Immune defects in patients with pulmonary *Mycobacterium abscessus* disease without cystic fibrosis

Milou M.F. Schuurbiers¹,⁵, Mariolina Bruno ²,⁵, Sanne M.H. Zweijpfenning¹,⁵, Cecile Magis-Escurra¹, Martin Boeree¹, Mihai G. Netea²,³, Jakko van Ingen ⁴, Frank van de Veerdenok² and Wouter Hoefsloot ¹

Affiliations: ¹Radboud University Medical Centre, University Centre of Chronic Diseases Dekkerswald, Dept of Pulmonary Diseases, Nijmegen, The Netherlands. ²Dept of Internal Medicine, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands. ³Dept for Genomics and Immunoregulation, Life and Medical Sciences Institute (LIMES), University of Bonn, Bonn, Germany. ⁴Radboud University Medical Centre, Dept of Medical Microbiology, Nijmegen, The Netherlands. ⁵These authors contributed equally.

Correspondence: Sanne M.H. Zweijpfenning, Radboud University Medical Centre, University Centre of Chronic Diseases Dekkerswald, PO Box 9101, 6500HB Nijmegen, The Netherlands. E-mail: sanne.zweijpfenning@radboudumc.nl

ABSTRACT The prevalence of *Mycobacterium abscessus* infections in non-cystic fibrosis (CF) patients has increased in recent years. In this study, we investigate whether immune defects explain the apparent susceptibility to this opportunistic infection in non-CF patients.

We performed stimulations of peripheral blood mononuclear cells and whole blood from 13 patients with *M. abscessus* pulmonary disease and 13 healthy controls to investigate their cytokine production after 24 h and 7 days.

Patients were predominantly women (54%) with a mean age of 59 years; 62% had nodular bronchiectatic disease. Many patients had predisposing pulmonary diseases, such as COPD (46%), and asthma (23%). Patients with COPD showed an impaired interleukin (IL)-6 response to *M. abscessus* and a reduced IL-17 response to *Candida*, together with a *M. abscessus*-specific enhanced IL-22 production. Patients without COPD showed higher levels of interleukin-1 receptor antagonist (IL-1Ra), an anti-inflammatory molecule. Within the non-COPD patients, those with bronchiectasis showed defective interferon (IFN)-γ production in response to *Candida albicans*.

In conclusion, susceptibility to *M. abscessus* is likely determined by a combination of immunological defects and predisposing pulmonary disease. The main defect in the innate immune response was a shift of the ratio of IL-1β to IL-1Ra, which decreased the bioactivity of this pathway in the adaptive immune response. In the adaptive immune response there was defective IL-17 and IFN-γ production. Patients with COPD and bronchiectasis showed different cytokine defects. It is therefore crucial to interpret the immunological results within the clinical background of the patients tested.
Introduction

*Mycobacterium abscessus* is a nontuberculous mycobacterium notorious for causing difficult-to-treat pulmonary infections. Due to the extreme drug resistance intrinsic to these bacteria, *M. abscessus* has rightfully been dubbed an "antibiotic nightmare" [1]. In the last decade, an increasing prevalence of *M. abscessus* infections has been reported in many countries [2]. *M. abscessus* comprises three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii*. The isolation prevalence of each subspecies varies depending on geographical location [3, 4].

Cystic fibrosis (CF), non-CF bronchiectasis, COPD and low body mass index (BMI) are the best-known predisposing conditions for nontuberculous mycobacteria (NTM) pulmonary disease (NTM-PD) [2, 5]. However, only a small subset of patients with these chronic structural lung diseases acquire *M. abscessus* infections. In these apparently immunocompetent patients, the presence of minor defects in the immune response might explain their increased susceptibility [6]. For CF patients, such subtle immune defects were found [7].

The host immune response against NTM compromises three steps. First, mononuclear phagocytes recognise the mycobacterial cell wall via Pattern Recognition Receptors, and they internalise the mycobacterium [8]. Second, mononuclear phagocytes release pro-inflammatory cytokines interleukin (IL)-1β, tumour necrosis factor-α (TNF-α), IL-6, IL-12 and IL-18, which activate alveolar macrophages, dendritic cells and monocyte-derived macrophages [9]. Third, the infected dendritic cells migrate to the locoregional lymph nodes to present the mycobacterial antigen to naive T-cells, which activates the polarisation of effector T-cells and the production of T-cell-derived cytokines: IFN-γ, IL-17 and IL-22 [10]. These adaptive cytokines will in turn further activate macrophages to eliminate the mycobacterium [11].

The aim of this study was to describe a cohort of non-CF patients with pulmonary *M. abscessus* disease and perform a prospective evaluation of their immune responses.

Methods

Recruitment and characterisation of study subjects

All patients who had at least one positive respiratory sample for *M. abscessus* in our Mycobacteriology Reference Laboratory database from January 1, 2010 through April 1, 2018 were reviewed. An additional search for mycobacterial disease in our local diagnosis registry was performed to complement the patient search. Patients with *M. abscessus* pulmonary disease according to the American Thoracic Society (ATS) diagnostic criteria and no CF were included [12]. Written informed consent was obtained from all subjects participating in this study. For the immunological study, we recruited a healthy volunteer without an immunologically relevant medical history for each patient; in total 13 patients could be tested for immunological responses. The ethical board provided a waiver for this study due to its minimally invasive character.

Data collection

Demographic and clinical data were extracted from electronic patient files. Chest computed tomography scans were reviewed by a radiologist and subsequently by a pulmonologist of our NTM reference clinic (WH, SZ).

Treatment and outcome

Treatment outcome was classified according to the NTM-NET definitions [13]. Culture conversion was defined as three consecutive negative mycobacterial cultures from respiratory samples, collected at least 4 weeks apart, during antimycobacterial treatment. The time of conversion was defined as the date of the first negative culture.

Microbiology

Isolates were identified to subspecies level by sequencing of the *erm*(41) and *hsp65* genes. Antimicrobial susceptibility testing was performed using broth microdilution, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [14].

Immunology

Prospective patient recruitment and blood sampling

Peripheral blood was obtained from 13 patients with a (history of) *M. abscessus* disease and 13 healthy volunteers. EDTA plasma was obtained by venous blood sampling and used for whole blood and peripheral blood mononuclear cells (PBMCs) stimulation tests within 2 h after collection. Not all patients were available for immunological testing.
Whole blood assay

Diluted Whole Blood Assays for Cytokine Responses to Innate Stimuli was performed as described previously [15]. Peripheral whole blood was diluted 1:4 in RPMI 1640 Dutch-modified culture medium (Life Technologies/Invitrogen, Breda, The Netherlands) and pipetted into a microtitre plate (48-well plate (500,000), 100 µL of whole blood). The following cytokine-inducing stimuli were added: heat-killed M. abscessus bacteria (means of infection (MOI) 2; 2 mycobacteria per immune cell) of the patient’s own isolate (1×10^6 cells·mL^−1), M. abscessus CIP 104536 type strain (1×10^6 cells·mL^−1), Candida albicans (clinical isolate UC820) (1×10^6 cells·mL^−1) and Aspergillus fumigatus (clinical isolate V05) (1×10^6 cells·mL^−1). The whole blood samples were incubated at 37°C for 48 h for measurements of TNF-α, IL-1β and IFN-γ production.

Isolation and stimulation of PBMCs

Human PBMCs, as described previously [16], were isolated from EDTA blood using Ficoll-Paque density gradient centrifugation (GE Healthcare, Little Chalfont, UK) and resuspended in RPMI 1640 Dutch-modified culture medium (Life Technologies/Invitrogen, Breda, The Netherlands) at a density of 5×10^6 cells·mL^−1. The isolated PBMCs (500,000/well, 100 µL) were seeded on a round-bottom 96-well plate (Corning Inc., Corning, NY, USA) and supplemented with a stimulus accompanied by 10% human serum in the 7 days condition and in the Aspergillus stimulation experiment. The cells were stimulated with RPMI only as a negative control, the patients’ heat-killed M. abscessus (1×10^6 cells·mL^−1, MOI 2), M. abscessus CIP 104536 type strain (1×10^6 cells·mL^−1, MOI 2), M. avium ATCC 700898 type strain (1×10^6 cells·mL^−1, MOI 2), heat-killed A. fumigatus V05 type strain (1×10^6 cells·mL^−1) and heat-killed C. albicans UC820 type strain (1×10^6 cells·mL^−1). The cell cultures were incubated at 37°C for 24 h for subsequent measurement of the production of TNF-α, IL-1β, interleukin-1 receptor antagonist (IL-1Ra) and IL-6, and for 7 days to detect IFN-γ, IL-17 and IL-22.

Cytokine measurements

Culture supernatants were collected at the fixed time points and frozen at −20°C until cytokine assessment. Cytokine production was determined in supernatants using commercial ELISA kits for TNF-α, IL-1β, IL-1Ra, IL-6, IL-17, IL-22 (R&D Systems, Minneapolis, MN, USA) and IFN-γ (Sanquin, Amsterdam, The Netherlands) following the instructions of the manufacturer. IL-1β to IL-1Ra ratio, as a measure of IL-1 bioactivity, was calculated by plotting the levels of IL-1β and IL-1Ra in response to M. abscessus stimulation.

Testing for underlying defects responsible for IFN-γ deficiency

To find the underlying defect responsible for the IFN-γ deficiency, we performed additional experiments by stimulating PBMCs with the two major Type 1 T-helper (Th1)-polarising cytokines, IL-12 (10 ng·mL^−1) and IL-18 (50 ng·mL^−1) for 7 days. In addition, using PBMCs from three patients and three controls we used either IL-12 or IL-18 separately with the addition of stimulus phytohaemagglutinin (PHA, 10 µg·mL^−1) or E. coli lipopolysaccharide (LPS, 1 ng·mL^−1), or a combination of both.

Statistical analysis

For the retrospective analyses, data are presented as mean (SD) for continuous variables and as number (percentage) for categorical variables. All data are analysed using SPSS version 23.0 (SPSS Inc., Chicago, IL, USA). Immunological results are presented as mean±SEM. Correlation analysis between clinical characteristics and cytokine secretion is performed using Spearman’s rank correlation coefficient and the 95% confidence interval is calculated accordingly. A p-value of <0.05 is considered statistically significant. Generation of graphs and statistical analyses are performed using GraphPad Prism 5 (La Jolla, CA, USA).

Results

13 patients without CF met the diagnostic criteria for M. abscessus pulmonary disease and were included in the current study (supplementary figure S1); their baseline characteristics are shown in table 1. The predominant radiographic manifestation was nodular bronchiectatic disease (61.5%). The baseline pulmonary function is presented in supplementary table S2. Ten patients (76.9%) used inhalation steroids at diagnosis.

Microbiology

Five patients had M. abscessus subsp. abscessus (38.5%); other patients had either M. abscessus subsp. boletii or M. abscessus subsp. massiliense but were not further typed. Four patients had acid-bacilli positive sputum smears (30.8%).

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Antimicrobial treatment and response
Antibiotic treatment was started in seven patients; three patients completed treatment, one patient is still on treatment, one patient discontinued treatment due to adverse effects and two patients died (supplementary figure S1). Antibiotic therapy was individualised based on drug susceptibility results and patient tolerance (supplementary figure S3). Adverse events were reported by all patients, and at least one drug was stopped because of adverse events in 86% of patients (S9).

Cytokine responses
Innate immunity: reduced bioactivity of IL-1β
When PBMCs were stimulated with *M. abscessus*, the IL-1β to IL-1Ra ratio proved significantly reduced in patients (figure 1a). There was a trend in lower IL-1β production in patients compared to healthy controls (p=0.0721; supplementary figure S4A). IL-1Ra production in response to *M. abscessus* was marginally higher in patients compared to controls, and it did not reach statistical significance. Interestingly, *Aspergillus* induced IL-1Ra was significantly higher in patients compared to healthy controls (supplementary figure S4B). The induction in whole blood and PBMCs of TNF-α and IL-6 was not significantly different between patients and controls (supplementary figure S4C-D, S5C-D).

Adaptive immunity: defective IL-17 and IFN-γ production
A significantly reduced IL-17 (p<0.05; figure 1b) and IFN-γ production (p<0.05; figure 1c) in patients compared to controls was measured in PBMC in response to *C. albicans* stimulation. The whole blood stimulation with *C. albicans* confirmed the lower IFN-γ production in patients (p<0.01; figure 1d). No significant differences in IL-22 production were documented between patients and controls (supplementary figure S6A). To find the underlying defect responsible for the IFN-γ deficiency, we performed additional experiments by stimulating PBMCs in the presence of the Th1-polarising cytokines for 7 days. A trend for reduced IFN-γ production upon IL-12 + IL-18 stimulation (p=0.0659) was
observed in patients compared to controls (supplementary figure S7). A graphic summary of the immune defect is given in figure 2a.

**COPD and bronchiectasis are correlated with different immune defects**

A high variability in cytokine production between patients was shown. Since COPD was the most frequent comorbidity in our patient cohort (46.2%), we divided our patients into two clinical groups based on a formal diagnosis of COPD and compared the cytokine concentrations between those two groups and the healthy controls. In the innate response, COPD patients showed a lower *M. abscessus*-induced IL-6 production, compared to healthy controls (*p*=0.0291; figure 3a), while in the non-COPD group IL-6 levels were similar to those in controls. In the non-COPD group, *Candida*-stimulated PBMCs produced more IL-1Ra than healthy controls after 24 h (*p*=0.0261; figure 3b). As for the adaptive cytokine response, in the non-COPD patient group a more prominent IFN-γ deficiency in response to *C. albicans* was seen (*p*<0.01; figure 3c). On the contrary, IL-17 deficiency in response to *Candida* stimulation was more prominent in the COPD group (figure 3d). Also, COPD patients showed a significantly increased IL-22 production with *M. abscessus* (figure 3e). To characterise the patients in the non-COPD group this group is stratified based on non-COPD bronchiectasis. Non-COPD patients with bronchiectasis still showed a significant defect in IFN-γ (*p*=0.0109) and a trend towards higher IL-1Ra production (*p*=0.0552) compared to healthy controls.

**FIGURE 1** Innate and adaptive immune responses of non-cystic fibrosis pulmonary *Mycobacterium abscessus* patients (*n*=13) and healthy controls (*n*=13). a) Ratio of cytokine *M. abscessus*-induced production of interleukin (IL)-1β and interleukin-1 receptor antagonist (IL-1Ra) between patients and controls. IL-17 (b) and interferon-γ (IFN-γ) (c, d) production upon 7 days stimulation of peripheral blood mononuclear cells (b, c) or whole blood (d) with RPMI, reference *Mycobacterium abscessus* [clinical isolate CIP 104536] [1×10⁶ cells·mL⁻¹], *M. abscessus* from patient’s own isolate [1×10⁶ cells·mL⁻¹], *M. avium* [clinical isolate ATCC 700898] [1×10⁶ cells·mL⁻¹], *Aspergillus fumigatus* [clinical isolate V05] [1×10⁶ cells·mL⁻¹] and *Candida albicans* [clinical isolate UC820] [1×10⁶ cells·mL⁻¹]. Graphs represent mean±SEM, *: *p*<0.05, **: *p*<0.01, two-tailed Mann–Whitney test.
(figure 3f and g). An overview of the above-mentioned stratification and the consequent possible immunological defect is given in figure 2b. To understand whether the radiological disease pattern would determine a difference in the immunological profile, we also stratified our patient cohort according to the nodular bronchiectatic disease and fibrocavitary disease type. However, except for a significantly lower \textit{M. abscessus}-induced IL-6 in the nodular bronchiectatic disease as compared to healthy controls (figure 3h), no differences in immune response were seen for nodular bronchiectatic disease versus fibrocavitary disease (supplementary figure S6 B-C).

**Discussion**

To our knowledge, this is the first study in which assessment of cytokine responses was combined with an extensive research of clinical characteristics and treatment outcome in non-CF patients with pulmonary

\[ \text{IL-12} \quad \text{IL-1Ra} \quad \text{IL-1} \beta \quad \text{IL-12} \quad \text{IL-18} \quad \text{IFN-γ} \]

\[ \text{Non CF pulmonary \textit{M. abscessus} disease} \]

\[ \text{No COPD} \]

\[ \text{COPD} \]

\[ \text{Inhaled steroids?} \]

\[ \text{AAT?} \quad \text{Smoking?} \]

\[ \text{IL-17} \quad \text{IL-6} \]

\[ \text{IL-22} \]

\[ \text{Legend} \]

\[ \text{Immune imbalance} \]

\[ \text{Hypothesised mechanism} \]

\[ \text{Underlying factor} \]

\[ \text{IL-1Ra} \quad \text{Bronchiectasis} \]

\[ \text{Ag presentation?} \]

\[ \text{IFN-γ} \]

\[ \text{(figure 3f and g). An overview of the above-mentioned stratification and the consequent possible immunological defect is given in figure 2b. To understand whether the radiological disease pattern would determine a difference in the immunological profile, we also stratified our patient cohort according to the nodular bronchiectatic disease and fibrocavitary disease type. However, except for a significantly lower \textit{M. abscessus}-induced IL-6 in the nodular bronchiectatic disease as compared to healthy controls (figure 3h), no differences in immune response were seen for nodular bronchiectatic disease versus fibrocavitary disease (supplementary figure S6 B-C).} \]
FIGURE 3 Peripheral blood mononuclear cells (PBMCs) immune profile comparing different clinical subgroups. a) Innate immune cytokine production upon 24 h stimulation of PBMCs reference *M. abscessus* (clinical isolate CIP 104536) (1×10^6 cells·mL^{-1}); interleukin-1 receptor antagonist (IL-1Ra) (b), interferon-γ (IFN-γ) (c), interleukin (IL)-17 (d) and IL-22 (e) production upon PBMC stimulation between healthy controls (n=13), chronic obstructive pulmonary disease (COPD) (n=6) and non-COPD (n=7) patients; IL-1Ra (f) and IFN-γ (g) production upon PBMC stimulation between healthy controls (n=13), COPD (n=6) and non-COPD with bronchiectasis (n=5) patients. Please note that non-COPD patients include patients with bronchiectasis; a-e (n=5), since not all non-COPD patients had bronchiectasis; (h) IL-6 production upon PBMCs stimulation between healthy controls (n=13), patients with fibrocavitary disease [Cavitary, n=4] and patients with nodular bronchiectasic disease (NB, n=8). Graphs represent mean±SEM, *: p<0.05, two-tailed Mann–Whitney test. TNF-α: tumour necrosis factor-α.
M. abscessus disease. In our cohort, we found defects in both the innate immunity (a reduced bioactivity of IL-1β) and in the adaptive immunity (defective IL-17 and IFN-γ production). Clinical phenotypes such as COPD and bronchiectasis are linked with different cytokine defects.

**Innate immunity: reduced bioactivity of IL-1β**

In this cohort, IL-1β/IL-1Ra ratio was lower in patients than controls, which can explain a higher susceptibility to M. abscessus disease. The importance of IL-1β signalling for host response against NTM is demonstrated by the finding that activation of NLRP3 inflammasome restricts M. kansasii disease [17]. Haverkamp et al. [18] performed single nucleotide polymorphism (SNP) analysis of 81 Dutch children with NTM lymphadenitis, and they found a positive association between NTM lymphadenitis and SNPs in the IL-1β gene that influences IL-1β mRNA levels. Perhaps a lower bioactivity of IL-1β could be the underlying cause of the imbalance of IL-1Ra and IL-1β which leads to less activation of macrophages.

**Adaptive immunity: defective IFN-γ and IL-17 production**

We report a significant defect in IFN-γ and in IL-17 production in patients compared to controls upon Candida stimulation. IFN-γ signalling is crucial for NTM host response [19]. Mutations in receptors or transcription factors for IFN-γ are associated with increased susceptibility to mycobacterial disease. To find the underlying defect responsible for the IFN-γ deficiency, we performed additional experiments, but we were not able to find whether the defect was more IL-12 or IL-18 dependent (supplementary figure S7).

In our cohort deficient IL-17 production seems to be a general defect, as it is not specific to M. abscessus, but evident upon C. albicans stimulation. Becker et al. [7] showed lower IL-22 levels while our study found higher IL-22 in COPD patients; this difference is probably due to variations in patient groups between the two studies, and the results are not significant in both studies. Becker et al. [7] showed that IL-22 levels were lower in the non-abscessus NTM-patients, but for the M. abscessus patients with CF, levels were similar or even higher as compared to controls, similarly to our results. Since higher IL-22 levels induce lung damage and susceptibility to infection, sustained levels of IL-22 upon *M. abscessus* stimulation might be peculiar to patients suffering from pulmonary *M. abscessus* disease. The IL-17 defect in non-CF patients is in line with the study of Becker et al. [7], in which an *M. abscessus*-specific IL-17 deficiency, rather than an IFN-γ defect, was suggested to play a crucial role in pulmonary *M. abscessus* disease in patients with CF. Moreover, another study showed that the IL-23/IL-17 axis in response to *M. avium* complex (MAC) is attenuated in patients with MAC-lung disease [20]. In conclusion, NTM pulmonary disease might be associated with reduced Th17 immunity as well as with reduced Th1 immunity.

**Correlation with BMI**

NTM disease is associated with a certain phenotype known as Lady Windermere syndrome. Women with this syndrome typically have a low BMI, mitral valve prolapse and pectus excavatum. Also, previous studies showed that patients with pulmonary NTM disease have lower BMI and less body fat [21, 22]. When correlating IFN-γ production in whole blood after 48 h with the BMI of our patient cohort, we found a significantly positive association (supplementary figure S8), meaning that patients with a lower BMI have a more pronounced IFN-γ defect, possibly because of a defect of gamma delta-T-cells or natural killer cells [22].

**COPD: the great immunological divide**

We found two patterns of immune defects in two categories of *M. abscessus* patients: those with and those without COPD. Patients with COPD show an impaired IL-6 response to *M. abscessus* and a reduced IL-17 response to *Candida*, together with an *M. abscessus*-specific enhanced IL-22 production, which worsens lung epithelial damage. The causes of those immunological alterations might be the use of inhalation steroids, cigarette smoking or an acquired deficit of antiprotease for the excessive IL-22 response. It is known that IL-22 is significantly increased in the sputum of stable COPD [23], and the absence of IL-22 does not affect the outcome of *M. tuberculosis* infection [24]. However, a higher level of IL-22 can contribute to T-helper imbalance, and the specific IL-22 production in response to *M. abscessus* might worsen the inflammation-mediated lung damage in COPD patients. Furthermore, Jong et al. [25] also showed a defect in IL-6 production specifically for *M. abscessus* in COPD patients.

On the other hand, patients without COPD show higher levels of IL-1Ra, which is an anti-inflammatory molecule that blocks the bioactivity of the IL-1 pathway and might subsequently increase susceptibility to *M. abscessus*. It is suggested that reduced body fat leads to reduced leptin levels and higher levels of adiponectin, which is known to suppress the expression of TNF-α and induces the expression of IL-1Ra and IL-10 [5].
Within the non-COPD patients, those with bronchiectasis show an IFN-γ defect in response to Candida stimulation, which might be related to impaired signalling of the polarising cytokines IL-12 and IL-18 or to the antigen presentation.

**Clinical characteristics**

In our cohort the high rate of COPD and the predominance of the *M. abscessus* subsp. *massiliense* stand out in comparison to cohorts from the USA, Taiwan and Japan [26–28]. The majority of patients had nodular bronchiectatic disease (61.5%), and the number of patients with cavitary lesions in our study (38.5%) is in line with previous studies (14–42%) [29–31]. These aspects may partly explain the high sputum culture conversion rate in this cohort (78%), which is similar to series from South Korea where culture conversion rates of up to 82% in *M. abscessus* subsp. *massiliense* pulmonary disease were observed, but only up to 26% for *M. abscessus* subsp. *abscessus* [32].

**Limitations**

One of the limitations of this study is the relatively small cohort: this was due to the rarity of *M. abscessus* pulmonary disease or to the underlying COPD/bronchiectasis.

In addition, it is difficult to discern whether the immune defects are causally related to *M. abscessus* disease or to the underlying COPD/bronchiectasis.

In conclusion, susceptibility to *M. abscessus* is probably determined by a combination of immunological defects and predisposing pulmonary disease. The main defect in the innate immune response was a shift in the ratio of IL-1β to IL-1Ra which decreased the bioactivity of this pathway. In the adaptive immune response we recorded a defective IL-17 and IFN-γ production. Patients with COPD and bronchiectasis showed different cytokine defects. It is therefore crucial to interpret the immunological results within the clinical background of the patients tested. Deciphering which patients are at risk of pulmonary *M. abscessus* disease and understanding the nature of the immune response to *M. abscessus* in patients will hopefully enable us to develop more effective adjunctive strategies to treat this disease and potentially prevent its occurrence.

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