GM-CSF knockout mice for preclinical testing of agents with antimicrobial activity against \textit{Mycobacterium abscessus}

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Objectives: Of the non-tuberculous mycobacteria, \textit{Mycobacterium abscessus} is particularly refractory to antimicrobial therapy and new agents with activity against these pathogens are urgently needed. The screening of candidate antimicrobial agents against \textit{M. abscessus} requires a relevant and reproducible animal model of chronic infection. Granulocyte-macrophage colony-stimulating factor knockout (GM-CSF KO) mice were used to develop a new animal model of chronic pulmonary \textit{M. abscessus} infection that can be used for preclinical efficacy testing of antimicrobial drugs.

Methods: GM-CSF KO mice were infected with a clinical isolate of \textit{M. abscessus} via intrapulmonary aerosol delivery using a microsprayer device. The clinical condition, histology and cfu of \textit{M. abscessus}-infected GM-CSF KO mice were evaluated over a period of 4 months. Mice were treated with azithromycin (100 mg/kg) by oral gavage and the clinical condition, histology and bacterial burden was determined after 2 weeks of treatment.

Results: We show that pulmonary infection of GM-CSF KO mice with \textit{M. abscessus} results in a chronic pulmonary infection that lends itself to preclinical testing of new antimicrobial drugs against this bacterium. Azithromycin treatment of \textit{M. abscessus}-infected GM-CSF KO mice resulted in a lower bacterial burden in the lungs and spleen, weight gain and significant improvement in lung pathology.

Conclusions: Intrapulmonary aerosol infection of GM-CSF KO mice with \textit{M. abscessus} is a useful animal model for studying pathogenesis as well as pre-clinical testing of new compounds against \textit{M. abscessus} in acute or chronic phases of infection.

Keywords: \textit{M. abscessus}, mouse model, drug treatment, macrolides

Introduction

There is increasing incidence and prevalence of infections due to the non-tuberculous mycobacteria (NTM). While members of the \textit{Mycobacterium avium} complex remain the most common cause of these infections, \textit{Mycobacterium abscessus} and closely related species of rapidly growing mycobacteria are of particular importance due to inherent resistance to many antimicrobial drugs. Macrolides, including azithromycin and clarithromycin, are active \textit{in vitro} against \textit{M. abscessus}. However, treatment of \textit{M. abscessus} infections with azithromycin can lead to induction of the erythromycin ribosome methyltransferase gene (erm), resulting in macrolide resistance.\footnote{Emergence of resistance may explain the lack of efficacy of this treatment in some cases and such patients often require treatment with parenteral drugs, which can be poorly tolerated and expensive, and vary in their efficacy. This scenario has sparked a new interest in preclinical discovery programmes directed at NTM infections.} 

In addition to discovering effective agents against \textit{M. abscessus}, there is also a need for reliable animal models of chronic \textit{M. abscessus} infection to allow the preclinical testing of new drugs. However, unlike the preclinical testing of drugs against \textit{M. tuberculosis}, such animal models do not exist for \textit{M. abscessus} infection. Prior to these studies we used previously reported animal models for \textit{M. abscessus} infection.\footnote{Host susceptibility to infection by \textit{M. abscessus} is highly dependent on complex host–pathogen interactions and a successful defence against mycobacterial infections relies on immune cells and cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF). For the purpose of our studies we used GM-CSF knockout (GM-CSF KO) mice to develop a new animal model of chronic pulmonary \textit{M. abscessus} infection. We show that a}
pulmonary infection of GM-CSF KO mice with M. abscessus results in a chronic pulmonary infection that lends itself to preclinical testing of new antimicrobial drugs against this bacterium.

Methods

Mice

Mice lacking the GM-CSF gene (GM-CSF KO mice) were generated by Dranoff et al. and expressed undetectable amounts of GM-CSF. Dr. B. C. Trapnell (Division of Pulmonary Biology, Children’s Hospital Medical Center, Cincinnati, OH, USA) provided GM-CSF KO mice. Mice were bred at Colorado State University Biosafety Level-2 (BSL-2) facilities and maintained in a specific, pathogen-free BSL-3 facility during infection with M. abscessus. All animals had free access to water and standard mouse chow. Animals were cared for in line with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and NIH guidelines, and the Colorado State University Institutional Animal Care and Use Committee approved all experimental protocols.

Bacteria

M. abscessus #21 was originally isolated from a patient who immigrated to the USA from Asia with a remote history of pulmonary tuberculosis. Dr. M. Jackson of Colorado State University and Dr. Nancy Madinger from the University of Colorado Hospital Clinical Microbiology Laboratory generously provided Colorado State University with M. abscessus #21. For speculation, the mycobacterial cultures were grown in Middlebrook 7H9-OADC broth supplemented with 0.05% Tween 80 on 7H11-OADC agar. Standard PCR and sequencing strategies were used to amplify and analyse partial sequences of the hsp65 (441 bp) and rpoB (723 bp) genes. For MIC determination and a time–kill study for azithromycin against M. abscessus #21, we used microbroth dilution in cation-adjusted Mueller–Hinton broth, pH 7.3. For this purpose, the bacterium was grown at 37°C with shaking in cation-adjusted Mueller–Hinton broth. Aliquots of broth (100 μL) were removed at the specified timepoints and serial 1:10 dilutions were plated and enumerated after 3–4 days of growth. The MIC values were converted to a log10 cfu/mL scale and plotted. The M. abscessus #21 stock was propagated and passaged four times in 7H9 broth supplemented with OADC and 0.05% Tween 80 and frozen in aliquots at −80°C until use.

Infection of mice

Mice were infected via the intrapulmonary aerosol route with 5 × 10^5 to 1 × 10^6 cfu/lung of M. abscessus #21. The bacteria were delivered via intrapulmonary aerosol using a microsprayer device (MicroSprayer, model IA-C; PennCentury, Philadelphia, PA, USA) attached to an FMU-250 high-pressure syringe device (PennCentury) as previously described. Briefly, mice were anaesthetized using an isoflurane and oxygen mix, the MicroSprayer tip was placed in the laryngeal vestibule and the bacterial suspension was sprayed. Three mice were sacrificed at day 0 post-infection to determine bacterial uptake. Other groups of mice (n = 5) were euthanized after 5, 15, 20 and 30 days and at 2 and 4 months of infection. The lungs and spleen were collected and prepared for bacterial load determination and histological analysis. The number of viable bacteria in the lungs and spleen was determined by plating serial dilutions of individual whole lung and spleen homogenates on nutrient Middlebrook 7H11 agar and counting bacterial colony formation after 48–72 days of incubation at 37°C. The data were expressed as the log10 value of the mean number of colonies counted. Mice were monitored on a daily basis, their weights were recorded and they were euthanized when they displayed symptoms associated with disease or had lost 20% of their original weight.

Results

We first sought to confirm that the clinical isolate used in these studies was an M. abscessus strain. For this purpose we performed a molecular investigation of the M. abscessus #21 clinical isolate by sequencing of the hsp65 and rpoB genes, as previously reported by others for characterization of M. abscessus. These results demonstrated that the clinical isolate #21 used in this study was a bona fide M. abscessus strain. Figure 1 shows the nucleotide sequence alignment of clinical isolate #21 partial sequences for the hsp65 and rpoB genes. The reference strains used in the alignment were Mycobacterium chelonae strain ATCC 35752, M. abscessus strain ATCC 19977, M. abscessus subsp. bolletii CIP 108541 and M. abscessus subsp. massiliense CIP 108297 (Figure 1a). The colony morphology of the stock of bacteria used in this study was predominately smooth when grown on agar plates (Figure 1b). The stock of bacteria was also tested in a time–kill study (Figure 1c) and an MIC study for its susceptibility to azithromycin. The time–kill study demonstrated that azithromycin had bacteriostatic activity against M. abscessus #21 and that the MIC was 1 mg/L, indicating the bacterium was azithromycin susceptible.

Prior to these studies, we used existing animal models for M. abscessus. However, in our experience the pulmonary infection was rapidly cleared and failed to establish a chronic state of infection. For the purposes of our studies we elected to use the GM-CSF KO mouse model of infection because previous studies demonstrated these mice are highly susceptible to mycobacterial infection. Several small trials using GM-CSF KO mice were performed to determine the approximate dose of infection with M. abscessus #21, and the age and weight at which GM-CSF KO mice should be used for infection. The weight of the mice, more importantly than age, appeared to be a critical factor in overcoming the infection. Mice weighing ~20 g succumbed to acute infection within 72 h, whereas mice weighing a minimum of 20 g were able to overcome the acute phase of the infection and had prolonged survival. Thus, all experiments described below were performed using mice with a minimum weight of 20 g and were

Histology

The accessory lung lobe from each mouse was fixed in 4% paraformaldehyde for 48 h inside the BSL-3 laboratory. Paraffin-embedded lung was sectioned at 5 μm and stained by routine haematoxylin and eosin and acid-fast methodology. Histopathological evaluation was performed by a certified pathologist.
Mouse model of M. abscessus

(a) hsp65

(b) rpoB

Figure 1. (a) Nucleotide sequence alignment of partial sequences of the hsp65 and rpoB genes. The reference strains used in the alignment are: MCH, M. chelonae strain ATCC 35752; MAB, M. abscessus strain ATCC 19977; MBO, M. abscessus subsp. bolletii CIP 108541; and MMA, M. abscessus subsp. massiliense CIP 108297. (b) M. abscessus #21 colonies after plating on 7H11 agar plates showing that most of the colonies had a smooth morphotype, with only one colony having a rough morphotype. (c) Time - kill kinetic study of M. abscessus #21 in the presence of azithromycin (squares) at four times the MIC (4 mg/L) compared with growth in the control tube with no antibiotics (diamonds). The organism was grown at 37°C with shaking in cation-adjusted Mueller - Hinton broth (pH 7.3) and 100 μL aliquots of broth were removed at the specified timepoints for plating of serial 1:10 dilutions and enumeration after 3 - 4 days of growth. Colony numbers were converted into a log10 CFU/mL scale and plotted. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
infected with $5 \times 10^5$ to $1 \times 10^6$ *M. abscessus* #2 bacilli per mouse delivered as an intrapulmonary aerosol.

The acute and chronic stages of infection in GM-CSF KO mice after pulmonary infection were monitored over 4 months and the bacterial loads in the lungs and spleen were determined at several timepoints over the course of infection. The bacterial loads in the lungs and spleens after 5, 15, 20 and 30 days and at 2 and 4 months of infection are shown in Figure 2(a). Three mice were sacrificed at day 0 post-infection to determine bacterial uptake. When compared with day 0 of infection, the bacterial load in the lungs appeared higher after day 5 then decreased to a plateau of $\sim 10^5$ cfu for the remaining 2 months of infection. The bacterial load increased again between 2 and 4 months of infection. A small increase in splenic mycobacterial burden was seen during the first week of the infection, which was subsequently cleared over the long course of the experiment. Interestingly, the morphotype—smooth or rough—of the colonies that appeared after plating the lung homogenates on agar differed depending on the time at which lungs were harvested during the infection. Thus, plating lung homogenates harvested at early timepoints of the infection on agar developed into colonies (90%–98%) with a smooth morphotype. However, plating lung homogenates harvested at 4 months of the infection on agar led to the development of colonies with a predominantly rough morphotype.

The body weight of the mice was monitored for 4 months during the course of infection. Weight loss occurred during the first week of the infection, but the weight plateaued for the remainder of the study (Figure 2b). There was great variability during the first week of the infection. Two groups displayed loss of >20% of their body weight. The bacterial load in these animals was higher than $10^5$ cfu.

Histopathology was evaluated at pre-determined endpoints of days 0, 10 and 28 of infection and in chronic stages at 4 months of infection (Figure 3). Further samples from each timepoint were stained to determine whether they were acid-fast. Acid-fast bacilli were found in all samples collected. Mice with rapid weight loss and acute infection had abundant acid-fast staining of bacilli in sections obtained from the lungs (Figure 3a and b). At 4 months of infection, mice with chronic infection were bright, alert and overweight, but still presented with large gross lesions (Figure 3g). The majority of acid-fast bacteria were found within macrophages in the lungs (Figure 3d and e), although some bacteria appeared free in the alveoli (Figure 3e).

While no inflammation was evident initially after infection on day 0, the most severe inflammation over the course of infection was evident in the acute stages at day 10 (Figure 3c). Inflammation was centred on small bronchioles and effaced alveolar architecture. Inflammatory cell populations consisted of sheets of macrophages with admixed intact and degenerate neutrophils. Occasional inflammation was present within airway lumens, and alveoli adjacent to the histiocytic inflammation contained eosinophilic fluid. By day 28 of infection, however, the inflammation subsided and consisted of macrophages and neutrophils in smaller numbers around airways and a higher proportion of lymphocytic inflammation. Chronic infection at 4 months consisted of accumulations of large macrophages within alveoli containing abundant cytoplasmic foamy vacuolation. Also, airway-centric lymphocytic inflammation was present with rare bronchioles displaying destruction of connective tissue and attenuation or loss of surface epithelium, consistent with early bronchiectasis (Figure 3f).

In the drug treatment trial, therapy with azithromycin was started on day 10 post-infection. As shown in Figure 4 treatment with azithromycin was effective and demonstrated significant activity as a single agent. The lung bacterial load of GM-CSF KO mice with chronic *M. abscessus* infection demonstrated a 3 log$_{10}$ decline in cfu after treatment with azithromycin for 2 weeks when compared with the untreated control group. In addition, the resulting lung pathology of GM-CSF KO mice with chronic *M. abscessus* infection revealed near complete resolution of inflammation and lack of bacilli by acid-fast staining when compared with similar sections from the untreated control group (data not shown).

**Discussion**

In this study the experimental pulmonary exposure to *M. abscessus* in GM-CSF KO mice resulted in both acute and chronic infection. GM-CSF is a cytokine functioning as a haematopoietic growth factor for the generation of macrophages and dendritic cells. We are unaware of a chronic model of *M. abscessus* using this arm of the adaptive immune system to demonstrate that it is responsible for control of the infection. The clinical condition, histology and cfu of mycobacteria were monitored over time and we demonstrated that the pulmonary bacterial loads increased in the 5 days after...
infection and then began to slightly decrease over a period of
2 months. After 2 months, the bacterial load began to increase
again. *M. abscessus* was isolated from the spleen, but the majority
of the infection and associated pathology was manifested in the
lungs. Low body weight was associated with severe infection, mor-
tality and overwhelming acute mycobacterial pneumonia. During
the chronic stages of infection, the majority of bacilli found in the
lungs were intracellular within foamy macrophages. Drug treat-
ment with 2 weeks of azithromycin (100 mg/kg) was effective
and was associated with diminished bacterial burden, weight
gain and significant improvement in lung pathology. This animal
model will be useful for studying pathogenesis as well as for
early pre-clinical testing of new compounds against *M. abscessus*
in acute or chronic phases of infection.
A mouse model of acute *M. abscessus* infection exists\(^1\) which evaluates the efficacy of a compound immediately after infection, when bacilli are thought to be actively replicating. However, we are unaware of a reproducible animal model of *M. abscessus* infection that allows the assessment of chronic *M. abscessus* pulmonary infection. The GM-CSF KO mouse model was able to withstand both acute and chronic *M. abscessus* pulmonary infection. Drug efficacy testing in animal models capable of withstanding both acute and long chronic infection may yield better insights into bactericidal activities of potential drug candidates against *M. abscessus*.\(^{16}\)

In our study, mice that developed a chronic infection were bright, alert and overweight, but still presented with large gross lesions. However, the infection was acute and overwhelming in mice with a low body weight. Interestingly, non-tuberculous lung disease in humans is associated with a specific morphology of *M. abscessus* including, among others, a low body mass index and tall stature.\(^{15}\) Likewise, bronchiectasis is one of the cardinal manifestations in humans infected with *M. abscessus*\(^{16-18}\) and the histopathological assessment of the lung in mice with chronic infection in this study displayed evidence of bronchiectasis. Altogether, our results suggest that the course of *M. abscessus* infection in mice with GM-CSF deficiency is similar to that described for human infection with *M. abscessus*.\(^{16}\)

There is an extensive body of literature dealing with the significance of the rough and smooth *M. abscessus* morphotypes in terms of colonization and disease progression.\(^{14,19,20}\) In our study the morphology of the bacterial suspension used to infect the mice by the pulmonary aerosol was mostly smooth. However, the morphology of the colonies isolated from the lung of GM-CSF KO mice during the course of the infection transitioned from smooth to rough with progression from early to late timepoints. This is an important observation consistent with both the basic science observations and clinical observations of other groups.\(^{14,19,20}\) Previous studies have suggested that the smooth colony phenotype is relatively avirulent and tends to form a biofilm, thereby allowing it to colonize surfaces such as those that might be found in the airways. Mixed smooth and rough morphotypes are commonly observed, with greater percentages of rough morphology over time in chronic infection, which some believe is a more inflammatory and virulent form of the organism.\(^{14,19,20}\) Future studies in our laboratory will further investigate these aspects of the infection.

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**Figure 4.** Bacterial burden in the lungs (\(P = 0.041\)) (a) and spleen (\(P = 0.002\)) (b) of GM-CSF KO mice infected with *M. abscessus* with and without treatment with azithromycin (100 mg/kg). (c) Graph showing the mean weights (\(n = 5\)) obtained at different times of infection in mice with (squares) and without (diamonds) treatment with azithromycin.
The data presented here demonstrate that the cytokine GM-CSF is important for immune clearance of M. abscessus by the host. Thus, in the absence of GM-CSF, as in the GM-CSF KO mice used in these studies, the mice either succumbed to the acute infection or the infection persisted to a chronic stage. Similar results have been demonstrated previously with M. tuberculosis infections. Mice lacking GM-CSF were highly susceptible to M. tuberculosis infection and died within 35 days of infection. In contrast, when interferon-γ knockout mice were infected with M. abscessus there was rapid clearance of the bacteria (data not shown). However, others reported successful establishment of infection in this model. It appears that early development and recruitment of granulocytes and macrophages by GM-CSF may prove important in preventing infection by mycobacterial species such as M. abscessus and M. tuberculosis. This notion is further supported by clinical data showing that alteration in the pathways of the GM-CSF cytokine are highly involved in mycobacterial infections and thus therapies targeting these pathways in conjunction with other anti mycobacterial agents may result in improvement of disease outcome.

Multidrug therapy with a macrolide backbone such as azithromycin or clarithromycin is commonly used to treat patients with chronic M. abscessus infection. In these studies we chose azithromycin to demonstrate a proof of concept that the GM-CSF KO mouse model is suitable for drug efficacy testing. Thus, 2 weeks of azithromycin (100 mg/kg) treatment for chronically M. abscessus-infected GM-CSF KO mice was effective and associated with diminished bacterial burden, weight gain and significant improvement in lung histology. We propose that model be investigate for preclinical in vivo efficacy testing of new agents against M. abscessus and also be tested against other NTM. The pathological findings of foamy macrophage lipid pneumonia and airway-centric disease during chronic infection in this model are reflective of human infection.

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Transparency declarations

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References