A Rhesus Macaque Model of Pulmonary Nontuberculous Mycobacterial Disease

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Abstract

In this study, we sought to develop a nonhuman primate model of pulmonary *Mycobacterium avium* complex (MAC) disease. Blood and bronchoalveolar lavage fluid were collected from three female rhesus macaques infected intrabronchially with escalating doses of *M. avium* subsp. *hominissuis*. Immunity was determined by measuring cytokine levels, lymphocyte proliferation, and antigen-specific responses. Disease progression was monitored clinically and microbiologically with serial thoracic radiographs, computed tomography scans, and quantitative mycobacterial cultures. The animal subjected to the highest inoculum showed evidence of chronic pulmonary MAC disease. Therefore, rhesus macaques could provide a robust model in which to investigate host–pathogen interactions during MAC infection.

Keywords: nontuberculous mycobacteria; *Mycobacterium avium*; macaque; T cells; granuloma

Clinical Relevance

In this study, our findings suggest that intrabronchial infection of immune-competent rhesus macaques with *Mycobacterium avium* is possible, as evidenced by radiographic findings and bacterial load in the animal infected with the highest dose. This article sets the stage for other researchers to further develop this model to study pulmonary nontuberculous mycobacteria (NTM) disease, which would greatly facilitate efforts aimed at understanding the mechanisms underlying the age-related increase and gender bias in incidence of NTM disease. This, in turn, will aid in the development of diagnostics, vaccines, and therapeutics.

Nontuberculous mycobacteria (NTM) are ubiquitous organisms that can cause chronic, progressive lung disease in humans (1). Although disseminated NTM disease is seen almost exclusively in immunosuppressed individuals, the vast majority of pulmonary NTM disease in the United States is due to *Mycobacterium avium* complex (MAC) and occurs in seemingly immunocompetent patients, disproportionately affecting females, older

persons, and those with bronchiectasis or chronic obstructive lung disease (2–7). Incidence rates are between 5/100,000 (general population) and 30/100,000 (>80 years old) patient-years, and are on the rise (6).

Given the ubiquitous MAC exposure, it is unclear why relatively few patients acquire NTM disease and why pulmonary NTM infections are indolent in some patients, and progress more rapidly in others (8). The

studies of NTM pathogenesis and host immunity to NTM have been hampered by the lack of a robust animal model that recapitulates the hallmark of human nondisseminated MAC pulmonary disease. Intratracheal or nasal inoculation of golden Syrian hamsters, immunocompetent C57BL/6 and BALB/c mice, and immunocompromised beige mice results in rapid dissemination from the lungs to other organs (9–12). Consequently, our

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understanding of the host immune response during NTM infection has largely been derived from the study of disseminated mycobacterial disease (13). In this study, we sought to develop a rhesus macaque model of pulmonary NTM disease by carrying out a dose escalation study of intrabronchial infection of *M. avium* 101. In addition to characterizing disease progression, we also determined the innate and adaptive immune response to acute NTM infection.

Materials and Methods

Bacteria and Bacterial Lysate

M. avium subsp. *hominissuis* strain 101 (MAC) was cultured in 7H9 broth (see the online supplement). A bacterial lysate was prepared by homogenization of approximately 10^9 /ml MAC in a bullet blender (Next Advance, Averill Park, NY), followed by centrifugation and filtration of the supernatant through a 0.2- μ m filter.

Animal Infection and Sample Collection

The use of nonhuman primates was approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee (protocol no. 0826). Three bilaterally oophorectomized female rhesus macaques (12–13 yr of age) were inoculated with 5 ml of MAC (6.8×10^8 , 10^7 , and 10^6 CFU) within the right caudal lobe via bronchoscopy. Blood and bronchoalveolar lavage (BAL) samples were collected (see the online supplement). Animals infected with the two higher doses were killed at 124 days postinoculation (dpi) after immune responses returned to baseline levels to determine any lung histological changes that were not detected by radiographic examination. Animals were killed in accordance with the guidelines of the American Veterinary Medical Association.

Luminex Analysis

Plasma and BAL supernatant were analyzed using a 29-plex nonhuman primate magnetic bead panel (LifeTech, Grand Island, NY).

Flow Cytometry

Innate immune cell populations were identified as described previously (14). To

measure T and B cell frequency and proliferation, peripheral blood mononuclear cells (PBMC) and BAL cells were first stained with anti-CD4 (Tonbo Biosciences, San Diego, CA), CD8 β (Beckman Coulter, Brea, CA), CD20 (BioLegend, San Diego, CA), IgD (Southern Biotech, Birmingham, AL), and CD27 (Tonbo Biosciences), then fixed and permeabilized before staining with anti-Ki67 (BD Pharmingen, San Diego, CA). To determine the frequency of MAC-specific T cells, 10^6 PBMC and BAL cells were stimulated with 10 μ l MAC lysate, anti-CD3 (positive control), or media alone (negative control) for 16 hours in the presence of brefeldin A. Cells were stained with anti-CD4 and CD8 β , then permeabilized and intracellularly stained with anti-IFN γ , TNF- α , and macrophage inflammatory protein-1 beta (MIP1 β) (15). Samples were acquired using the LSRII (BD Biosciences) and data analyzed using FlowJo (Treestar, Ashland, OR).

Assessment of Antibody Response

IgG antibody titers were measured by ELISA using plates coated overnight at 4°C with 100 μ l/well of a 1:200 dilution of MAC lysate (15).

Imaging

Animals were placed supine, two-view thoracic radiographs (anterior, lateral) were obtained every 2 weeks, and a chest computed tomography (CT) scan was made immediately before necropsy.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized and stained with hematoxylin and eosin, or anti-CD68 (monocytes), anti-CD20 (B-cells), or anti-CD3 (T cells) (Dako, Carpinteria, CA) (see the online supplement). 3,3'-Diaminobenzidine chromagen with hematoxylin counterstain (Vector, Burlingame, CA) was used to visualize staining. Images were obtained using an Axioplan microscope (Carl Zeiss, Jena, Germany) with a Spot Insight camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Microbiologic Assessments

BAL supernatant (100 μ l) and immune cells lysed with Triton X-100 were serially diluted, plated on 7H10 agar, and incubated

for 3 weeks at 37°C. Diseased lung tissue (0.2 g) was homogenized using a Mini-Beadbeater-1 (Biospec Products, Bartlesville, OK), digested with type IV collagenase, then serially diluted and plated onto 7H10 agar. Colonies were confirmed to be MAC by 16S rRNA and heat-shock protein 65 sequencing (see the online supplement).

Results

Increased Bacterial Burden in Lung and Opacity

Postmenopausal females are at an increased risk of obtaining pulmonary NTM (PNTM) disease (3, 5, 16). In addition, MAC is often first identified and limited within the right middle lobe or lingual in human disease (17). To ensure a successful recapitulation of PNTM in our animal model, three bilaterally oophorectomized female rhesus macaques (12–13 yr old) were inoculated with escalating doses of MAC in the right caudal lung. Only animal 22060, inoculated with the highest dose (6.8×10^8 CFU), developed disease, as evidenced by a focal opacity within the right caudal lobe at 14 dpi (Figure 1B). This infiltrate initially became more dense and then decreased in size and density with near resolution by necropsy (Figure 1C). MAC burden was also first detected in BAL supernatant 14 dpi (1.04^3 CFU/ml), reached a peak of 2.4^3 CFU/ml at 42 dpi, and then declined to a nadir at 70 dpi (Table 1). During the study period, the animal exhibited no fever or weight loss and an occasional unproductive cough. No viable bacteria were detected in PBMC homogenates collected at peak of infection, or from lymph nodes and spleen collected at necropsy from animal 22060.

Inflammation and Increased Frequency of Dendritic Cells and Monocytes after MAC Infection in BAL

To begin to characterize the host response to MAC, we measured levels of 29 cytokines, chemokines, and growth factors in BAL supernatant and plasma using a nonhuman primate-specific multiplex kit. Within BAL supernatant, only animal 22060 showed changes in soluble mediators. Specifically, increased levels of IL-6, IL-12, IFN γ , TNF- α , and MIP-1 β were detected 42 dpi

Figure 1. Increased bacterial burden induces robust cytokine levels and increased frequency of dendritic cells and monocytes in bronchoalveolar lavage (BAL). (A–C) Chest X-rays of animal 22060 that received 10^8 CFU at baseline (A), 14 (B), and 112 (C) days postinfection (dpi). (B) A box delineates lower right lobe opacity, suggestive of inflammatory process at 14 dpi. (D) Levels of IL-6, IL-12, IFN γ , TNF- α , and macrophage inflammatory protein-1 β (MIP1 β ; pg/ml) in BAL supernatant after *Mycobacterium avium* complex (MAC) infection in animal 22060 (infected with the highest dose). (E and F) Frequency of dendritic cells (E) and monocytes (F) in BAL was measured at the indicated time points using flow cytometry.

(Figure 1D), whereas levels of the remaining factors were either unchanged or below detection level (data not shown). Levels of IL-12, IFN γ , TNF- α , and MIP-1 β , either immediately (IL-12) or gradually (IFN γ , TNF- α , and MIP-1 β) returned to

baseline after 42 dpi, whereas levels of IL-6 continued to increase, peaking at 70 dpi and returning to baseline 112 dpi (Figure 1D). Despite the lack of disseminated infection, we detected changes in plasma soluble mediators only

in animal 22060. IL-6, IL-12, and IFN γ levels peaked at 28 (IL-6), 14 (IL-12), and 21 (IFN γ) dpi (data not shown). Given the observed changes in cytokines and chemokines, we next determined changes in frequency of dendritic cells (DCs) and monocytes in BAL, as recently described (14). In the BAL, the highest (22060) and middle (28493) dose animals showed an increase in DCs at 42 dpi, and all three animals showed changes in monocyte frequency at 42–56 dpi (Figures 1E and 1F).

Table 1. Bacterial Burden in Bronchoalveolar Lavage and Lung Tissue

Days after Infection	Animal 22060 6.8×10^8 CFU/ml	Animal 28493 6.8×10^7 CFU/ml	Animal 29152 6.8×10^6 CFU/ml
Bronchoalveolar lavage			
Baseline	0	0	0
14	1.0×10^3	0	0
28	1.5×10^2	0	0
42	2.4×10^3	0	0
56	1.5×10^2	0	0
70	1.0×10^1	0	0
84	0	0	0
112	0	0	0
124	0	0	0
Lung tissue			
124	8.8×10^1	0	0

MAC Infection Induces Robust T Cell and B Cell Response in the Lungs

To characterize the T cell response to MAC infection, we determined kinetics and magnitude of T cell proliferation in BAL and PBMCs by measuring number of Ki67⁺ cells using flow cytometry. We detected robust CD4 and CD8 T cell proliferation in

BAL starting at 14 dpi (Figures 2A and 2B). T cell proliferation peaked earlier in the two animals receiving the middle and low dose (14 dpi) compared with animal 22060 (28 dpi) (Figures 2A and 2B). T cell proliferation in the PBMCs occurred later (21–28 dpi) compared with BAL, and was comparable in all three animals (data not shown). Because not all T cell proliferation is antigen driven, we also measured frequency of antigen-specific T cells using intracellular staining.

As described for T cell proliferation, antigen-specific T cell responses were detected earlier (14 dpi) in the animals that were infected with the middle and low dose compared with the animal that received the highest dose (28 dpi) (Figures 2C and 2D). Overall CD8 T cell responses were lower than CD4 T cell responses (Figures 2C and 2D). Despite a robust T cell proliferation, we were unable to detect MAC-specific T cell responses in PBMCs (data not shown). In contrast to

T cells, B cell proliferation was only detected in PBMC at 21 dpi, and correlated with the detection of MAC-specific IgG, especially in high-dose animal 22060 (Figures 2E and 2F and data not shown).

Development of a Granulomatous Lesion in the Animal Infected with the Highest Dose

As described previously here for chest X-rays, only animal 22060 developed

Figure 2. MAC infection induces robust T cell and B cell response in the lungs. (A and B) T cell proliferative burst of CD4 (A) and CD8 (B) T cells in BAL after MAC infection was determined by measuring Ki67 expression using flow cytometry. (C and D) Frequency of MAC-specific CD4 and CD8 T cells were identified in BAL samples using intracellular cytokine staining for IFN γ (C) and TNF- α (D) after overnight stimulation with MAC lysate. (E) Proliferation of B cells in peripheral blood mononuclear cells was determined by measuring Ki67 expression using flow cytometry. (F) Antigen-specific IgG end-point titers in plasma were determined using ELISA.

Figure 3. High-dose infection of MAC results in the development of granuloma. (A) Computed tomography (CT) scan of animal 22060 reveals a granuloma, indicated by the box. (B) CT scan of animal 28493 shows no pathology. (C) Lesions in the lower right lobe (indicated by the box) and cross-section of the lower right lobe of animal 22060. (D) Lung and cross-section of the lower right lobe of animal 28493 with no overt pathology.

radiologic findings on CT scan characteristic of a granulomatous infection (Figure 3A). At necropsy, lung sections from animal 22060 showed extensive consolidation involving much of the caudal portion of the right caudal lobe, where the bacterial inoculum was deposited (Figure 3C), and necropsy tissue cultured from this area yielded 8.8×10^1 CFU/g of MAC (Table 1). In contrast, animal 28493, infected with the middle dose, showed no gross pathology (Figures 3B and 3D). Microscopic evaluation of the right caudal lung lobe from animal 22060 revealed characteristic granulomatous inflammation and histological evidence of bronchiectasis (Figure 4A). To better characterize the cellular infiltrate of animal 22060's granulomatous lesions, we performed immunohistochemistry staining using antibodies directed against CD68 (macrophages, Figure 4B), CD3 (T cells, Figure 4C), and CD20 (B cells, Figure 4D). The lung section from

animal 22060 revealed an abundant infiltration of macrophages (Figure 4B) that formed a central region bordered by T cells, which were frequently observed to be dispersed around macrophages and throughout the granuloma (Figure 4C). Aggregates of B cells were located in the outer layer of the granuloma (Figure 4D). In contrast, very few mononuclear cells were detected in lung sections from animal 28493 (Figures 4B–4D). In addition, no microscopic or microbiological evidence of dissemination to other tissues was detected in either animal, indicative of a pulmonary NTM infection.

Discussion

Determining the mechanisms underlying resistance and susceptibility to NTM infections has been hindered by the lack

of an animal model that faithfully recapitulates human PNTM disease. For the first time, we successfully established an isolated pulmonary infection in one of three inoculated rhesus macaques with characteristic clinical and radiologic features of human PNTM disease. Moreover, differences were observed in immune responses between the animal in which successful disease was established and in those that did not develop disease after inoculation.

Although all three animals used in this study showed an immune response, an infection dose of 6.8×10^8 was required for the presence of mild clinical symptoms. Interestingly, disease was limited to the right caudal lobe (site of initial infection), and did not disseminate to the left lobe or systemically. This recapitulates human disease, where MAC is often first identified and then remains limited within the right middle lobe or lingula for months or years without disseminating from the lung (17, 18), and sometimes resolving either spontaneously or during antibiotic therapy upon repeat imaging 3–6 months later (4). It is unclear if this animal would have spontaneously resolved this infection. At time of necropsy, the animal had improved microbiologically and radiologically, although it is possible that a low level of infection could have persisted with time, a situation akin to that observed in humans.

After infection, increased concentrations of IL-6, IL-12, IFN γ , TNF- α , and MIP-1 β in the BAL supernatant of animal 22060 at 42 dpi correlated with the highest bacterial burden in BAL supernatant. The augmented levels of IL-12, IFN γ , and TNF- α , coupled with the inability to detect T helper (Th) 2 cytokines (IL-4, IL-5, IL-10) or the Th17 cytokine IL-17 suggest that response to MAC is primarily Th1 in nature. The role of IL-12 and IFN γ in mediating a protective response to *M. avium* has been demonstrated in previous studies, where defects in these pathways were associated with an increased susceptibility to disseminated NTM infections in humans (19). The increased use of TNF- α receptor antagonist drugs in rheumatoid arthritis is associated with an increase in NTM infection, suggesting that TNF- α may also play a role in protection

Figure 4. Granuloma is composed of macrophages, T cells, and B cells. (A) Hematoxylin and eosin (H&E) staining of the right caudal lung sections of animals 28493 and 22060. The sections from 22060 include a granuloma. (B–D) Immunohistochemistry staining detected CD68 (B), CD3 (C), and CD20 (D) in adjacent sections. Scale bars, 500 μ m.

against NTM (20). The source of these cytokines in the BAL supernatant is still unknown. It is possible that they are produced by DCs and macrophages, the frequency of which within the bronchoalveolar space increased sharply 42 dpi.

The development of acquired immunity was evident by the development of MAC-specific T and B cell responses. Although robust CD4 and CD8 T cell proliferation was observed in the BAL

(14–28 dpi), CD4 T cell responses were, on average, larger than the observed CD8 T cells responses. Similarly, a depletion study of intranasally infected C57BL/10 by Saunders and colleagues (21) showed that CD4, but not CD8 T, cells are important for the production of IFN γ and clearance of bacterial burden. Studies in mouse models showed that increasing numbers of MAC fail to result in increased frequency of antigen-specific T cells (22). Although we observed delayed and decreased magnitude

of T cell responses with increasing inoculum dose, more animals will be needed to confirm these preliminary observations.

Previous studies demonstrated that mice pretreated with antibodies directed against mycobacterial antigen and infected with *M. tuberculosis* survived significantly longer and had reduced mycobacterial dissemination, as well as enhanced granulomatous response, in the lung compared with controls (23, 24). In humans, serologic responses correlate with disease, although it is unclear if they serve a protective role (25). MAC infection of nonhuman primates also induced B cell responses that seemed to correlate with inoculum dose.

Finally, gross pathology, CT scan, and microscopic evaluation revealed granulomatous lesions in the caudal right lobe of animal 22060. Immunohistochemistry showed massive infiltration, mostly by macrophages and T cells, which are the characteristic cellular components of granulomas after mycobacterial infection (26, 27). We observed a distinct pattern of distribution of macrophages, T cells, and B cells in which a central region of macrophages is interspersed with T cells and the next layer of granulomatous material contains aggregates of T and B cells (27).

In summary, our findings suggest that intrabronchial infection of immune-competent rhesus macaques with *M. avium* is possible, and that it can mimic human pulmonary nontuberculous *Mycobacterium* disease. The availability of pulmonary disease model would greatly facilitate efforts aimed at understanding the mechanisms underlying the age-related increase and sex bias in incidence of NTM disease, which, in turn, will aid in the development of diagnostics, vaccines, and therapeutics. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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