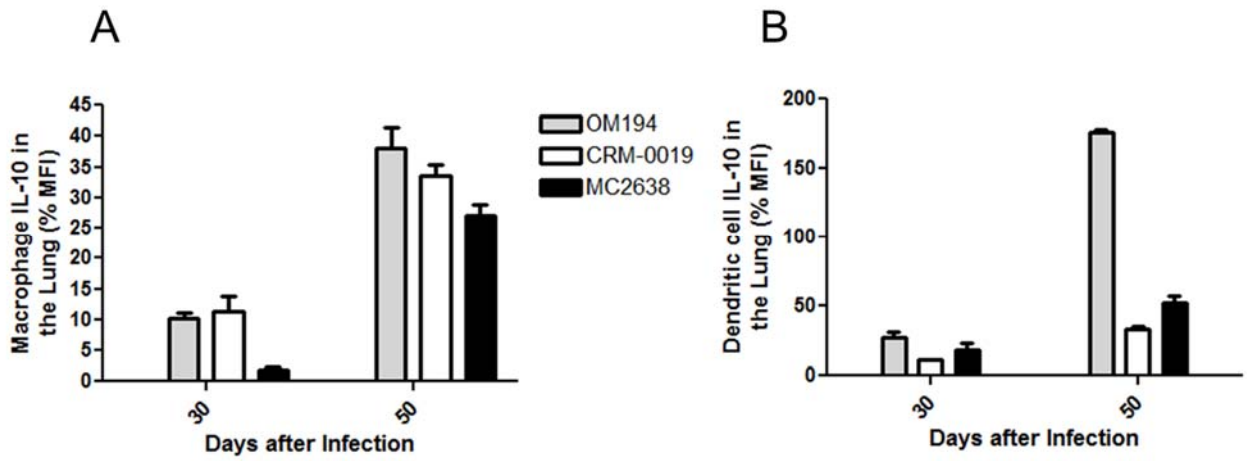

Figure 12. IL-10 Expression by Antigen Presenting Cells in the Lungs of GM/Rag-dblKO Mice upon Infection with *M. abscessus* Outbreak Strains. The level of expression of IL-10 by the macrophages (A) and dendritic cells (B) was much higher in the lungs of GM/Rag-dblKO mice infected with the outbreak strains *M. abscessus* sbsp. *massiliense* OM194 and CRM-0019 compared with GM/Rag-dblKO mice infected with the outbreak strain MC2638. Error bars represent ±SEM; n=5.

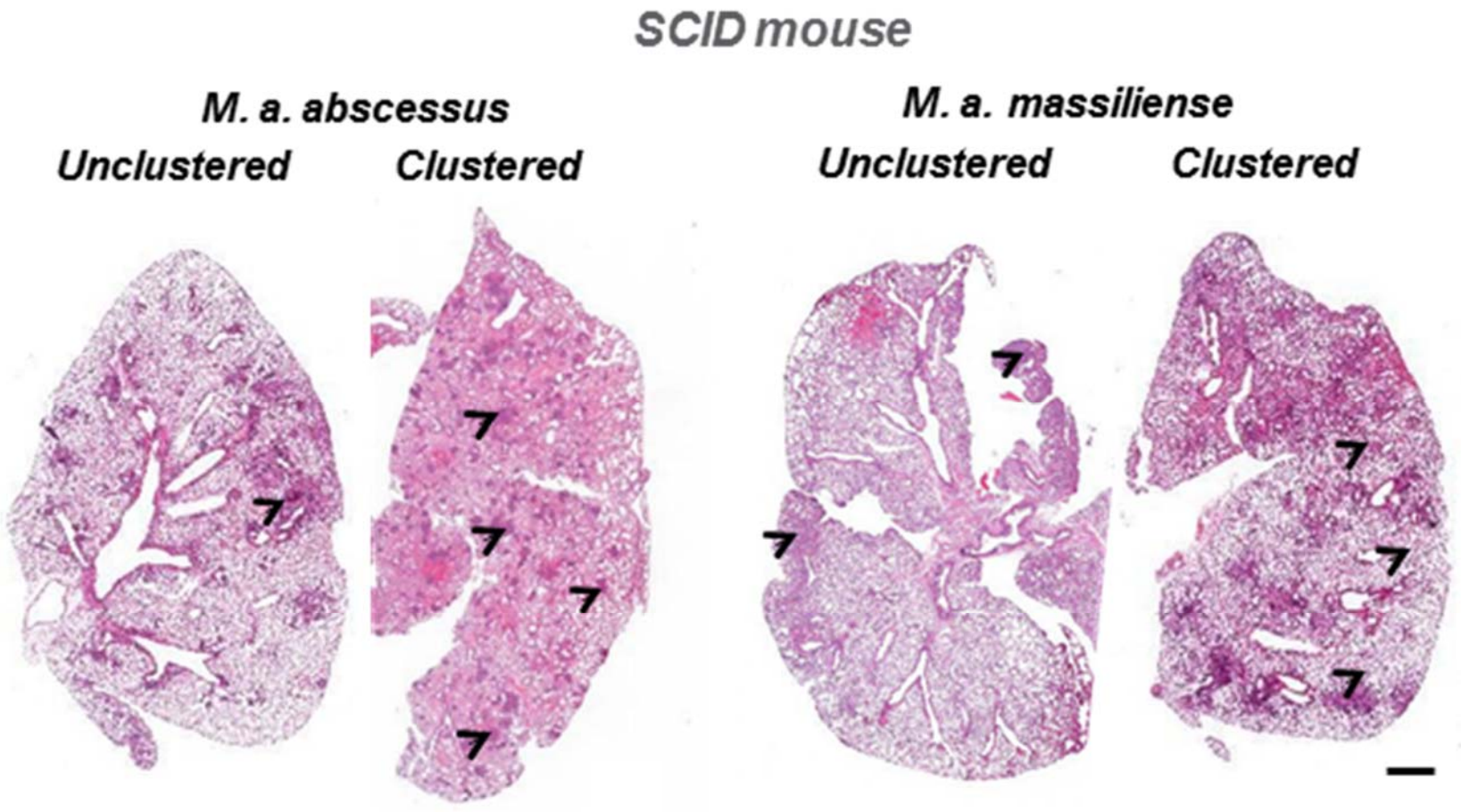

Figure 13. Lung Histopathology of SCID Mice Following Infection with Clustered versus Unclustered *M. abscessus* Isolates. Infection of SCID mice with clustered *M. a. abscessus* (blue) and *M. a. massiliense* (red) resulted in exacerbated granulomatous pulmonary inflammation (arrowheads) than that of unclustered strains. Scale bar, ×4 xx [80].

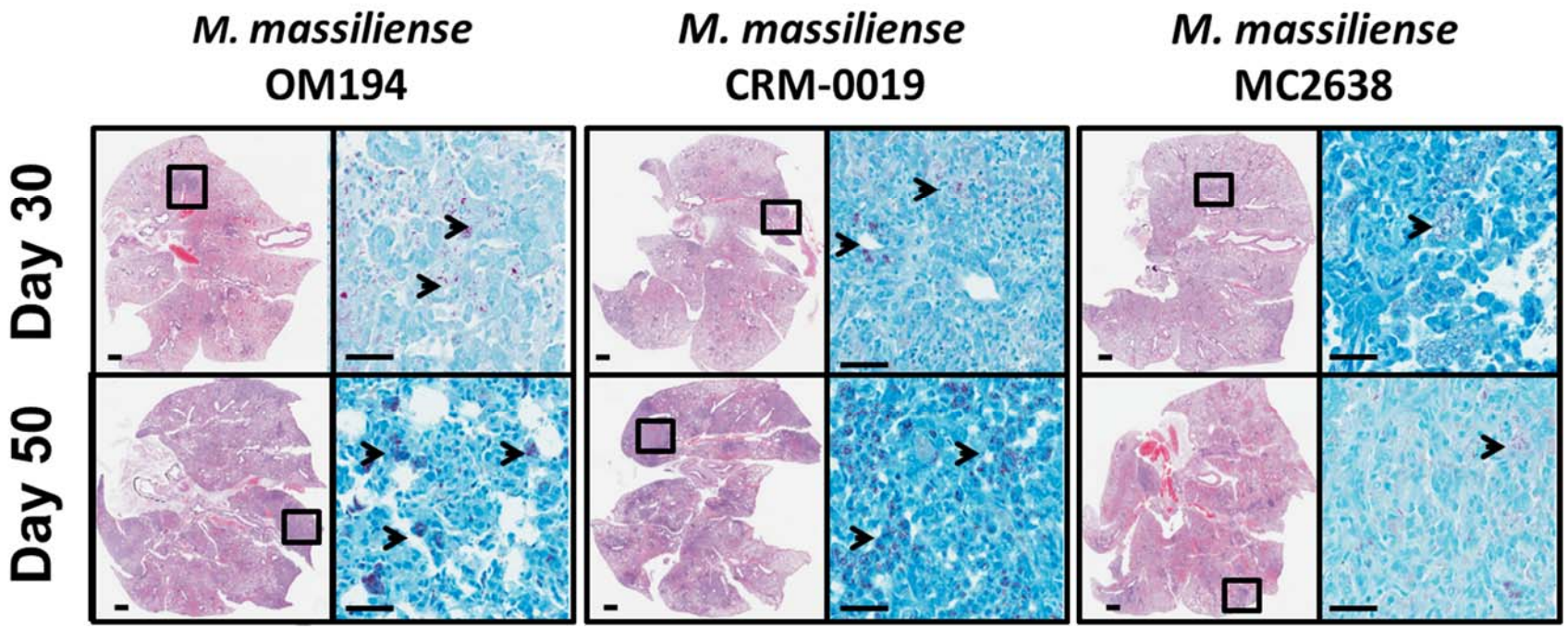

Figure 14. Granulomatous Inflammation in the Lungs of GM/Rag-dbIKO Mice Infected with Outbreak Strains of *M. abscessus*. Granulomatous inflammation is seen in the lungs of GM/Rag-dbIKO mice infected with the outbreak strains *M. abscessus* sbsp. *massiliense* OM194 (Cambridge), CRM-0019 (Brazil), and MC2638 (Seattle). The granulomas have coalesced, leading to lung consolidation.

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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
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