Virulence and Immune Response Induced by *Mycobacterium avium* Complex Strains in a Model of Progressive Pulmonary Tuberculosis and Subcutaneous Infection in BALB/c Mice

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The genus *Mycobacterium* comprises more than 150 species, including important pathogens for humans which cause major public health problems. The vast majority of efforts to understand the genus have been addressed in studies with *Mycobacterium tuberculosis*. The biological differentiation between *M. tuberculosis* and nontuberculous mycobacteria (NTM) is important because there are distinctions in the sources of infection, treatments, and the course of disease. Likewise, the importance of studying NTM is not only due to its clinical significance but also due to the mechanisms by which some species are pathogenic while others are not. *Mycobacterium avium* complex (MAC) is the most important group of NTM opportunistic pathogens, since it is the second largest medical complex in the genus after the *M. tuberculosis* complex. Here, we evaluated the virulence and immune response of *M. avium* subsp. *avium* and *Mycobacterium colombiense*, using experimental models of progressive pulmonary tuberculosis and subcutaneous infection in BALB/c mice. Mice infected intratracheally with a high dose of MAC strains showed high expression of tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase with rapid bacillus elimination and numerous granulomas, but without lung consolidation during late infection in coexistence with high expression of anti-inflammatory cytokines. In contrast, subcutaneous infection showed high production of the proinflammatory cytokines TNF-α and gamma interferon with relatively low production of anti-inflammatory cytokines such as interleukin-10 (IL-10) or IL-4, which efficiently eliminate the bacilli but maintain extensive inflammation and fibrosis. Thus, MAC infection evokes different immune and inflammatory responses depending on the MAC species and affected tissue.

Although the genus *Mycobacterium* was described over a century ago (1, 2), the main research focus has been on *Mycobacterium tuberculosis*, currently considered the most important human bacterial pathogen. Indeed, tuberculosis (TB) is the worldwide leading cause of death produced by bacterial disease and is one of the most important challenges to public health (3–5). However, there are a large number of species known as atypical or nontuberculous mycobacteria (NTM) (6), which include nearly 140 species (1, 2) and although they are not considered a public health problem, their importance is increasing due to their frequent association with immunosuppression, especially in HIV/AIDS patients, which is highly fatal (7–11). Diseases caused by NTM are known collectively as mycobacteriosis, and the symptoms include lung infection, lymphadenitis, soft tissue or skin lesions, and even disseminated disease (12).

The biological differentiation between *M. tuberculosis* and NTM is important because it implies fundamental differences in the source of infection, treatment, and the course of the disease. Likewise, the importance of studying NTM is not only because of its clinical relevance but also because of the involved mechanisms by which some species of the genus are pathogenic while others are not. Although the epidemiology of TB has been extensively studied, the incidence worldwide and the prevalence of mycobacteriosis remains poorly understood, partly due to the fact that NTM diseases are not usually reported to public health centers. Mycobacteriosis estimates are based on occasional laboratory isolates and, in most cases, are suspected of being caused by *M. tuberculosis* (13–15). In addition, because the vast majority of the NTM are naturally resistant to drugs used against *M. tuberculosis* (12), it is not unusual that they are being wrongly identified and reported as multidrug-resistant *M. tuberculosis* strains (16, 17).

*Mycobacterium avium* complex (MAC) contains clinically important NTM worldwide and is the second largest medical complex in the *Mycobacterium* genus after the *M. tuberculosis* complex. MAC strains are frequently isolated worldwide, and currently *M. tuberculosis* and MAC are the mycobacterial species that require the biggest efforts in care and treatment within the genus (12, 14, 18–20). MAC affects patients with chronic obstructive pulmonary disease, cystic fibrosis, and mainly immunosuppressed individuals with HIV/AIDS (21, 22). MAC is composed of a number of different serovars, strains, subspecies, and morphological forms that differ in virulence (21, 22, 27). MAC is considered the leading cause of highly fatal systemic bacterial infection that affects 40% of...
patients with HIV/AIDS and is the most common NTM pathogen group in the United States (12, 23). In most cases, it is not known which of the MAC species is the pathogen and, unlike the person-to-person transmission of *M. tuberculosis*, MAC transmission appears to occur from an environmental source (6, 24). *M. avium* is the most widely studied MAC species, is frequently isolated from drinking water, and could be the main source of infection for immunosuppressed individuals (25). Instead, pulmonary disease is the most common manifestation in immunocompetent individuals (26), whereas AIDS patients frequently present with a generalized infection (27).

*Mycobacterium colombiense* is a MAC species that was isolated from the sputum and blood of HIV/AIDS Colombian patients (28); this strain produced lymphadenopathy in immunocompetent children from France and Spain (29, 30) and was associated with pulmonary infections that complicated cases of cystic fibrosis (31). Therefore, this group of opportunistic pathogens have virulence mechanisms that allow them to adapt, survive, replicate, and produce disease in the host. However, the virulence and immune response evoked *in vivo* by members of these species has not been evaluated. The aim of the present study was to evaluate the virulence and immune response evoked by two MAC species (*M. avium* subsp. *avium* and *M. colombiense*) using experimental models of progressive pulmonary TB and subcutaneous infection in BALB/c mice.

**MATERIALS AND METHODS**

**Selection of study strains.** We used two different and well-characterized MAC species from the Spanish Type Culture Collection (CTC): *M. colombiense* CECT 3035 and *M. avium* subsp. *avium* CECT 7407. *M. tuberculosis* H37Rv (American Type Culture Collection [ATCC] 25618) was used as a control for comparison. Bacteria were grown in Middlebrook 7H9 broth (BD Difco) enriched with glycerol and albumin, catalase, and dextrose (Middlebrook ADC; BD Difco), in constant agitation at 37°C and 5% CO2 during 21 days for *M. tuberculosis* H37Rv and 15 days for MAC. The stock cultures were stored at −70°C in 50% glycerol until use.

**Experimental model of progressive pulmonary TB in BALB/c mice.** Virulence (as determined by survival, pulmonary histopathology, and bacterial load) and immune response induced by each isolate were evaluated in 8-week-old male BALB/c mice as previously described (32–35). Briefly, bacteria were grown as described above and, as soon as the culture reached the log phase, the bacilli were harvested, and the concentration was adjusted to 2.5 × 10⁷ viable bacilli per 100 μl of phosphate-buffered saline (PBS), as determined by fluorescein diacetate (Sigma-Aldrich) incorporation. Progressive pulmonary TB induction was performed as follows: mice were anesthetized with sevoflurane vapors and inoculated intratracheally using a sterile cannula (Thomas Scientific, catalog no. 1121A12, straight, 22G/11003), with 2.5 × 10⁷ bacilli in 100 μl of PBS.

Infected mice were kept in a vertical position until the effect of anesthesia passed. Two independent experiments were performed; in each experiment three groups of 50 mice were infected with either MAC species or *M. tuberculosis* H37Rv and 10 mice more from each group were left undisturbed to record survival from day 1 up to day 120 after infection. Six animals from each group were sacrificed by exsanguination at 1, 3, 7, 14, 21, 28, 60, and 120 days after infection. One lung lobe, right or left, was perfused with ethanol and prepared for histopathological studies. The other lobe and other samples (whole blood, mediastinal lymph nodes) were snap-frozen in liquid nitrogen and stored at −70°C for microbiological and immunological analyses. Infected mice were kept in cages fitted with microisolators connected to negative pressure. All procedures were performed in a class III cabinet in a biosafety level III facility according to the guidelines and approval by the Animal Experimentation Ethics Committee of the National Institute of Medical Sciences and Nutrition of Mexico.

**Experimental model of subcutaneous infection in BALB/c mice.** The experimental model was set up in 6- to 8-week-old male BALB/c mice. Bacteria were grown as described above and, as soon as the culture reached log phase, the bacilli were harvested, and the concentration was adjusted to 2.5 × 10⁷ viable bacilli per 100 μl of PBS, as determined by diacette of fluorescein incorporation (Sigma-Aldrich). As reported previously (36), groups of 50 animals were inoculated subcutaneously with 2.5 × 10⁷ bacilli suspended in 40 μl of PBS for each individual strain utilizing a sterile syringe and needle in each footpad, and 10 mice from each group were left undisturbed to record survival from day 1 up to day 120 after infection. Three animals from each group were sacrificed by exsanguination at 3, 14, 21, 28, 60, and 120 days after infection. One footpad, right or left, was prepared for histopathological studies, and the other footpad was snap-frozen in liquid nitrogen and stored at −70°C for microbiological and immunological analysis. Infected mice were kept in cages fitted with microisolators connected to negative pressure.

**Tissue preparation for histology and automated morphometry.** One lung lobe from each mouse was fixed by intratracheal perfusion with 10% formaldehyde dissolved in PBS for 24 h and then sectioned through the hilus and embedded in paraffin. Sections, 5 μm thick, were stained with hematoxylin and eosin for the histological and morphometric analysis using an automated image analyzer (Carl Zeiss, Ltd., Herts, United Kingdom) as previously described (33).

Regarding the subcutaneous tissue analysis, one footpad from each of the three mice per time point was obtained, immediately fixed by immersion in 10% formaldehyde dissolved in PBS during 24 h, and then sectioned longitudinally and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin and analyzed.

**Determination of CFU in infected tissues.** Right or left lung lobes, whole blood, mediastinal lymph nodes, and spleens from three mice at each time point and in two independent experiments were used for CFU counting. The tissues were homogenized with a Polytron (PT 3100-Laboratory homogenizer; Kinematica Dispensing and Mixing Technology) in sterile 50-ml tubes containing 1 ml of PBS (×1 Tween 80 (0.05%). Three dilutions of each homogenate were spread onto duplicate plates containing Middlebrook 7H10 agar (BD Difco) enriched with glycerol, albumin, oleic acid, dextrose, and catalase (Middlebrook OADC; BD Difco). Colonies were counted twice under a stereoscopic microscope (STEMI 2000 MICR-PA 085; Carl Zeiss) after 21 days of incubation for *M. tuberculosis* H37Rv and 15 days for MAC (33).

Three footpads, right or left, collected from infected and control mice at each time point were used for colony counting. Tissues were ground with a mortar and then homogenized with a Polytron; dilutions of each homogenate were spread onto duplicate plates containing Middlebrook 7H10 agar (BD Difco) enriched with glycerol, albumin, oleic acid, dextrose, and catalase (Middlebrook OADC; BD Difco). Colonies were counted twice under a stereoscopic microscope (STEMI 2000 MICR-PA 085; Carl Zeiss) after 21 days of incubation for *M. tuberculosis* H37Rv and 15 days for MAC (33).

**Kinetics of cytokines gene expression determined by real-time PCR in tissue homogenates.** Right or left lung lobes and footpads from three different mice per group were used to isolate total RNA using an RNeasy minikit (Qiagen Sample & Assay Technologies) according to the manufacturer’s recommendations. Total RNA quality and quantity were evaluated through spectrophotometry (using a 260/280 absorbance ratio) and on agarose gels. Reverse transcription was performed using Omniscript reverse transcriptase (Qiagen) from 100 ng of total RNA, 150 μg of Oligo(dt) 15 primer (Promega Corp.)/ml, 10 U of RNase inhibitor (Invitrogen/Life Technologies), 1× reverse transcriptase buffer, 0.5 mM concentrations of each deoxynucleoside triphosphate, and 4 U of Omniscript reverse transcriptase. Real-time PCR was performed using the 7500 real-time PCR system (Applied Biosystems) and a QuantiTect SYBR green PCR kit (Qiagen). Standard curves of quantified and diluted PCR product, as well as negative controls, were included in each PCR run. Specific primers were used for the following targets: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-CATTGTTGAAAGGTTGTCATG-3′ and 5′-GGAAGCCATGCGATGAGC-3′; tumor necrosis factor alpha (TNF-α), 5′-TGTTGCTGACCTCTACCC-3′ and 5′-GGCGAGAAA GGTGCTTG-3′; gamma interferon (IFN-γ), 5′-CCCTCACTGGCACAATACTCAT-3′ and 5′-GGTGACATGAAAATCTCCTGAC-3′, inducible
Virulence and Immune Response of MAC Strains in Mice

RESULTS

Survival, histopathology, and bacillary loads in mice infected by the intratracheal route. In order to characterize the *M. avium* and *M. colombiense* virulence in the model of pulmonary TB, groups of BALB/c mice were infected intratracheally with 2.5 × 10^5 bacilli of either MAC species or *M. tuberculosis* H37Rv strain as a comparative control. All of the animals infected with *M. avium* or *M. colombiense* survived after 4 months of infection. In contrast, mice inoculated with *M. tuberculosis* started to die at 8 weeks postinfection, and 50% survived after 120 days of infection (data not shown). These survival rates associated well with the CFU quantifications in lung homogenates where, by the third week postinfection, a lower bacterial load was found in mice infected with *M. colombiense* even though similar numbers of CFU had been detected in the three groups during the first week of infection (Fig. 1A). In comparison to animals infected with *M. tuberculosis*, at days 21, 28, and 60 postinfection, significantly lower bacterial loads were found in the lungs of mice infected with *M. colombiense*.

For digital automated morphometry, slides were scanned for each strain and tissue using an Aperio ScanScope (Aperio Technologies, Vista, CA). After saving each digital image, all of the lung area or the footpad inflammatory infiltrate were selected for analysis. Aperio ImageScope software (Aperio) was used with the application of the Aperio Pixel Count v9 algorithm, which is based on the spectral differentiation between brown (positive) and blue (counter) staining and provide a number of intensity positive pixels, a mean of intensity was determined from each slide that corresponded to the estimated concentration of each selected cytokine.

Statistical analysis. Two-way analysis of variance was used to determine the statistical significance of CFU and cytokines. A *P* value of <0.05 was considered significant.

Cytokine production determined by immunohistochemistry and digital automated morphometry. The same paraffin-embedded material prepared for histopathological studies was used to determine the local cytokine production by immunohistochemistry. Lung and footpad sections from mice infected with either MAC or *M. tuberculosis* H37Rv strain, obtained at early (day 21) and late (day 120) infection, were deparaffinized and maintained in 1× HCN buffer (HEPES, NaCl, and CaCl₂). Sections were washed with 1× HCN plus 0.05% Tween 20, and the endogenous peroxidase activity was blocked with 6% H₂O₂ dissolved in 1× PBS plus 0.1% sodium azide, followed by incubation for 1 h. After blocking with normal swine serum, tissue sections were incubated with primary antibodies overnight at 4°C at optimal dilutions, which had been determined previously. We used primary antibodies against TNF-α (rabbit polyclonal IgG clone H-156; Santa Cruz Biotechnology), IFN-γ (goat polyclonal IgG clone D-17; Santa Cruz Biotechnology), IL-4 (goat polyclonal IgG; Santa Cruz Biotechnology), and IL-10 (goat polyclonal IgG clone M-18; Santa Cruz Biotechnology). Secondary biotinylated antibodies (biotin–anti-rabbit IgG or biotin–anti-goat IgG) were used to detect the binding of the primary antibodies. Finally, horseradish peroxidase-conjugated avidin and 3,3-diaminobenzidine–hydrogen peroxide were used to develop the reaction. Tissue sections were counterstained with hematoxylin.

FIG 1 Lung bacillus loads in BALB/c mice infected by intratracheal injection. Mice were infected with *M. colombiense* (grey), *M. avium* (white), or *M. tuberculosis* strain H37Rv (black) and euthanized at different time points after infection, and the indicated organs were used to determine the number of CFU. Asterisks represent statistical significance (*P* < 0.005) comparing MAC strains with *M. tuberculosis* H37Rv.

A. Lung

B. Blood

C. Spleen

D. Mediastinal lymph nodes
or *M. avium*, being fewer in the former, while at day 120 almost undetectable live bacilli were observed in animals infected with either MAC species. In contrast, a progressive increase of bacillus loads in the lung was seen in mice infected with *M. tuberculosis*, raising its peak at day 120 postinfection (Fig. 1A). In blood, after 3 days of infection, mice infected with either MAC species showed a progressive increase of the bacillus load for 2 weeks, followed by a transient decrease by days 21 and 28 postinfection and a new increase at day 60 postinfection. At almost all of these time points, the bacillus loads in blood from mice infected with *M. avium* or *M. colombiens* were significantly higher than in mice infected with *M. tuberculosis* (Fig. 1B). A similar trend was observed in spleens and mediastinal lymph nodes (Fig. 1C and D, respectively), where, after the first week during late infection at days 60 and 120, higher bacillus loads were determined in animals infected with MAC strains than with *M. tuberculosis*.

The histopathological analysis of the lungs showed after 2 and 3 weeks of infection with MAC, extensive inflammatory infiltrate constituted by lymphocytes and macrophages located in the alveolar-capillary interstitium, as well as around venules and bronchioles (Fig. 2A and B). At this point during the infection, larger granulomas (12,703 ± 1,000 μm²) were formed than those produced by *M. tuberculosis* (4,025 ± 670 μm²); 2 weeks later, granulomas induced by MAC (9,215 ± 435 μm²) were similar in size to those in the lungs of *M. tuberculosis*-infected mice (9,963 ± 650 μm²). During late infection, granulomas progressively declined in number and size, being very few in number and small at day 120 postinfection with either MAC strain (2,844 ± 320 μm²). It is important to emphasize that there was little pneumonia in MAC-infected mice (<5%) and that the inflammatory response decreased substantially after 1 month of infection, with only occasional cuffs of lymphocytes around blood vessels and mild hyperplasia of the lymphoid tissue associated with bronchial epithelium observed at day 120 postinfection (Fig. 2D). As shown in Fig. 2D and E, at day 120 there was almost normal histological appearance in the lung of mice infected with *M. colombien* and *M. avium*. In contrast, *M. tuberculosis*-infected mice showed progressive pneumonia after 28 days postinfection, reaching its peak at day 120 when 80 ± 10% of the lung surface was affected (Fig. 2F).

**Cytokine gene expression and production in the lungs of infected mice.** TNF-α gene expression in mice infected with *M. tuberculosis* was rapid and higher during early infection and was followed by a progressive decrease during late disease (Fig. 3A). In contrast, whereas infection with *M. colombiens* induced the highest expression of TNF-α during early infection at days 3 and 7, and this peak was followed by a progressive decrease, in mice infected with *M. avium* TNF-α showed progressive expression peaking at day 120 (Fig. 3A). Similar kinetics were observed in iNOS gene expression (Fig. 3B). The kinetics of IFN-γ gene expression were similar among the three groups, with higher expression during early infection peaking at day 14, followed by progressive decrease, being higher in mice infected with *M. colombien* strain (Fig. 3C).

Regarding the expression of anti-inflammatory cytokines, mice infected with *M. colombien* showed the highest TGF-β expression from days 3 to 21, whereas animals infected with *M. avium* exhibited the highest TGF-β expression at days 28 and 60, and both MAC species induced higher TGF-β expression than did *M. tuberculosis* (Fig. 3D). *M. tuberculosis* infection induced progressive IL-4 expression, whereas *M. colombien* induced lower and stable IL-4 expression, and animals infected with *M. avium* exhibited higher IL-4 expression during early infection (Fig. 3E). During the first week of infection, *M. colombien* induced high expression of IL-10, whereas *M. avium* and *M. tuberculosis* induced...
similar mild expression during late infection at day 120, and both MAC species induced higher expression of IL-10 (Fig. 3F).

Considering that the cytokine gene expression determined by real-time reverse transcription-PCR cannot inform the cellular source and since total tissue homogenates used to isolate RNA may not reflect protein levels, we performed immunohistochemistry and digital quantitative image analysis of lung sections comparing early infection, specifically examining when protective immunity against *M. tuberculosis* is maximal in this murine model (day 21) and for late disease (day 60) (37). Activated macrophages were the principal source of TNF-α, being 2-fold higher after 21 days of infection in animals infected with *M. tuberculosis* than with either MAC strain, whereas similar low TNF-α production was seen at day 120 postinfection (Fig. 4). At day 21 postinfection, a trend similar to that seen with TNF-α was observed for IFN-γ production, with lymphocytes being the most common immunostained cells, while at day 60 of infection mice infected with *M. tuberculosis* showed lower IFN-γ production than at day 21, but it was still significantly higher than in mice infected with either MAC strain. Regarding anti-inflammatory cytokines, low production of both IL-10 and IL-4 was determined at day 21 of infection with either MAC strain or *M. tuberculosis*, while at day 60 the lungs of mice infected with *M. tuberculosis* showed 2-fold more IL-10 and IL-4 production than in mice infected with MAC, with lymphocytes being the predominant immunostained cells located in the pneumonic areas in *M. tuberculosis* infection and in perivascular inflammation in MAC-infected animals (Fig. 4).

Survival, histopathology, and bacillary loads in mice infected subcutaneously. Although it is not common, *M. avium* may penetrate the subcutaneous tissue following traumatic inoculation through the skin (37, 38). To characterize the *M. avium* and *M. colombiense* virulence in subcutaneous tissue infection, groups of BALB/c mice (60 per group) were infected in both footpads with either MAC strains or *M. tuberculosis* H37Rv as comparative control. All of the animals survived after 4 months of infection (data not shown).

Both NTM strains showed progressive increases in the bacillus burden, reaching a peak at day 28 that was higher than in the *M. avium* infection; this peak was followed by a pronounced decrease until day 120, when the lowest level was detected (Fig. 5A). In contrast, animals infected with *M. tuberculosis* showed bacillus burdens characterized by a progressive decrease throughout the time points measured, and these burdens were consistently lower than in mice infected with either MAC strain (Fig. 5A). In the regional lymph nodes (inguinal) at any time point measured—except at day 28 postinfection—the bacillus loads were higher in animals infected with MAC than in mice infected with *M. tuberculosis* strain H37Rv. Late during infection, at days 60 and 120, almost 5-fold more CFU were counted in MAC-infected mice than in *M. tuberculosis*-infected mice, the counts being highest in mice infected with *M. avium* (Fig. 5B).

Extensive and progressive chronic inflammatory infiltrate was seen in the footpad subcutaneous tissue of mice infected with MAC strains at day 60 (data not shown) and 120 when some...
animals showed focal areas of necrosis and fibrosis, with numerous lymphocytes infiltrating not only the connective tissue but also the muscle and adipose tissues (Fig. 5C and D). In contrast, since the third day of infection, M. tuberculosis induced mild inflammation that consisted of lymphocytes and macrophages spread in the connective tissue, with occasional granulomas seen after 28 days (data not shown). However, the infection did not spread beyond the limb with any of the mycobacterial strains studied. As shown in Fig. 5E, at day 120 of M. tuberculosis infection, scarce chronic inflammatory infiltrate in the connective tissue and around the blood vessels was observed.

**Cytokine gene expression and production in the subcutaneous tissues of infected mice.** The expression of the proinflammatory cytokines TNF-α and IFN-γ in the subcutaneous tissues of mice infected with M. colombiense was rapid, stable, and highest throughout the course of infection; iNOS exhibited similar kinetics but with fewer transcripts. Subcutaneous infection with M. avium induced low and stable expression of TNF-α, iNOS, and
IFN-γ during early infection, similarly to *M. colombiense* during late infection at days 60 and 120 (Fig. 6A to C). Animals subcutaneously infected with *M. tuberculosis* exhibited the lowest levels of transcripts encoding TNF-α and iNOS proinflammatory factors (Fig. 6A and B). Although the levels of transcripts for IFN-γ in the footpads of mice infected with the three strains were rather similar throughout the first month, late in the infection (day 120) the levels were higher in footpads infected with *M. tuberculosis* than in those infected with MAC (Fig. 6C). With regard to the levels of transcripts encoding anti-inflammatory cytokines, during the first month of infection there was similar low expression in animals infected with either strain. At days 60 and 120, there was higher expression, with the levels being similar in the case of TGF-β, and animals infected with *M. avium* showed the highest IL-10 expression but relatively low numbers of transcripts and similar higher levels for IL-4 in MAC infections (Fig. 6).

Quantitative immunohistochemistry analysis showed at day 21 postinfection a similar high expression of TNF-α production in mice infected with *M. tuberculosis* or either MAC strain, with macrophages being the most commonly immunostained cells, while at day 60 of infection animals infected with *M. tuberculosis* showed significantly lower TNF-α production than did MAC-infected mice, the highest levels being detected in *M. avium*-infected footpads (Fig. 7). Animals infected with *M. avium* showed the highest production of IFN-γ at days 21 and 60 of infection, while *M. colombiense* induced similarly high production: 2-fold higher than *M. tuberculosis* at day 60 of infection (Fig. 7). Regarding anti-inflammatory cytokines, at day 21, higher production of IL-10 was determined in the footpads of mice infected with *M. tuberculosis* or *M. avium* than in the footpads of mice infected with *M. colombiense*, while at day 120 postinfection, similar low production levels were observed in the footpads of MAC- or *M. tuberculosis*-infected animals. A comparable trend was observed for IL-4 production (Fig. 7).

**DISCUSSION**

The *Mycobacterium avium* complex (MAC) consists of nine recognized species and a variety of strains that may be members of undescribed taxa (12, 28, 38–41). MAC can induce four distinct clinical syndromes: pulmonary disease, lymphadenitis, disseminated disease, and skin disease (12). Human infection by MAC is believed to be initiated by the respiratory airways or the intestinal tract. We used intratracheal infection with a high bacillus dose in BALB/c mice in order to reproduce one of the most common infection routes in humans and compared two MAC species and *M. tuberculosis* H37Rv, which has been extensively studied in this mouse model (32, 33, 35, 37). When BALB/c mice are infected with *M. tuberculosis* H37Rv, a 7’ helper cell type 1 response is developed that peaks at 2 to 3 weeks, temporarily controlling bacterial growth (33). After this control, bacterial proliferation re-commences, accompanied by increasing anti-inflammatory cytokine expression, such as TGF-β, IL-10, and IL-4, and decreasing IFN-γ, TNF-α, and iNOS expression, along with extensive tissue damage and death of the animals (33, 37). Both MAC species induced different disease evolution with total mice survival and scarce pneumonia (<5%) without necrosis or fibrosis. However, both MAC strains induced moderate inflammatory infiltrate around middle-size blood vessels and airways during the first month of infection, as well as granulomas. During the late stage of the infection, the inflammation was almost totally cleared. In addition, mild perivascular inflammatory cuffs and hyperplasia of the lymphoid tissue associated with bronchial mucosa with some intra-alveolar activated macrophages were seen. MAC-infected mice showed an increase in CFU during the first 2 weeks of infection, followed by a progressive decline that was almost undetectable after 4 months of infection. Although the bacillus load kinetics were similar, mice infected with *M. avium* had higher bacterial burdens than animals infected with *M. colombiense*, indicating a...
higher virulence of the former, but both MAC strains were clearly attenuated compared to *M. tuberculosis* H37Rv. It is interesting that in spite of its attenuation, higher bacillus loads were observed in the blood of mice infected with either MAC species than in *M. tuberculosis*-infected animals. During the late stage of the infection with MAC, there were also higher bacillus loads in the spleen and mediastinal lymph nodes. It appears that MAC strains disseminate more efficiently than *M. tuberculosis* H37Rv and, perhaps due to this ability, systemic MAC infections are common in immunodeficient patients.

The cytokine expression profiles in infected lungs were quite different. Intratracheal infection with *M. colombiense* induced rapid and very high expression of TNF-α during the first and second weeks of infection, followed by a progressive decrease. This TNF-α expression was highest at days 3 and 7 postinfection and exhibited a trend similar to that of iNOS expression. In contrast, *M. avium* induced low and stable TNF-α expression during the first month of infection, followed by high expression during late infection, peaking at day 120. TNF-α production in *M. avium* determined by immunohistochemistry, however, was similar to *M. colombiense*. The iNOS gene expression kinetics were similar to those of TNF-α, suggesting that nitric oxide may participate in the control of bacillus growth.

Adaptive immunity to *M. avium* is centered on CD4+ T cells and IFN-γ production. Depletion studies have shown that CD4+ but not CD8+ T lymphocytes are required for adaptive immunity against *M. avium* (45). Antibody blocking of IFN-γ exacerbates *M. avium* infection (45), and mice deficient in the expression of this cytokine are more susceptible to the infection (46, 47). Our results showed that the lungs of animals infected with *M. avium* displayed IFN-γ expression kinetics and protein production similar to that of mice infected with *M. tuberculosis*, exhibiting higher expression during early infection, followed by a progressive decrease. *M. colombiense* induced slightly higher IFN-γ production in the earlier stage of the infection rather than later, suggesting that this strain is more efficient at maintaining Th1 responses than does *M. avium*, an observation that could indicate a higher immunogenicity and lower virulence for *M. colombiense* than for *M. avium*. What was not expected was high IFN expression throughout the course of the infection in BALB/c mice infected with *M. tuberculosis*. The reason for this finding is unknown. An uninfected control group was not included in the present study that would have allowed us to evaluate this result.

The type 2 response mediated by IL-4, IL-13, IL-10, and TGF-β does not seem to have a significant role in determining susceptibility to infection (48). *M. avium* infection in C57BL mice does show that nitric oxide is relatively ineffective in *M. avium* removal, since most strains are not susceptible to its toxic effects (44), our results showed that iNOS gene expression kinetics are similar to those of TNF-α, suggesting that nitric oxide may participate in the control of bacillus growth.

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![Graph showing cytokine expression kinetics](image-url)
not induce detectable IL-4 responses, and antibody or genetic depletion of IL-10 have little or no impact on the course of *M. avium* infection (49). Although it was reported that disease was more severe in transgenic mice that overproduce human IL-10 (50), our results suggest that these cytokines should participate in clearing inflammation during the late stage of the infection when MAC bacillus loads are very low. In consequence, this high expression will not have a detrimental effect on bacillus growth control due to their antagonistic effect on Th1 cells. This coincides with previous observations in BALB/c mice that did not develop granuloma necrosis after intravenous infection with virulent *M. avium* ATCC 25291, and this mouse phenotype was reverted after the genetic disruption of the IL-10 gene (51). In addition, in response to the stress caused by the immune response and hypoxia, the majority
of mycobacteria are thought to enter a dormant state in human TB; however, it is not known whether NTM, as MAC members, can develop this state during pulmonary infection. Detection of culturable \textit{M. tuberculosis} from latently infected individuals is extremely difficult. Our results in this model of pulmonary infection in BALB/c mice suggest that the MAC species used here (\textit{M. avium} and \textit{M. colombiense}) developed a viable but not culturable state (52) with mild levels of pro- and anti-inflammatory cytokines (TNF-$\alpha$ versus IL-10) characterized by minimal pulmonary inflammation and very low bacillus burdens. Additional experiments are needed to investigate this hypothesis.

MAC can also enter the host by the intradermal route through cuts and skin punctures (53, 54). Experimental work showed that intradermal bacillus administration resulted in infection of cervical and/or axillary lymph nodes in both BALB/c and nude mice, suggesting that skin lesions may also be responsible for the cervical lymphadenitis seen in humans (53). Our results confirmed and extended these findings by the demonstration that subcutaneous infection in the footpads of BALB/c mice induced local bacillus growth. This growth was efficiently controlled after 4 months of infection and was able to disseminate to local lymph nodes (inguinal) and shown to be higher in mice infected with \textit{M. avium}. One significant difference with pulmonary infection despite low bacillus loads was the extensive and constant inflammation with fibrosis produced by MAC infection in the subcutaneous tissue. This tissue response corresponds with the high and stable expression and production of TNF-$\alpha$ and IFN-$\gamma$ during the later stage of infection with MAC along with the production of relatively low anti-inflammatory cytokines. Although MAC induced a high expression of TNF-$\alpha$ and IFN-$\gamma$ during late subcutaneous infection, iNOS expression was slightly higher. Nitric oxide is an inhibitory factor in the production of fibrosis, as shown in iNOS-deficient mice infected with mycobacteria that developed more fibrosis (55). Thus, the fibrosis observed in the subcutaneous tissue during the late stage of the MAC infection in BALB/c mice could be influenced by the low expression of iNOS.

Few mycobacterial species, most of which are pathogenic for humans, produce a unique array of complex cell wall-associated lipids, such as phthiocerol dimycocerosates (PDIMs) and phenolglycolipids (PGLs), two groups of molecules shown to be important virulence factors (56, 57). Experimental studies demonstrated that \textit{M. tuberculosis} strains deficient in the production or surface localization of PDIMs are markedly attenuated for growth in the lungs of intravenously or intranasally infected mice (58–60), and both PDIMs and PGLs are required for full virulence of \textit{M. marinum} in the zebrafish model (61). Members of \textit{M. tuberculosis} complex also produce p-hydroxybenzoic acid derivatives (p-HBADs), which are precursors of PGL biosynthesis (62). Mutants of \textit{M. tuberculosis} that lack production of some or all forms of p-HBADs were shown to induce histological differences in lung tissue of infected BALB/c mice with extensive and diffuse inflammation (63). These \textit{M. tuberculosis} mutants also induce an increased secretion of the proinflammatory cytokines TNF-$\alpha$, IL-6, and IL-12 compared to the \textit{M. tuberculosis} strain Mt103 (63). These studies could indicate that while PGL and PDIM are associated with virulence, p-HBAD is associated with tissue damage. The biosynthesis of PDIM/PGL/p-HBAD is a very complex pathway that involves more than 15 enzymatic steps and more than 27 genes, most clustered on a 70-kb region of the chromosome (56, 64), and MAC members lack several important genes necessary for the biosynthesis of these lipid molecules (65–67; see also the Virulence Factors of Pathogenic Bacteria database [http://www.mgc.ac.cn/cgi-bin/VFs/compvfs.cgi?Genus=Mycobacterium]) (Fig. 8). We hypothesized...
that MAC members and *M. tuberculosis* have different pathogenicities and trigger different immune responses and inflammation as a result of differences in their cell envelopes. Thus, in the murine model of infection with MAC strains, the low virulence could be associated with the lack of production of PDIMs and/or PGLs, and the high chronic inflammation could be associated with the lack of production of p-HBADs.

In conclusion, MAC strains differ in their level of virulence and type of immune response. *M. colombiense* and *M. avium* demonstrate low virulence in BALB/c mice infected by the intratracheal route. Infection in subcutaneous tissue by either MAC strain was also efficiently controlled, but they each induced high expression of proinflammatory cytokines during the later stage of the infection and relatively low production of anti-inflammatory cytokines, producing extensive and constant inflammation. In addition to the mycobacterial antigenic constitution and the genetic background of mice, the site of infection is important in the type of evoked immune response.

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