CD4⁺ T Cells but Not CD8⁺ or γδ⁺ Lymphocytes Are Required for Host Protection against Mycobacterium avium Infection and Dissemination through the Intestinal Route

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Disseminated Mycobacterium avium infection is common in AIDS patients that do not receive anti-AIDS therapy and in patients for whom therapy fails. M. avium is commonly acquired by ingestion, and a large number of AIDS patients have M. avium in their intestinal tracts. To better understand the dynamics of the infection in patients with AIDS, we studied orally infected mice. To determine if immunocompetent mice challenged orally with M. avium can develop protection against the infection, and if so, which cell population(s) is responsible for the protection, we exposed wild-type as well as CD4⁺/−, CD8⁺/−, and γδ⁺/− knockout mice to low concentrations of M. avium strain 101 given orally, followed by treatment with azithromycin. After 1 month, the mice were challenged with kanamycin-resistant M. avium 104. Only CD4⁺ T cells appeared to be required for protection against the second challenge. Both CD4⁺ and CD8⁺ T cells produced comparable amounts of gamma interferon after the first exposure to the bacterium. Tumor necrosis factor alpha was elevated in CD4⁺ T cells but not in CD8⁺ T cells. Following exposure to a small inoculum of mycobacteria orally, wild-type mice did not develop disseminated infection for approximately 4 months, although viable bacteria could be observed in the mesenteric lymph nodes. The ingestion of small numbers of M. avium cells induces a protective immune response in the intestines against subsequent infection. However, the bacteria remain viable in intestinal lymph nodes and might disseminate later.

Disseminated infections caused by Mycobacterium avium are commonly identified in patients with AIDS that do not receive modern anti-human immunodeficiency virus therapy, as well as in those for whom therapy fails (15, 21). Although initially M. avium was assumed to be acquired by the respiratory tract, the advent of the AIDS epidemic raised our appreciation of the gastrointestinal tract as a main route of M. avium infection for this group of individuals (12, 22).

M. avium is an environmental organism that can be isolated from water and soil (15, 21). A large percentage of the population of patients admitted to hospitals for diverse reasons have serum antibodies against M. avium, suggesting that those individuals were previously exposed to the bacterium (7, 18), most likely through the intestinal tract.

The reason that healthy individuals, in contrast to AIDS patients, do not develop disseminated M. avium infections despite the widespread presence of the bacterium in the environment has not been determined. A number of studies (mainly with mice) have shown that the CD4⁺ T cells have a major role in the specific host defense against M. avium (1, 25), supporting the idea that the absence of CD4⁺ T lymphocytes in patients with AIDS is the main reason for the infection. In addition, using both in vitro and in vivo systems, it has been shown that natural killer (NK) cells participate in the innate response against the pathogen (3, 8). It has been demonstrated that AIDS patients have quantitative and qualitative deficiencies in NK cells (10). In contrast, the protective role of CD8⁺ T lymphocytes has not been established for a mouse model by the use of both specific cytotoxic antibodies against the T-cell population and knockout mice (19, 28, 32). Despite the fact that γδ⁺ T cells have been suggested to be involved in the defense of the respiratory and intestinal mucosae, studies have also failed to show a role for γδ⁺ T lymphocytes in mice infected with M. avium by the respiratory route (33). In vitro studies, however, suggested that γδ⁺ T cells can be cytotoxic to M. avium-infected macrophages (4). Discovering whether γδ⁺ T cells have any protective role in mice will require future studies using alternative designs.

Since a large majority of healthy individuals have contact with M. avium present in the environment without developing disease, it can be assumed that an effective host response must exist. We hypothesized that constant contact with small numbers of M. avium cells can induce a protective host defense by stimulating a specific T-cell response. It is also not known if M. avium acquired by immunocompetent hosts is killed or persists in a latent form in host cells, in a comparable manner to Mycobacterium tuberculosis, which remains viable for many years in thoracic lymph nodes (14). Observations of the AIDS population indicate that not all individuals with disseminated M. avium disease have the bacterium colonizing the intestinal tract (11). Although culture techniques are still suboptimum, it is possible that in a percentage of the population, the reactivation of the M. avium infection occurs from an internal source such as mesenteric lymph nodes. We therefore evaluated the individual roles of CD4⁺ T cells, CD8⁺ T cells, and γδ⁺ T cells

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in the host defense against *M. avium* acquired by the gastrointestinal route, and we attempted to identify the fate of the bacteria and the route of dissemination in the immunocompetent host.

**MATERIALS AND METHODS**

**Mycobacteria.** *M. avium* strain 101 and *M. avium* strain 104 are clinical isolates from the blood of AIDS patients. MAC 104, which is naturally resistant to kanamycin (MAC 104 Km²), was selected by plating 10⁶ *M. avium* 104 cells on 7H11 agar containing 200 μg/ml of kanamycin. *M. avium* 101 is susceptible to 200 μg/ml of kanamycin at an inoculum of 10⁶ bacteria. The resistant phenotype was determined to be stable (data not shown). In preliminary experiments, the strain was shown to be equally virulent in both human and murine macrophages (data not shown). For our experiments, bacteria were grown in Middlebrook 7H11 agar (Difco Laboratories, Detroit, Mich.) supplemented with oleic acid-albumin-dextrose acid-catalase (Difco, Detroit, Mich.) and used after 2 weeks of quarantine.

**Infection.** 7H11L6 mice (6 to 8 weeks old, approximately 20 g) were given *M. avium* 101 (100 organisms) by gavage on day 1 and day 8. The mice were then treated with azithromycin (200 mg/kg of body weight/day) orally from day 14 to day 28. On day 35, some mice were harvested and the presence of *M. avium* was evaluated by plating splenic and intestinal homogenates onto Middlebrook 7H11 agar. The remaining mice were challenged orally with 10⁶ CFU of MAC 104 Km². After 4 weeks, the mice were harvested, the terminal ileum, spleen, and liver tissues were homogenized, and the suspension was plated onto 7H11 agar plates (6, 9). Macrophages in Lab-Tek slides were fixed and infected monolayers were maintained at 37°C for 2 h. The cell suspension, containing macrophages, epithelial cells, and lymphocytes (approximately 30% of the number of macrophages), was washed, and infected monolayers were maintained at 37°C. Supernatants for the measurement of IL-10 were obtained at 24 h. Uninfected macrophages or T cells from uninfected mice were used as controls. The sensitivities of the assays for IFN-γ, TNF-α, and IL-10 were 12 ng/ml, 10 ng/ml, and 1 ng/ml, respectively.

**M. avium persists in mesenteric lymph nodes.** To investigate if bacteria are killed or survive in macrophages in immunocompetent mice without evidence of infection, we infected C57BL/6 mice orally with 100 GFP-*M. avium* 104 cells and monitored them for 6 months. At 1, 2, 4, and 6 months, mice were harvested and the presence of *M. avium* was examined in mesenteric lymph nodes and spleens by quantifying the bacterial load as well as by PCR amplification using 23S rRNA primers and fluorescence microscopy. Mesenteric lymph nodes were removed and cells were separated as previously described (20). The cell suspension was then seeded into both 24-well tissue culture plates (Gibco) and Lab-Tek slide chambers (Nunc Inc., Naperville, Ill.) to enrich for macrophages. The monolayers were washed with HBSS after 1 h to remove unattached cells, and macrophages were maintained in culture in the presence of RPMI-1640 medium supplemented with 10% FBS (Sigma). Monolayers in 24-well tissue culture plates were then lysed with sterile water, and lysates were serially diluted and plated onto 7H11 agar plates (6, 9). Macrophages in Lab-Tek slides were fixed with 2% paraformaldehyde for 20 min, washed, mounted, and observed under a Nikon fluorescence microscope for the presence of GFP-expressing bacteria.

Some monolayers were lysed and genetic material was obtained as previously described (13). To determine the viability of bacteria within macrophages, we lysed the monolayers with sterile water and performed a live-dead assay as previously described (5).

**23S rRNA and primers.** To identify the presence of *M. avium* in mesenteric homogenates, we used the 23S rRNA gene. Lymph nodes were homogenized, and the DNAs in the homogenate were purified as previously reported (20). Briefly, the lysate was suspended in 400 μl of TE buffer (10 mM Tris-Cl, pH 8.0, and 1 mM EDTA) and mixed with 400 μl of phenol-chloroform-isooamyl alcohol (pH 8.0) (Sigma). The mixture was added to 500 μl of 0.1-mm-diameter glass beads (Sigma) and vigorously shaken with a mini-bead beater for 3 min at room temperature. The organic and aqueous phases were separated by centrifugation. The DNA was precipitated with 1/10 volume of sodium acetate (pH 5-2) and 2 volumes of ethanol. After sedimentation, the nucleic acid pellet was dissolved in 50 μl of TE buffer.

The mycobacterial DNA was amplified by PCR, using the following primers specific to the 23S rRNA gene, kindly provided by Kevin Nash (Children’s Hospital, University of Southern California, Los Angeles, Calif.): 23.1, 5′GACG TAAC GACTTCT CAACTGT 3′; and 23.2, 5′ GICA CTA GAG GTT CGT CCT GC 3′. The 100-μl amplification reaction consisted of 50 nM Tris-Cl, 50 mM KCl, 1.5 mM MgCl₂, 2.5 U of Taq DNA polymerase (Eastman Kodak, Rochester, N.Y.), and 10 μl of DNA. The conditions used were 94°C for 1.5 min, 60°C for 2 min, and 72°C for 3 min. Each reaction was subjected to 35 cycles in a 480 Thermal Cycler (Perkin-Elmer, Norwalk, Conn.). Amplification products were analyzed by electrophoresis in a 3% agarose gel.

**M. avium dissemination.** To investigate if dissemination occurred through the infection of blood monocytes/macrophages, we orally infected 6-week-old C57BL/6 Ilb2 CD8 KO mice and wild-type C57BL/6 mice with 10⁸ *M. avium* 104 cells and determined the numbers of bacteria in the spleen, liver, and terminal ileum after 1 day, 2 weeks, and 4 weeks. At these time points, mice were harvested and their organs were homogenized and plated onto Middlebrook 7H10 agar as previously reported (6).

**RESULTS**

**Infection of knockout mice.** Previous studies using the intravenous route of infection have determined that the presence of CD4 lymphocytes is required for host protection against *M. avium* (19, 28). Oral inoculation with *M. avium* resulted in infections of wild-type (WT), CD4⁻/⁻, CD8⁻/⁻, and γδ⁻/⁻ mice (Fig. 1). When mice were given small doses of oral bacteria with the intent to trigger a specific immune response and subsequently reinfected with *M. avium*, WT mice preexposed to *M. avium* controlled the infection in a significant manner, while the immunized CD4⁻/⁻ KO mice showed the same level...
of infection as mice that were not exposed to mycobacteria (Fig. 1). Some level of protection against subsequent infection, however, was observed when either CD8⁻/⁻ or γδ⁻/⁻ mice were used for the same experimental protocol. As shown in Fig. 1, mice deficient in CD8⁺ T lymphocytes developed protection against an M. avium challenge following exposure to a low concentration of bacteria, which was also observed when mice deficient in γδ T cells were infected with M. avium (Fig. 1).

**Production of cytokines.** To determine if prior infection with *M. avium* triggers T-cell activation, we infected mice with 100 CFU of *M. avium* 101 on days 1 and 8, and 4 weeks after the first challenge, we purified CD4⁺ and CD8⁺ T lymphocytes from mesenteric lymph nodes and exposed them to *M. avium* for 24 h. To ensure that macrophage preparations were not contaminated with lipopolysaccharide, we performed some of the assays with polymyxin B (Sigma) as a control. No difference was observed between assays with and without the presence of polymyxin B (data not shown). Supernatants were then obtained, and the concentrations of IFN-γ, IL-10, and TNF-α were determined. As shown in Table 1, both CD4⁺ T and CD8⁺ T cells from mice exposed to *M. avium* at a low concentration were able to produce IFN-γ in a comparable manner, while CD4⁺ T cells produced larger amounts of TNF-α.
infected mice (but not from uninfected mice) produced IL-10. Bacteria taken up were found in the 2 in. of ileum. The smaller number of bacteria in the ileum than in the spleen disseminated equally in both the wild-type and KO strains of M. avium. We have shown that M. avium uses blood mononuclear phagocytes to disseminate, we can therefore conclude that M. avium is not limited to the spleen by its ability to grow in 7H11 agar. The supernatants were then removed, and monolayers were seeded onto Lab-Tek slides in the presence of RPMI-1640 supplemented with 10% heat-inactivated FBS. The suspensions were then seeded onto Lab-Tek slides in the presence of RPMI-1640 supplemented with 10% heat-inactivated FBS. The supernatants were then removed, and monolayers were fixed and mounted for fluorescence microscopy with a GFP filter. We observed that M. avium was present in the mesenteric lymph nodes but not in the spleens until later time points (Table 3). In addition, we observed that M. avium was present within phagocytic cells (Fig. 2). The spleens and lymph nodes (five per mouse) were also prepared, and DNAs were extracted. PCR amplification determined that M. avium was present in mesenteric lymph nodes after 1 month of infection and in the spleen 4 months following oral challenge. The bacteria were viable in the organs, as determined by their ability to grow in 7H11 agar.

### DISCUSSION

Healthy individuals are exposed to microbes that are present in the environment. M. avium is encountered in soil and water, and therefore the large majority of the population should have a similar exposure. We have shown that M. avium is not limited to the spleen by its ability to grow in 7H11 agar.

### TABLE 2. Oral infection and dissemination of bacteria to the liver and spleen in mice infected with M. avium

| Mouse strain (time of infection) | No. of bacteria (CFU/g) | Terminal ileum | Liver | Spleen |
|----------------------------------|-------------------------|----------------|-------|--------|
| C57BL/6 control (1 day)          | 5 ± 0.3 x 10^3         | 6 ± 0.3 x 10^4 | 2.1 ± 0.6 x 10^4 |
| C57BL/6 KO (1 day)               | 4 ± 0.4 x 10^2         | 5 ± 0.4 x 10^4 | 3.0 ± 0.5 x 10^4 |
| C57BL/6 control (2 wks)          | 2.0 ± 0.3 x 10^3       | 4.3 ± 0.4 x 10^5 | 2.8 ± 0.3 x 10^5 |
| C57BL/6 KO (2 wks)               | 4.4 ± 0.4 x 10^3       | 6.4 ± 0.2 x 10^5 | 3.2 ± 0.6 x 10^5 |
| C57BL/6 control (4 wks)          | 8.6 ± 0.3 x 10^3       | 1.8 ± 0.6 x 10^6 | 6 ± 0.2 x 10^6 |
| C57BL/6 KO (4 wks)               | 1.1 ± 0.2 x 10^4       | 1.7 ± 0.5 x 10^6 | 3.9 ± 0.9 x 10^6 |

* The experiment was repeated twice. The data represent means ± standard errors for 24 mice in each group.

* Spleens weighed between 0.06 and 0.07 g on day 1 and between 0.26 and 0.38 g at 2 and 4 weeks.

### TABLE 3. Amounts of bacteria and PCR amplification of 23S rRNA from mesenteric lymph nodes and spleens of mice infected with M. avium

| Time point (mo after infection) | Tissueb | Cytokines (ng/ml) | PCR amplification result |
|---------------------------------|---------|------------------|-------------------------|
| Before infection                |         |                  |                         |
| 1                               | Lymph node | Undetected       | +                       |
|                                | Spleen    | Undetected       | −                       |
| 2                               | Lymph node | 26 ± 3           | +                       |
|                                | Spleen    | Undetected       | −                       |
| 4                               | Lymph node | 32 ± 2           | +                       |
|                                | Spleen    | Undetected       | −                       |
| 6                               | Lymph node | 64 ± 6           | +                       |
|                                | Spleen    |                  | −                       |

* Uninfected mice were negative for M. avium 23S rRNA at all time points. Four animals were used for each time point. The mice were not treated with azithromycin.

* Spleens weighed between 0.07 and 0.08 g.

* The limit of detection was 20 CFU per g of tissue.
had exposure to the bacterium. Epidemiologic studies have confirmed this assumption, with most individuals showing *M. avium*-specific antibodies in the serum (7).

The results of research during the last several years have demonstrated that AIDS patients develop disseminated *M. avium* infection following ingestion or inhalation of the bacterium (12, 15, 21, 22). In addition, *M. avium* has been shown to interact with the intestinal-tract mucosa and to translocate the mucosal barrier (29). For humans, a large number of bacteria can be seen infecting macrophages in the lamina propria (12).

For this study, we employed a mouse model of oral infection to address the following questions. (i) If the majority of individuals are exposed to and infected with *M. avium*, what prevents them from developing the disease? (ii) Once the bacterium crosses the intestinal mucosae of healthy individuals, what is the fate of the organism?

Our results show that CD4+ T cells, but not CD8+ T cells or γδ+ T cells, are required for host protection against *M. avium* infection by the gastrointestinal route, and they agree with the observation for AIDS patients that seems to indicate that CD4+ T cells are mainly responsible for the protective adaptive response against the bacterium. Our study indicates that continued infection by a small number of *M. avium* organisms results in degrees of protection against infection. This observation agrees with previous work by Fattorini and colleagues showing that the delivery of a small number of *M. avium* organisms by the nasal or intraperitoneal route induced protection against a subsequent challenge in BALB/c mice (16). This exposure was followed by the appearance of subpopulations of CD4+ and CD8+ T cells specific for *M. avium* in the intestinal tract. Although the two populations of T cells in our study could produce IFN-γ upon exposure to *M. avium*, only CD4+ T cell KO mice, and not CD8+ T cell KO mice, developed disseminated infection secondary to an oral challenge. Previous studies have shown that CD8+ T cells do not appear to have a role in the host defense against *M. avium* in mice (19, 28, 32), in contrast with the evidence for *M. tuberculosis* infection (17). The lack of CD8+ T cell participation in the host defense was observed in studies using the respiratory as well as the systemic route of infection (19, 28, 32). Our data confirm past observations but add results for a challenge through the gastrointestinal tract. Mouse CD8+ T cells, in contrast to human CD8+ T cells, do not express granulysin, which has been associated with the host defense against *M. tuberculosis* (34). Whether the absence of granulysin is the reason for the lack of a role for CD8+ T cells in mice is presently unknown. Inter-

FIG. 2. Presence of GFP-*M. avium* in the mesenteric lymph nodes of mice infected for 6 months. Mesenteric lymph nodes were obtained from mice that had been infected for 6 months, and the suspension was placed on a Lab-Tek slide for adherence of the macrophages. After 1 h, the chamber was washed and subsequently fixed with 2% paraformaldehyde for 20 min. Monolayers were washed with HBSS, mounted, and observed for GFP-expressing bacteria under a Nikon microscope using a GFP filter. *M. avium* was not detected in uninfected mice by PCR amplification.
estingly, intestinal CD8+ T cells were sensitized to M. avium antigens and produced IFN-γ when exposed in vitro to the bacterium. However, they did not confer any measurable protection against infection. A recent study of Salmonella enterica serovar Typhimurium infection of mice evidenced that dissemination from the mesenteric lymph nodes was prevented by the presence of IFN-γ (24). In the case of M. avium infection, we suggest that IFN-γ is likely important for the prevention of dissemination, but other factors are potentially required. Studies of mice have established the importance of IFN-γ in the host defense against M. avium (1, 25), perhaps following secretion by NK cells as well as CD4+ T cells. However, the optimum host response requires TNF-α (31). In fact, our results suggest that TNF-α production by CD4+ T cells might be, at least in part, the explanation for the protection offered by CD4+ T cells. In addition, the slightly higher level of IL-10 produced by CD8+ T cells than that produced by CD4+ T cells might have an impact. Macrophages from infected mice do not produce TNF-α in large amounts during established infections, which supports the idea that M. avium infection suppresses cytokine production. The lack of contribution from γδ T cells to the host defense was also surprising. This population of lymphocytes is mainly found in the mucosa and submucosa and is assumed to participate in the host defense against invasive pathogens (36). Our data, however, support previous observations with a mouse model of lung infection (33).

Once in the intestinal mucosa, M. avium must use either the blood (transport within cells or direct translocation across endothelial cells) or the lymphatic system to disseminate. Using CD18 KO mice with a significant defect in translocating across the endothelial wall, we observed that tissue macrophages and blood monocytes are not the primary form of dissemination of infection. Studies with S. enterica serovar Typhimurium employing the same mouse system have shown that macrophages can transport Salmonella to distant organs (35). Our evidence suggests that after crossing the intestinal barriers, M. avium uses either the lymphatic system or is capable of invading the endothelial wall of the mesenteric vessels, although because CD18 KO leukocytes still can enter tissue, we cannot absolutely rule out macrophages as a source of dissemination.

For our study, the lungs were excised as a possible organ to which M. avium disseminates. The data were not conclusive, and additional work would be necessary to address relevant areas of this issue (data not shown).

M. avium was able to establish persistent infections, although dissemination took a long time to occur in mice infected with a small inoculum. Recent work has demonstrated that M. avium invades the mucosal epithelium but does not trigger chemokine production (30). In vivo, it can be documented by the lack of an inflammatory response early in the infection (23). This likely represents a pathogenic trait by which the bacterium can establish a niche before being exposed to phagocytic cells. Once the infection spreads, bacteria would infect mesenteric lymph node macrophages. Immunosuppression would likely be associated with M. avium dissemination. This observation perhaps explains why some AIDS patients develop disseminated disease without evidence of a source of infection. In addition, it also indicates that many individuals are potential carriers of the infection and may develop infections in joints, bones, kidneys, and other sites following dissemination. The model is amenable to genetic analysis, which would allow us to dissect bacterial factors associated with persistence and to explain the failure of the immune system to control the infection.

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