Role of γδ T Cells in Immunopathology of Pulmonary Mycobacterium avium Infection in Mice

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Several studies have shown that γδ T cells influence granuloma development after infection with intracellular pathogens. The role of γδ T cells in controlling the influx of inflammatory cells into the lung after Mycobacterium avium infection was therefore examined with gene-disrupted mice (K/O). The mice were infected with either M. avium 724, a progressively replicating highly virulent strain of M. avium, or with M. avium 2-151 SmT, a virulent strain that induces a chronic infection. γδ-K/O mice infected with M. avium 2-151 SmT showed early enhanced bacterial growth within the lung compared to the wild-type mice, although granuloma formation was similar in both strains. γδ-K/O mice infected with M. avium 724 showed identical bacterial growth within the lung compared to the wild-type mice, but they developed more-compact lymphocytic granulomas and did not show the extensive neutrophil influx and widespread tissue necrosis seen in wild-type mice. These data support the hypothesis that isolates of M. avium that induce protective T-cell-specific immunity are largely unaffected by the absence of γδ T cells. Whereas with bacterial strains that induce poor protective immunity, the absence of γδ T cells led to significant reductions in both the influx of neutrophils and tissue damage within the lungs of infected mice.

Mycobacterium avium is the most common cause of disseminated bacterial infection among human immunodeficiency virus (HIV)-infected individuals in the United States and Europe (2, 8, 13). Infection typically occurs late in the course of the AIDS disease, when CD4+ T-cell counts are below 100/μl (7, 25). Successful treatment of M. avium infection is difficult, not only because of the suppressed state of the host’s immune system but also because of a lack of effective antibiotics to treat the infection and the concurrent development of resistance to currently available drugs (17, 18, 22).

Infection of HIV-positive individuals with M. avium is thought to occur through the respiratory or gastrointestinal tract, with systemic spread being a common feature of this disease (7, 19). In the HIV-negative population M. avium generally presents as a pulmonary disease, and both systemic and pulmonary infection cause disabling disease in infected individuals (9, 15). M. avium infection typically leads to the generation of large, diffuse granulomatous lesions within infected tissue, such as the lung and lymph nodes. Characteristically, these lesions are filled with foamy macrophages, neutrophils, and a smaller percentage of lymphocytes. Tissue necrosis and fibrosis are common features of pulmonary M. avium infections (11, 20).

Recent studies in mice have suggested that the inflammatory response generated after infection with several intracellular pathogens, including Mycobacterium tuberculosis, is controlled in part by γδ T cells (10, 27, 29). While they constitute only a minor T-cell population (1 to 5%) within lymphoid organs, γδ T cells are a predominant T-cell population within epithelial tissues, including the skin, gut, and airways (16). They accumulate in infected tissue, including lesions from leprosy patients, and in vitro they produce an extensive array of cytokines and chemokines after antigenic stimuli (3, 6). γδ T cells recognize a range of mycobacterial proteins in vitro, including low-molecular-weight proteins and nonprotein ligands, often without the need for previous antigen processing and presentation (4, 6, 30). In addition, γδ T cells taken from patients with AIDS that are infected with mycobacteria recognize mycobacterial antigens in vitro (28). A recent epidemiological study found that γδ T-cell numbers were increased in HIV-M. avium-coinfected individuals but not in patients with HIV-M. tuberculosis coinfections, thus suggesting a role for γδ T cells in response to M. avium infection in patients with AIDS (24).

To investigate the hypothesis that γδ T cells control the influx of inflammatory cells into the lung after M. avium infection, gene-disrupted (γδ-K/O) mice were infected with M. avium, and the disease progression was monitored. In these experiments the mice were infected with either M. avium 724 or a smooth transparent isolate of M. avium 2-151 (2-151 SmT). Strain 724 is a highly virulent M. avium isolate that grows progressively within mice (12). Strain 2-151 SmT is also a virulent isolate, which induces strong protective immunity resulting in a chronic infection (12). Mice were infected by aerosol exposure to a low bacterial dose in order to mimic one of the natural routes of infection in humans. We report here that the contribution of γδ T cells to immunity to M. avium varies depending upon the infective strain used. Infection of γδ-K/O mice with M. avium, while having only a transient influence upon the growth of bacteria, significantly affected the inflammatory response generated within the lungs. γδ-K/O mice developed lesions containing a higher proportion of lymphocytes and, in the case of infection with M. avium 724, they did not show the extensive neutrophil influx nor the development of casedated lesions seen in wild-type-infected mice.

MATERIAL AND METHODS

Mice. Breeding pairs of T-cell receptor Cγ gene mutant mice (C57BL/6-J-Tcrd (Mom) and JR2120) (23) were obtained from the Jackson Laboratories (Bar Harbor, Maine) and bred in the Laboratory Animal Resources Center at Colorado State University. Wild-type controls (C57BL/6/J) were obtained from the Jackson Laboratories as required. Age- and sex-matched mice were kept under barrier conditions in the ABL-3 bioshield facility throughout the experiment.
The specific-pathogen-free nature of each colony was shown by testing sentinel animals; these were determined to be negative for 12 known mouse pathogens.

**Bacteria and infection.** *M. avium* 724 and 2-151 SmT were grown from laboratory stocks in Proskauer-Beck liquid medium to mid-log phase, aliquoted, and then frozen at −70°C. Mice were infected with approximately 500 bacteria by using a Middlebrook Airborne Infection apparatus (Middlebrook, Terre Haute, Ind.) as previously described (26). The numbers of viable bacteria in the lung, spleen, and liver were determined at various time points by plating serial dilutions of organ homogenates on nutrient Middlebrook 7H11 agar and counting the bacterial colonies after 14 days of incubation at 37°C. The data are expressed as the log$_{10}$ value of the mean number of bacteria recovered per organ (n = four animals).

**Histology.** Tissues from four mice per experimental group were infused with fresh 10% formaldehyde in phosphate-buffered saline. Sections made from paraffin blocks were stained with hematoxylin and eosin. Consecutive sections were stained for acid-fast bacilli by the Kinyoun staining procedure. Sections were examined by a veterinary pathologist without prior knowledge of the experimental groups.

**Statistical analysis.** Differences between the mean of experimental groups were analyzed by using the Student t test. Differences were considered significant when P was < 0.05.

### RESULTS

**Variation in the growth of two strains of *M. avium* in γδ T-K/O mice.** Strains 724 and 2-151 SmT are virulent serotype-2 strains of *M. avium* that have been extensively studied in this laboratory. Both generate protective T-cell immunity early during the course of the infection, and as a result the growth of 2-151 SmT is restrained, giving rise to a chronic disease state. In contrast, however, mice infected with 724 gradually lose their expression of acquired immunity (9a), for reasons as yet completely unknown, allowing the infection to grow progressively.

To test the hypothesis that γδ T cells are involved in these mechanisms, we examined the course of infection with these two bacterial strains in mice lacking this cell population. Wild-type mice infected with *M. avium* 724 showed progressive bacterial growth within the lung and dissemination to the liver and spleen (Fig. 1). γδ-K/O mice showed identical bacterial growth in the lung, although dissemination into these organs was significantly delayed (P < 0.05).

Mice infected with *M. avium* 2-151 SmT developed a chronic bacterial infection, with bacilli initially growing to a number 1 log higher in the lungs of the γδ-K/O mice (P < 0.05) (Fig. 1). By day 120, however, the differences in bacterial loads between

### TABLE 1. Lung histology in wild-type and γδ-K/O mice infected aerogenically with *M. avium* 724 or 2-151 SmT

<table>
<thead>
<tr>
<th>Time postinfection (days)</th>
<th><em>M. avium</em> 2-151 SmT</th>
<th><em>M. avium</em> 2-151 SmT</th>
<th><em>M. avium</em> 2-151 SmT</th>
<th><em>M. avium</em> 2-151 SmT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>γδ-K/O</td>
<td>Wild type</td>
<td>γδ-K/O</td>
</tr>
<tr>
<td>40</td>
<td>Minimal granulomatous pneumonia with few lymphocytic cells</td>
<td>Minimal granulomatous pneumonia with few lymphocytic cells</td>
<td>Minimal lymphocytic interstitial pneumonia</td>
<td>Minimal lymphocytic interstitial pneumonia</td>
</tr>
<tr>
<td>60</td>
<td>Mild granulomatous pneumonia with few lymphocytic cells</td>
<td>Mild granulomatous pneumonia with increased number of lymphocytic cells</td>
<td>Minimal lymphocytic interstitial pneumonia</td>
<td>Minimal lymphocytic interstitial pneumonia</td>
</tr>
<tr>
<td>90</td>
<td>Mild to moderate granulomatous pneumonia with increased number of lymphocytic cells</td>
<td>Moderate granulomatous pneumonia with markedly increased number of lymphocytic cells</td>
<td>Severe granulomatous pneumonia with caseation and few lymphocytic cells</td>
<td>Marked granulomatous pneumonia with increased number of lymphocytic cells</td>
</tr>
<tr>
<td>120</td>
<td>Moderate granulomatous pneumonia with increased number of lymphocytic cells</td>
<td>Moderate to marked granulomatous pneumonia with markedly increased number of lymphocytic cells</td>
<td>Severe granulomatous pneumonia with caseation and few lymphocytic cells</td>
<td>Severe granulomatous pneumonia with increased number of lymphocytic cells</td>
</tr>
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the two mouse strains were no longer significant. Moreover, no differences were seen in the pattern of dissemination of *M. avium* 2-151 SmT between the wild-type and the γδ-K/O mice.

**Differences in the inflammatory response generated in γδ-K/O mice.** The lungs of wild-type and γδ-K/O mice infected with *M. avium* had significant differences in lesion type, with the severity differing between the two bacterial strains (Table 1). Wild-type mice infected with *M. avium* 724, while showing progressive bacterial growth, did not mount a strong inflammatory response to the invading bacteria during the first 60 days of infection. There was some thickening of the alveolar septae by lymphocytes and macrophages along with scattered small foci of lymphocytes, but no large rafts of macrophages or lymphocytes were present (Fig. 2A). By day 90, the lungs of the wild-type mice had extensive granulomas with severe caseation surrounded by a thick wall of degenerative neutrophils that were in turn surrounded by a rim of epithelioid macrophages (Fig. 2C, enlarged in Fig. 3A to C). Acid-fast staining of consecutive lung sections revealed extensive numbers of bacilli within the epithelioid macrophage layer surrounding the wall of degenerative neutrophils (Fig. 3D).

In contrast, γδ-K/O mice developed a markedly different granulomatous response to infection with *M. avium* 724. Although the initial response was similar to that seen in the controls, the γδ-K/O mice failed to develop the caseous lesions prominent in the wild-type mice. Granulomas in the K/O mice were composed primarily of lymphocytes and macrophages, with only small pockets of neutrophils present (Fig. 2B, enlarged in Fig. 3E). Acid-fast staining showed the bacilli within the macrophages (Fig. 3F). At 120 days postinfection, granulomatous involvement in the lungs of the γδ-K/O mice had increased, but granulomas were still composed primarily of lymphocytes and macrophages with only small numbers of neutrophils (Fig. 2D).

The significant differences seen in granuloma formation in the lungs of wild-type and γδ-K/O mice were not evident in the other organs investigated. Examination of the liver revealed that both mouse strains developed multifocal granulomatous
FIG. 3. Representative high-magnification photomicrograph of lung tissue from mice infected with *M. avium* 724. (A) Section of a caseated lesion in a wild-type mouse at 90 days postinfection. Note the concentrated laminated rings of degenerative neutrophils (middle) and epithelioid macrophages (top) surrounding the central caseation (bottom). The thick-bar area is magnified in panel B; the thin-bar area is magnified in panel C. (B) Higher magnification of the neutrophilic lamina (bottom) with the epithelioid macrophages (top). (C) The center of the caseated area contains amorphous debris. (D) Acid-fast bacilli in macrophages. This is a magnification of the area of panel A just above the thick bar. (E) Section of a noncaseated lesion in a γδ-K/O mouse at 90 days postinfection. Note that the lesion is composed primarily of macrophages and lymphocytes. (F) Acid-fast bacilli in macrophages from a γδ-K/O mouse at 90 days postinfection. The panel is a magnification from an area similar to that shown in panel E. Panels A, B, C, and E were stained with hematoxylin and eosin; panels D and F were stained with Kinyoun’s stain. Bar, 10 μm.
hepatitis (Fig. 4). Granuloma formation in the spleen and kidney also did not differ significantly between the two mouse strains (data not shown).

While wild-type mice infected with *M. avium* 724 clearly showed evidence of caseation and necrosis, such was not the case in mice infected with *M. avium* 2-151 SmT. Thickening of the alveolar septae by macrophages and lymphocytes was visible earlier in these mice, often by 20 days postinfection, and small granulomas composed primarily of lymphocytes and macrophages began to form by around day 40. These granulomas continued to increase in size throughout the infection, but caseation was never observed (Fig. 5). γδ-K/O mice infected with *M. avium* 2-151 SmT developed granulomas with a higher lymphocytic proportion than those seen in the wild-type mice, and these differences were especially apparent by 90 days postinfection, when the lymphocyte influx into the lungs appeared to peak (Fig. 5D). Neutrophil influx into the lungs of wild-type and γδ-K/O mice was only minor over the 120-day time course examined, with no major differences apparent in response to *M. avium* 2-151 SmT infection between the two mouse strains.

**DISCUSSION**

This study shows that, when bacterial growth remains unchecked, as in the *M. avium* 724 infection, the presence of γδ T cells in the host can be detrimental and appears to accelerate the destructive pathogenic response. If, however, the bacterial infection remains chronic, as in the 2-151 SmT infection, then the γδ T cells appear to play no significant role in disease progression.

The key observation reported here concerns the necrotic nature of the lesions in strain 724-infected mice. In *M. avium*-infected individuals a high bacterial burden within the lungs is usually accompanied by extensive tissue necrosis and fibrosis (2, 11, 13, 15, 20). Like the lesions seen in humans, wild-type mice infected with *M. avium* 724 developed lesions composed predominantly of neutrophils and macrophages. Perhaps as a consequence of the high bacterial numbers in this animal model, extensive tissue necrosis was also seen, with some necrotic lesions progressing to a caseated state.

An indication of the mechanism involved in the generation of this pathogenic response is provided by the observations reported here. Specifically, while the bacterial loads in the wild-type and γδ-K/O mice were equivalent, the lesion development was significantly different. In contrast to the extensive degenerating lesions seen in the wild-type mice, the lung lesions of the γδ-K/O mice infected with strain 724 consisted of small granulomas containing mixtures of macrophages and lymphocytes. By comparing histological samples from different time points it was evident that progression of the pathogenic response was slower in the γδ-K/O mice. By day 120 of the experiment, lung lesions in the K/O mice had increased to a size similar to those of the wild-type mice 1 month earlier. However, the lesions were composed of vast fields of epithelioid macrophages, with a few scattered aggregates of lymphocytes and with no overt necrosis evident.

While the absence of γδ T cells diminished the necrosis and tissue damage seen within the strain 724-infected mice, this was not the case in *M. avium* 2-151 SmT-infected animals. Both wild-type and γδ-K/O mice infected with 2-151 SmT developed similar lymphocytic lesions, which were composed predominantly of macrophages and lymphocytes, except that lesions in the γδ-K/O mice appeared to be more lymphocytic in nature.

One possible explanation for the differences in lesion formation induced by the two strains of *M. avium* may be in the immune response each one induces. Protective immunity to *M. avium* requires an inflammatory cell influx to surround and contain infected macrophages and a protective T-cell response to activate macrophages to kill infecting bacilli. *M. avium* 2-151 SmT induces both the influx of inflammatory cells to surround infected macrophages and, as previously shown, a strong acquired immune response (14) that controls bacterial growth such that a chronic disease state then ensues. Mice aerogenically infected with strain 724 show no ability to contain bacterial growth. Early in the infection only a mild interstitial pneumonia was seen within the lungs of infected mice, and cells were not recruited to surround infected macrophages. It appears, therefore, that a protective acquired immune response was not generated. Indeed *M. avium* 724 grows identically in both CD4 K/O and wild-type mice (28a). Studies in this laboratory (9a) found that intravenous infection with 724 does appear to generate acquired immunity during the early course of this infection.
of the infection but, for reasons that are currently unclear, this specific resistance is then gradually lost, thus allowing the infection to grow progressively and eventually kill the animal.

We postulate that in mice aerogenically infected with strain 724, once the bacterial load reaches a critical threshold, γδ T cells, driven by recognition of mycobacterial antigens (4, 5, 31) and/or recognition of damaged or infected self (21), produce a cytokine-chemokine response that stimulates the influx of inflammatory cells, particularly macrophages into infected tissue. These macrophages are detrimental to the mouse since they serve as host cells for the pathogen and, because of the absence of protective αβ T cells, they remain inactivated and gradually degenerate, causing local tissue damage and an influx of neutrophils. In the absence of γδ T cells, this inflammatory cell influx is diminished and less tissue damage is seen.

In M. avium 2-151 SmT-infected mice, where a protective acquired immune response is generated (1), macrophages entering infected tissues become activated and are able to control bacterial growth. In the absence of γδ T cells, more lymphocytes were seen in lesions in the lung, suggesting that the presence of γδ T cells dampens the recruitment of lymphocytes to the infected tissue. In 2-151 SmT-infected mice, because of the presence of a protective acquired immune response, the macrophages recruited by γδ T cells become activated and are able to control bacterial growth. As the bacterial growth is controlled the amount of tissue damage and mycobacterial debris is also reduced. In this situation therefore, unlike in the strain 724-infected tissue, there was a much lower stimulus for the recruitment of neutrophils and the development of necrosis and caseation.

In support of this hypothesis, studies investigating the role of γδ T cells in other infections have shown repeatedly that γδ T cells, while often not directly affecting the growth of the infective pathogen, still control inflammatory processes in response to infection (10, 27, 29). In the absence of γδ T cells, infected mice generally developed larger more-diffuse lesions, with an increase in tissue necrosis and abscess formation being common.

Indeed, in M. tuberculosis infection the absence of γδ T cells led to the development of a pyogranulomatous inflammatory...
response with lesions containing increased numbers of neutrophils and large foamy macrophages, distinct from the lymphocytic granulomas formed in the wild-type mice (10). While this data appears to contradict our data, we would contend that it is the recruitment of macrophages by γδ T cells into a nonimmune site that leads to the potential for larger more necrotic lesions. Thus, in strain 724-infected mice, γδ T cells stimulate a macrophage influx, but no protective αβ T-cell response is present to activate these macrophages, and as a result bacterial growth continues and the macrophages degenerate, stimulating a neutrophil influx which increases the tissue damage seen. In contrast, in M. tuberculosis-infected mice, γδ T cells still stimulate a macrophage influx; however, these cells are activated by the protective αβ T-cell response, and bacterial growth is controlled. In the absence of γδ T cells, macrophage recruitment is diminished and so we see the rapid growth of M. tuberculosis in a low macrophage environment which results in rapid tissue damage and the subsequent recruitment of neutrophils.

Finally, this animal model, if extrapolated, may have implications for HIV-positive patients infected with M. avium. The implication here is that a failure of acquired cellular immunity expressed by CD4 T cells allows an opportunistic M. avium infection to disseminate and grow. Meanwhile, the γδ T-cell response inadvertently promotes this process by amplifying the recruitment of macrophages into lesions, but the absence of IFN-γ-secreting CD4 T cells results in astronomic bacterial loads and pathologic lesions. This model is therefore similar to the disease progression in HIV-positive patients with disseminated M. avium infections and may be useful in identifying mechanisms of pathogenesis.

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