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ORIGINAL ARTICLE

Alpha-1-antitrypsin (AAT) anomalies are associated with lung disease due to rapidly growing mycobacteria and AAT inhibits *Mycobacterium abscessus* infection of macrophages

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Abstract

Rapidly growing mycobacteria (RGM) are ubiquitous in the environment but cause lung disease in only a fraction of exposed individuals. This variable susceptibility to disease implies vulnerability to RGM infection due to weakness in host defense. Since most persons who contract RGM lung disease have no known host defense defect, it is likely that uncharacterized host deficiencies exist that predispose to RGM infection. Alpha-1-antitrypsin (AAT) is a host factor that may protect individuals from respiratory infections. Therefore, we assessed AAT protein anomalies as a risk factor for RGM lung disease. In a cohort of 100 patients with RGM lung disease, *Mycobacterium (M.) abscessus* was the most prevalent organism, isolated in 64 (64%) subjects. Anomalous AAT proteins were present in 27% of the cohort, which is 1.6 times the estimated prevalence of anomalous AAT proteins in the United States population ($p = 0.008$). In *in vitro* studies, both AAT and a synthetic inhibitor of serine proteases suppressed *M. abscessus* infection of monocyte-derived macrophages by up to 65% ($p < 0.01$). AAT may be an anti-RGM host-defense factor, and anomalous AAT phenotypes or AAT deficiency may constitute risk factors for pulmonary disease due to RGM.

Introduction

Non-tuberculous mycobacteria (NTM) are found widely in soil and water [1]. Excluding persons infected with human immunodeficiency virus type 1, NTM infection usually presents as a chronic pulmonary disease characterized by bronchiectasis, parenchymal nodules, or cavitation. The prevalence of NTM pulmonary disease in persons without immunodeficiency appears to be increasing, and in many areas of the United States (US) NTM pulmonary disease prevalence exceeds that of *M. tuberculosis* [2,3]. The most common pathogenic NTM organisms in the US are *M. avium* complex and members of the rapidly growing mycobacteria (RGM) group such as *M. abscessus* [2–4]. These

observations suggest that lung disease due to RGM is an emerging public health problem [5].

Since RGM are widespread in the environment, but cause disease in a minority of exposed persons, host defense defects that predispose these individuals to infection probably exist. Known host risk factors for RGM pulmonary disease include cystic fibrosis, chronic aspiration, immunodeficiency, and previous mycobacterial disease. However, risk factors have not been identified in recently reported cases [2], suggesting the existence of additional uncharacterized host defense defects.

Alpha-1-antitrypsin (AAT) is the most abundant inhibitor of endogenous serine proteases in the circulation [6]. Persons with normal AAT possess a

complement of two M-type AAT alleles and the corresponding protein product is designated the protease inhibitor (Pi) MM phenotype (PiMM or MM). An abnormal *AAT* gene may result in reduced AAT levels, dysfunctional AAT protein, or both. Severe AAT deficiency is associated with Z- or S-type AAT variants (e.g. ZZ or SZ) [7–11], and persons with Z or S AAT may develop precocious and severe emphysema due to unopposed host-derived serine protease activity. However, AAT heterozygotes with 1 normal M-type AAT protein and 1 anomalous AAT protein (e.g. PiMS or PiMZ) may also have compromised AAT function. Reports linking AAT heterozygosity to asthma, pulmonary fibrosis, emphysema, bronchiectasis, and decline in pulmonary function support this concept [12–14]. Recent studies show that AAT possesses antimicrobial and anti-inflammatory properties that may defend against bacterial infections in the lung [15–17]. Interestingly, AAT replacement therapy given to patients with AAT deficiency may reduce the risk of respiratory infections [18]. Since AAT may possess host defense function against pulmonary infection, we examined a cohort of patients with RGM lung disease for anomalous AAT variants and AAT deficiency. In companion *in vitro* studies, we quantified the effect of exogenous AAT and of a synthetic inhibitor of serine proteases in *M. abscessus* infection of human macrophages.

Materials and methods

Materials

Clinical grade AAT (Aralast®) was purchased from Baxter Corporation, Deerfield, IL. Ala-ala-pro-val-chloromethylketone (AAPV-CMK), a synthesized inhibitor of serine proteases, was purchased from Bachem, Torrance, CA. Human albumin was purchased from ZLB Bioplasma AG, Berne, Switzerland. Monocyte derived macrophage (MDM) culture medium consisted of RPMI-1640 medium (Cambrex Bioscience, Walkersville, MO) containing 10% (v/v) fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA). ADC Enrichment Broth was obtained from the Remel Company, Lenexa, KS.

Patient characteristics

All patients referred to the National Jewish Medical and Research Center (NJMRC) with RGM pulmonary disease between July 1996 and February 2003 were identified by retrospective record review. Age, gender, and race information was recorded. Diagnosis of RGM lung disease required the presence of

3 criteria: 1) more than 3 months of pulmonary or constitutional signs or symptoms including fever, sweats, fatigue, cough, or sputum production; 2) imaging studies showing bronchiectasis, centrilobular nodules, or cavities; and 3) two or more separate isolations of a RGM from sputum or bronchial alveolar lavage, or a single lung biopsy showing granulomas and growth of a RGM [4]. RGM species were identified using previously published criteria [19].

AAT phenotype and serum concentration

Serum AAT phenotype was determined using isoelectric focusing within vertical polyacrylamide gels. The degree of gel migration of AAT from subjects was compared to migration of control M, S, Z, B, C, F, G, and I AAT proteins that were run simultaneously. Subject AAT proteins were considered identical to co-migrating control AAT variants. Serum AAT levels were quantified using rate nephelometry (Beckman Coulter, Fullerton, CA).

M. abscessus preparation

M. abscessus was obtained from the American Type Culture Collection (Manassas, VA). The mycobacteria were grown to log phase at 37°C under agitation in 7H9 medium consisting of Middlebrook 7H9 broth (Fisher, Pittsburgh, PA) supplemented with 10% (v/v) ADC Enrichment Broth (resulting in 0.5% bovine serum albumin (BSA), 0.2% glucose, 0.087% NaCl, and 0.0004% catalase, 0.2% (v/v) glycerol, and 0.05% (v/v) Tween 80. The *M. abscessus* stock culture was stored at a concentration of 1.0 McFarland turbidity standard (10^8 bacilli/ml) at –80°C.

MDM infection with *M. abscessus*

The study design and consent procedure were approved by the National Jewish Medical and Research Center Institutional Review Board. Following Institutional Review Board approval and informed consent, venous blood from healthy, non-smoking volunteers was aspirated into syringes containing sodium heparin (50 units/ml blood final concentration, Becton Dickinson, Franklin Lakes, NJ). Peripheral blood mononuclear cells (PBMC) were isolated from blood by centrifugation over a cushion of Ficoll-Hypaque as previously described [20]. PBMC were suspended at 1×10^6 /ml in culture medium and 0.5 ml (5×10^5 PBMC) aliquots were added to wells in 24-well polystyrene tissue culture plates. PBMC monocytes were differentiated into MDMs by adherence for 5 to 7 d at 37°C, 5% CO₂.

In previous studies using this protocol, we determined that 10% of PBMC added to culture wells differentiate into MDM.

On the d of MDM infection, culture medium was aspirated from wells and replaced with fresh medium alone (control), with medium containing 5 mg/ml AAT, medium containing 50 μ M AAPV-CMK, or with medium containing 5 mg/ml albumin (0.5 ml final volume for each culture). After 3 h of incubation (all incubations were at 37°C, 5% CO₂), the RGM organism *M. abscessus* was added to the cultures at an estimated multiplicity of infection of 10 *M. abscessus* bacilli to 1 macrophage. After 1 h of incubation to allow *M. abscessus* to infect the MDMs, all medium with free bacteria was removed. D 0 cultures were lysed immediately to liberate and quantify cell-associated *M. abscessus*, while parallel cultures received fresh medium alone (no AAT, AAPV-CMK, or albumin) and were lysed after 4 or 8 d of incubation. Cultures were lysed by washing the cells twice with a 1:1 solution of phosphate buffered saline:RPMI with 10% FBS, adding 0.5 ml of 0.25% sodium dodecyl sulfate for 1 min, then adding 0.5 ml 7H9 medium. A lysate aliquot was obtained and subjected to 6 serial 10-fold dilutions in 7H9 medium. Five μ l of each lysate dilution was inoculated in triplicate onto 7H10 agar plates composed of reconstituted 7H10 Middlebrook base (Fisher) supplemented with 0.09% (w/v) N-Z Amine A from milk, 0.5% glycerol, 0.04% Tween-80, 0.5% BSA Fraction V, 0.2% glucose, 0.087% NaCl, and 0.0004% catalase (all from Sigma-Aldrich, St. Louis, MO). After incubating the agar plates at 37°C for 4 to 5 d, the number of *M. abscessus* colony-forming units (CFU) in the plates was quantified and the mean CFU calculated. This represented the number of *M. abscessus* organisms that infected the MDM cultures. The effect of AAT, AAPV-CMK, and albumin on MDM viability was assessed by adding 1:20 Trypan blue:deionized water into culture wells after 0, 4, or 8 d of incubation. Dye uptake was assessed microscopically.

Statistical analysis

This was a retrospective cohort study of all patients with RGM lung disease at a single institution in the time period 1996–2003. Outcomes include anomalous (non-MM) AAT phenotypes and serum AAT concentration. Results in our cohort were compared to US population estimates.

The prevalences of anomalous (non-MM) AAT phenotypes in the study cohort were compared to US population prevalences using a 1-sample proportion (*Z*) test. In MDM experiments, *M. abscessus* CFU in each AAT, AAPV-CMK, or albumin-con-

taining culture was expressed as a percent of *M. abscessus* CFU in the corresponding control culture (medium alone, set at 100%). A linear mixed model was constructed to fit percent *M. abscessus* CFU as a function of time. The final model included a random intercept term for subjects, and a first order autoregressive covariance structure was used to model the correlated responses within subjects over time. Modeling for percent *M. abscessus* CFU was performed after converting percent values to log_e values, and the results were converted back to percent data for presentation. These transformations resulted in data estimates comprising geometric means with asymmetric 95% confidence intervals (CIs). Data are graphed as means with 95% CIs or upper limit of the 95% CI where indicated. When noted, data are presented as mean \pm standard error of the mean (SEM). *p* < 0.05 was considered statistically significant.

Results

Patient characteristics

Between 1996 and 2003, a total of 100 patients with pulmonary RGM disease were evaluated at NJMRC. The mean ages of female and male subjects were 61 and 64 y, respectively, and 85 (85%) subjects were female. 93% were Caucasian, 6% were Asian, and 1% non-white Hispanic.

Microbiology

As shown in Table I, the most common RGM isolate was *M. abscessus*, which was the sole species isolated in 34 (34%) subjects. *M. abscessus* was isolated with 1 or 2 other RGM species in 30 (30%) subjects. *M. chelonae* was the sole isolate in 25 (25%) subjects, and *M. chelonae* was isolated with 1 or 2 additional RGM species in 26 (26%) subjects. *M. abscessus* or *M. chelonae* was isolated alone or with other mycobacteria in the vast majority (94, or

Table I. Mycobacterial isolates in 100 subjects with RGM lung disease.

Isolated RGM species	Number of subjects (<i>n</i> = 100)
<i>M. abscessus</i>	34
<i>M. chelonae</i>	25
<i>M. fortuitum</i>	6
<i>M. abscessus</i> and <i>M. chelonae</i>	22
<i>M. chelonae</i> and <i>M. fortuitum</i>	5
<i>M. abscessus</i> and <i>M. fortuitum</i>	4
<i>M. abscessus</i> , <i>M. chelonae</i> , and <i>M. fortuitum</i>	4

94%) of the cohort. *M. fortuitum* was the least abundant RGM, isolated alone in 6 (6%) cases, and with 1 or 2 other species in an additional 13 (13%) subjects.

Prevalence of AAT anomalies

73 (73%) subjects with RGM pulmonary disease had the normal MM phenotype, whereas 27 (27%: 95% CI 18%–36%) possessed anomalous non-MM AAT phenotypes (Table II). The non-MM AAT phenotypes included 16 MS, 4 MZ, 2 MF, 1 MC, 1 MB, 1 MG, 1 MI, and 1 ZZ. Since US population estimates document 17% prevalence for all non-MM AAT phenotypes [8], the prevalence of non-MM AAT phenotypes in our cohort with RGM lung disease is 1.6 times that of the US (Figure 1A, $p = 0.008$).

Z and S AAT proteins are most closely associated with AAT deficiency [7–11]. We therefore analyzed AAT phenotypes in our cohort that included Z or S AAT proteins. Z or S AAT was observed in 21 (21%: 95% CI 13%–29%) of our subjects, including MS ($n = 16$), MZ ($n = 4$), and ZZ ($n = 1$). Since these 3 AAT phenotypes occur in 8.7% of the US population [8], these phenotypes were 2.4 times more prevalent in our subjects with RGM lung disease (Figure 1B, $p < 0.0001$). Serum AAT concentrations were also measured in our cohort. The mean serum AAT concentration in the 27 subjects with anomalous AAT was 1.8 mg/ml, and only 3 of these 27 subjects had AAT serum concentrations below the normal range of 1.5 to 3.5 mg/ml.

Effect of exogenous AAT and AAPV-CMK on *M. abscessus* infection of MDM

RGM pulmonary disease was associated with anomalous (non-MM) AAT phenotypes in our cohort, suggesting a role for AAT activity in blocking RGM lung infection. To explore the relationship between AAT and infection, we assessed AAT and serine protease inhibition in MDM infected with RGM in

vitro. Since *M. abscessus* was the most prevalent RGM that infected our cohort (Table I), we used the same organism to infect MDM to enhance the clinical relevance of these studies. Figure 2 shows MDM infection in 3 separate experiments conducted in medium alone, which served as controls. The mean \pm SEM number of *M. abscessus* organisms (in million CFU) that infected the cultures at d 0, d 4, and d 8 were 0.37 ± 0.27 , 1.65 ± 1.19 , and 3.34 ± 2.06 , respectively. The number of *M. abscessus* organisms (CFU) in each control culture was set at 100% and the CFU in cultures exposed to AAT or AAPV-CMK were expressed as percent of controls. Figure 2 (open bars) shows the effect of AAT on MDM infection with *M. abscessus*. AAT reduced MDM infection in d 0 cultures to 55% (95% CI 38%–78%) of control cultures (set at 100% and represented as a stippled bar, $p < 0.05$). Furthermore, the same duration of AAT exposure inhibited MDM infection after 4 or 8 d of culture. D 4 MDM infection was reduced to 43% (95% CI 30%–62%) of d 4 control ($p < 0.05$), and d 8 MDM infection was reduced to 35% (95% CI 25%–51%) of d 8 control ($p < 0.001$).

Since AAT is a serine protease inhibitor [6], blockade of host serine proteases was a likely mechanism by which AAT reduced MDM infection. To independently assess the effect of host serine protease blockade on MDM infection, experiments were conducted using the synthetic serine protease inhibitor AAPV-CMK. As shown in Figure 2 (closed bars), AAPV-CMK reduced *M. abscessus* infection of MDM to a degree similar to that of AAT. For d 0, d 4, and d 8 cultures, MDM infection in the presence of AAPV-CMK was 48% (95% CI 29%–78%, $p < 0.05$), 37% (95% CI 22%–60%, $p < 0.05$), and 32% (95% CI 19%–52%, $p < 0.001$) of control infections, respectively. Exposing MDM to 5 mg/ml human serum albumin did not affect infection of MDM with *M. abscessus* at d 0, d 4, and d 8 (not shown). AAT, AAPV-CMK, and albumin did not affect MDM viability using Trypan blue vital dye analysis (viability $> 95\%$ in 3 separate experiments for each molecule, data not shown).

Table II. Anomalous (non-MM) AAT phenotypes in subjects with RGM lung disease.

AAT phenotype	Number (%) of subjects
MS	16 (16%)
MZ	4 (4%)
MF	2 (2%)
MC	1 (1%)
MB	1 (1%)
MG	1 (1%)
MI	1 (1%)
ZZ	1 (1%)

Discussion

We studied a cohort of 100 consecutive patients referred to NJMRC for RGM pulmonary disease. This cohort was predominantly Caucasian (83%) and female (85%), similar to previously described cohorts [21]. *M. abscessus* was the most common RGM species isolated, while *M. chelonae* was the second most common isolate.

The prevalence of anomalous (non-MM) AAT phenotypes is 17% in the US population [8]. Since anomalous AAT phenotypes were present in 27% of

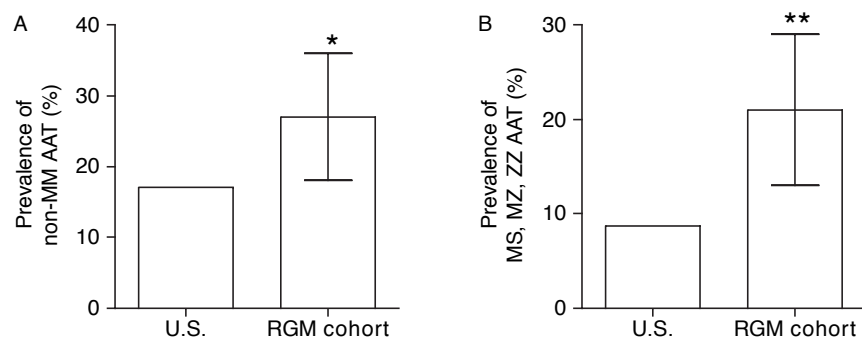


Figure 1. Prevalence of anomalous AAT phenotypes in subjects with RGM lung disease compared to the US population. In (A), the prevalence of all anomalous AAT phenotypes (non-MM AAT) in the cohort of persons with RGM lung disease (RGM cohort) is compared to the US population (US). In (B), the prevalence of MS, MZ, and ZZ AAT phenotypes (MS, MZ, ZZ AAT) is compared. RGM cohort data in (A) and (B) are shown as means with 95% CIs, $*p = 0.008$ and $**p < 0.0001$ compared to the US population using 1-sample proportion Z test.

our cohort with RGM-induced chronic lung disease, anomalous AAT was observed 1.6 times more frequently in our subjects than in the US population [8]. 26 of the 27 subjects in our cohort with anomalous AAT phenotypes were heterozygous for 1 M and 1 non-M AAT protein, and 1 subject was homozygous for 2 Z AAT proteins. If we restrict analysis to Z- or S-containing AAT phenotypes in

our cohort (MS, MZ, and ZZ), the prevalence was 2.4 times that of the US population. As shown in Figure 1, the cohort increase for MS, MZ, and ZZ phenotypes (2.4 times the US population) exceeded the cohort increase for all anomalous (non-MM) AAT phenotypes (1.6 times the US population). Since Z and S AAT proteins are most closely associated with AAT deficiency and pulmonary

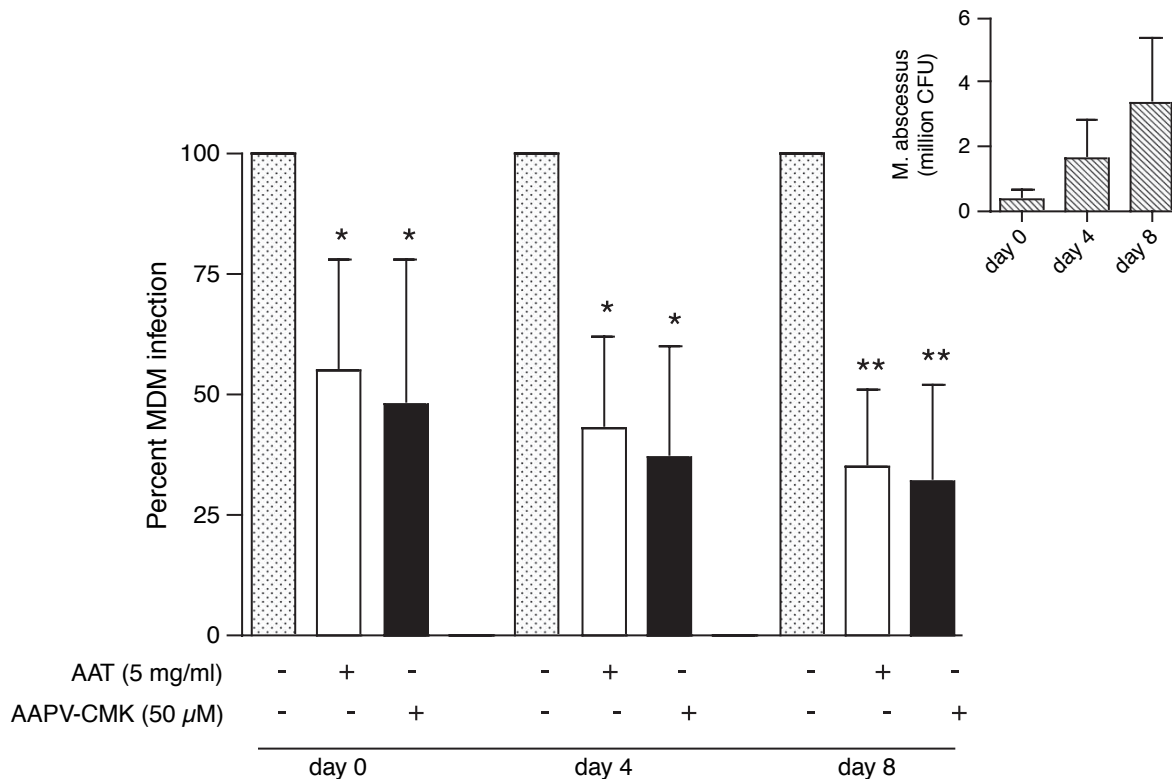


Figure 2. Effect of exogenous AAT and AAPV-CMK on *M. abscessus* infection of MDMs. The inset figure shows mean \pm SEM *M. abscessus* (million CFU) in d 0, d 4, and d 8 MDM control cultures conducted in medium alone. Mean \pm SEM million CFU on d 0, 4, and 8 were 0.37 ± 0.27 , 1.65 ± 1.19 , and 3.34 ± 2.06 , respectively. These control MDM infections were defined as 100%, and are represented in the larger figure as stippled bars. Percent MDM infection in the presence of AAT (open bars) or AAPV-CMK (closed bars) is shown in d 0, d 4, and d 8 cultures. Data are depicted as mean and upper limit of the 95% CI, as derived from the mixed model analysis. Data show experiments using cells from 3 different healthy donors. $*p < 0.05$, and $**p < 0.01$ compared to 100% in each time point using a mixed-effects modeling analysis.

disease, these observations suggest that RGM lung disease is coupled with more severe AAT anomalies. This observation leads us to speculate that enhanced AAT dysfunction is associated with an amplified risk for RGM lung disease. Confirming this conjecture will require further clinical investigation. No population estimates are available for the prevalences of heterozygous MF, MC, MB, MG, and MI AAT phenotypes (observed in 6 subjects in our cohort, Table II). Therefore, it is not possible to compare the prevalence of these specific cohort phenotypes to the US population.

The biological significance of heterozygous AAT phenotypes is poorly understood and may be underappreciated. Several studies suggest that persons heterozygous for AAT are at increased risk for pulmonary diseases [12–14], and this increased risk probably reflects reduced AAT serine protease inhibitor function. While most of our AAT heterozygous patients had normal serum AAT concentrations, the AAT anti-protease function of these anomalous proteins may be reduced.

Sustained RGM infection is initiated by phagocytosis of organisms, followed by an inability of phagocytes to eradicate intracellular RGM. This enables immune evasion due to RGM survival and replication within cells. We surmised that AAT protects healthy individuals from RGM lung disease by blocking infection of monocytic cells. Using an in vitro model of RGM infection, we showed that AAT significantly reduced *M. abscessus* infection of human MDM. This observation supports the concept that reduced AAT levels or defective AAT function enhances RGM phagocytosis and facilitates pulmonary disease. Interestingly, the AAT inhibition of *M. abscessus* infection in vitro was prolonged. Exposing MDM to AAT for 3 h prior to adding *M. abscessus* reduced MDM infection for the entire 8 d of culture (Figure 2). This occurred despite the absence of AAT in the cultures beyond d 0. For d 0, d 4, and d 8, AAT reduced *M. abscessus* infection of MDMs by means of 45%, 57% and 65%, respectively. Albumin did not affect *M. abscessus* MDM infection, indicating that AAT suppression of infection was specific. Since AAT is a prototype serine protease inhibitor, we independently tested the effect of serine protease inhibition in *M. abscessus* infection of MDMs. AAPV-CMK, a synthetic inhibitor of serine proteases, replicated the AAT inhibitory effect. AAPV-CMK inhibited infection in d 0, d 4, and d 8 cultures by means of 52%, 63%, and 68%, respectively (Figure 2). This suggests that the serine protease inhibitor function of AAT blocked *M. abscessus* infection.

These findings support the hypothesis that impaired host serine protease inhibitor activity due to

reduced or defective (anomalous) AAT predisposes persons to RGM pulmonary infection. Further epidemiological study is needed to confirm the generalizability of the association between RGM lung disease and AAT anomalies. Since AAT blocks RGM uptake by monocytes, our results suggest that AAT administered intravenously or by inhaled aerosol may be effective as a prophylactic agent or as a treatment in patients with RGM pulmonary disease. This approach to therapy, which blocks serine proteases of host origin, is probably unaffected by mycobacterial mutation. Therefore, such an approach to treatment may have the advantage of circumventing natural RGM resistance to antibiotics that target bacterial components [22]. The molecular mechanisms by which AAT inhibits RGM phagocytosis should be studied, and clinical investigation is required to determine whether exogenous AAT will benefit patients with RGM lung disease.

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