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# Growth of *Mycobacterium tuberculosis* in BCG-Resistant and -Susceptible Mice: Establishment of Latency and Reactivation

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**Growth of mycobacterial species is controlled by a gene, *Bcg* (candidate *Nramp*). *Bcg* acts at the macrophage level and is thought to control some aspect of macrophage priming for activation. Infection of *Mycobacterium bovis* BCG-susceptible (*Bcg*$^s$) mice with several different mycobacterial species results in the growth of the microorganisms, while the growth of the same organisms is controlled in BCG-resistant (*Bcg*$^r$) mice. The capacity of *Bcg* to control the growth of *M. tuberculosis* has not been extensively explored. The purpose of this investigation, therefore, was to compare the growth of *M. tuberculosis* in *Bcg*$^r$ and *Bcg*$^s$ mice. We found that the growth of tubercule bacilli was different in the lungs and spleens of *Bcg*$^r$ and *Bcg*$^s$ mice when they were inoculated with fewer then $10^3$ CFU of the mycobacterium. The differences in growth were more easily distinguished in the lungs then in the spleens. The growth of the microorganisms in both strains of mice peaked between 35 and 43 days, and a latent infection was established by 65 days after initial infection. Activation of the hypothalamic-pituitary-adrenal axis resulted in reactivation of the growth of *M. tuberculosis* in both *Bcg*$^r$ and *Bcg*$^s$ mice. Greater numbers of tubercule bacilli were isolated from lungs than from spleens following reactivation. The utility of this mouse model in the study of the establishment of latency and reactivation of *M. tuberculosis* is discussed.**

Control of the early growth of mycobacterial species has been shown to be controlled by a gene, *Bcg*, that maps to chromosome 1 in mice (12, 13, 22, 25). The *Bcg* gene also controls the growth of *Salmonella typhimurium* (*Ity*) and *Leishmania donovani* (*Lsh*) (2, 25). A candidate *Bcg* gene (*Nramp*) has recently been cloned in mice and humans (1, 5, 26). *Nramp* has the structural motif of a membrane transport protein. However, the function of *Nramp* in mediating resistance to a variety of intracellular pathogens remains unknown. Despite numerous reports that *Bcg* controls the growth of many mycobacterial species, it is not clear whether the gene also controls the growth of *Mycobacterium tuberculosis* (9, 12, 19, 20, 23, 24).

Most studies of the growth of *M. tuberculosis* in mice have utilized relatively large doses of bacteria, ranging from $10^4$ to $10^6$ CFU. This results in progressive growth of the bacillus (11, 17, 18). In contrast, studies utilizing *Bcg*$^r$ and *Bcg*$^s$ mice have shown that *Bcg* can control the growth of mycobacterial species only when small doses of bacteria are utilized (12, 22). Resistance has been defined as the ability to control the growth of the bacteria in the spleen following intravenous injection of $10^4$ CFU of *M. bovis* BCG. Several reports have indicated that the effect of *Bcg* on mycobacterial growth may be limited to the spleen, while others have also shown difference in the growth of mycobacteria in the lungs of infected *Bcg*$^r$ and *Bcg*$^s$ mice (10, 19).

During the course of these studies to determine if *Bcg* also controls the early growth of *M. tuberculosis*, we found that inoculation of small doses of the microorganisms resulted in the establishment of a latent infection. Since previous studies in our laboratory had shown that activation of the hypothalamic-pituitary-adrenal (HPA) axis results in an increase in the susceptibility of *Bcg*$^s$ mice to mycobacterial growth, we also determined if activation of the HPA axis also results in reactivation of the growth of *M. tuberculosis*. We found that the control of *M. tuberculosis* growth correlated with the *Bcg*$^r$ phenotype in both the spleens and lungs of congenic mice and that activation of the HPA axis resulted in reactivation of the growth of *M. tuberculosis*. This mouse model of latency and reactivation of *M. tuberculosis* provides a useful tool for the study of the changes in T-cell- and macrophage-mediated effector function that are important for establishment of latency and reinitiation of mycobacterial growth.

## MATERIALS AND METHODS

**Animals.** Male BALB/c.*Bcg*$^s$ and DBA/2 (*Bcg*$^r$) mice were purchased from Charles River (Wilmington, Mass.) when 6 to 8 weeks of age. Congenic C.D2*Ity*$^r$ (BALB/c.*Bcg*$^r$) mice were originally obtained from Michael Potter (National Cancer Institute, Bethesda, Md.) and bred in our facility (21). The mice were housed in microisolator cages (Lab Products, Maywood, N.J.) and given food and water ad libitum.

**Microorganisms.** *M. tuberculosis* (Erdman) was obtained from the American Type Culture Collection (ATCC 35801, TMC 107) and cultured on Lowenstein-Jensen agar slants (Difco, Detroit, Mich.). The bacteria were then transferred to Middlebrook 7H9 broth seed cultures. The mycobacteria were grown to a density of $5 \times 10^8$ CFU/ml of Middlebrook 7H9 broth and stored at −70°C until use. Prior to use, the microorganisms were thawed, briefly sonicated, and diluted in Hanks balanced salt solution. Mice were injected with 0.2 ml of the bacteria via the tail vein. The intravenous route was used because previous studies that defined the effect of *Bcg* used intravenous inoculation of the mycobacteria (12). The number of bacteria that were injected was confirmed by plate counting.

To determine the growth of *M. tuberculosis*, mice were weighed and then sacrificed at the times indicated in Fig. 1. The spleen and lungs were removed, weighed, and homogenized. The suspensions were serially diluted in Hanks balanced salt solution and then plated onto Middlebrook 7H11 agar plates supplemented with Middlebrook OADC enrichment (Difco). The plates were placed into gas-permeable plastic bags and incubated at 37°C in an atmosphere containing 10% $CO_2$ in air for 4 weeks or until visible colonies appeared. The results were calculated and expressed as CFU per gram of organ per gram of body weight.

**HPA axis activation.** The HPA axis was activated by restraining the mice within well-ventilated 50-ml conical centrifuge tubes as previously described by us (3). The mice were restrained for five daily 15-h cycles, rested for 2 days, and then restrained again for five 15-h cycles. Previous studies have shown that

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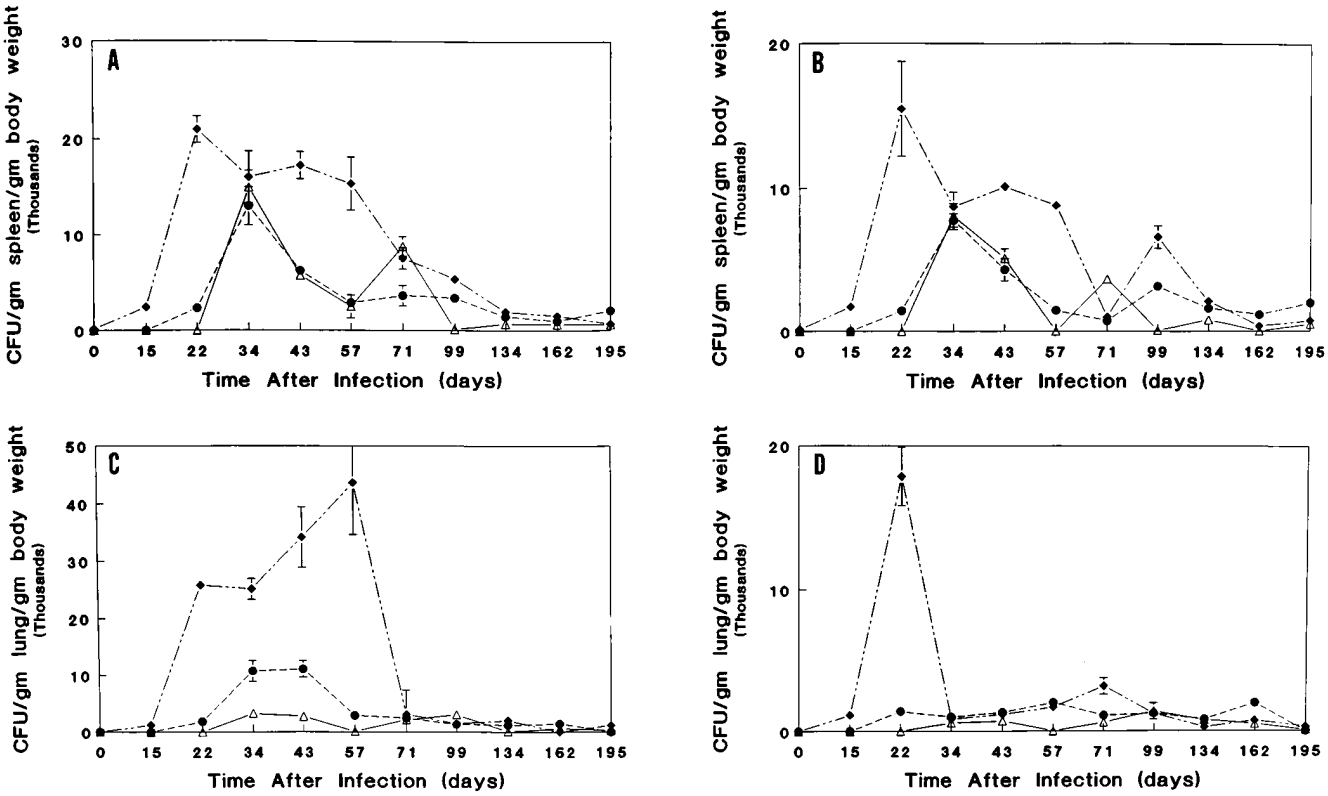


FIG. 1. Growth of *M. tuberculosis* in BCG-susceptible and -resistant mice. Mice were inoculated with $10^1$ to $10^3$ CFU of *M. tuberculosis* (Erdman) via the tail vein. The growth of the microorganisms was monitored by plate counting at the times indicated. Panels: A, BCG-susceptible BALB/c mouse spleen; B, BCG-resistant DBA/2 mouse spleen; C, BCG-susceptible BALB/c mouse lung; D, BCG-resistant DBA/2 mouse lung. The data represent the mean ± the standard deviation of five mice for each time point for each mycobacterial dose. Symbols: △, $10^1$ CFU; ●, $10^2$ CFU; ◆, $10^3$ CFU.

restraint for a single 18 cycle or for five 18-h cycles does not alter mycobacterial growth (3). The mice were placed in conventional housing at the end of each 15-h restraint cycle. During restraint, the mice were deprived of food and water. Control mice were therefore also deprived of food and water.

## RESULTS

**Growth of *M. tuberculosis* in BCG-resistant and -susceptible mice.** Figure 1 shows the results of experiments in which groups of five mice for each time point were injected with either $10^1$, $10^2$, or $10^3$ CFU of *M. tuberculosis*. Growth of the microorganisms was initially detected in the spleens and lungs of both BALB/c and DBA/2 mice within 15 days of inoculation. *M. tuberculosis* colonies were first detectable in mice receiving the larger number of microorganisms and detected later in mice injected with smaller numbers of bacteria. The pattern of growth in the spleens of BALB/c.*Bcg*$^s$ mice was similar to that observed in the spleens of *Bcg*$^r$ DBA/2 mice except that greater numbers of bacilli were isolated from the spleens of the BALB/c.*Bcg*$^s$ mice. Growth in the lungs was much different. Thus, while the appearance and growth of the bacilli were related to the inoculum size in the lungs of BALB/c.*Bcg*$^s$ mice, only the $10^3$-CFU inoculum dose grew in *Bcg*$^r$ DBA/2 mice. *M. tuberculosis* organisms were isolated from the lungs of the mice receiving smaller inocula, but no peak in growth was observed throughout the time course of the experiment. The results in Fig. 2 illustrate this by showing the pattern of *M. tuberculosis* growth after inoculation of 100 CFU. The *Bcg* gene effect was apparent in the spleen only at day 34. The numbers of *M. tuberculosis* bacilli remained low in the lungs of DBA/2 mice throughout the time course, while the numbers of bacilli peaked between days 34 and 43 in the lungs of BALB/c.*Bcg*$^s$ mice. In each case, the numbers of microorganisms returned to low levels by day 57 of infection.

To more precisely implicate the role of *Bcg*, the experiments were repeated with congenic BALB/c.*Bcg*$^r$ and BALB/c.*Bcg*$^s$ mice. The results in Fig. 3 show that greater numbers of microorganisms were isolated from the spleens and lungs of *Bcg*$^s$ mice than from those of *Bcg*$^r$ congenic mice. While twice as many tubercle bacilli were isolated from the spleens of *Bcg*$^s$ mice than from those of *Bcg*$^r$ mice, almost four times as many *M. tuberculosis* bacilli were isolated from the lungs of *Bcg*$^s$ mice than from the lungs of *Bcg*$^r$ mice.

**HPA axis activation results in reactivation of the growth of *M. tuberculosis*.** Since the latent disease state that we observed in mice was reminiscent of the disease in humans, we determined if temporary suppression of immunity by activation of the HPA axis would result in reactivation of *M. tuberculosis* growth. The results in Fig. 4 show that activation of the HPA axis resulted in reinitiation of *M. tuberculosis* growth in both the spleens and lungs of both BCG-resistant and -susceptible mice. The effect of HPA axis activation was apparent after the last restraint period (day 12 or 73 postinfection) and was more pronounced in the lungs then in the spleens of both strains of mice (note the differences in scale). While only twice as many microorganisms were isolated from the spleens 26 days following HPA axis activation (88 days postinfection), almost 10 times as many tubercule bacilli were isolated from the lungs of mice following HPA axis activation.

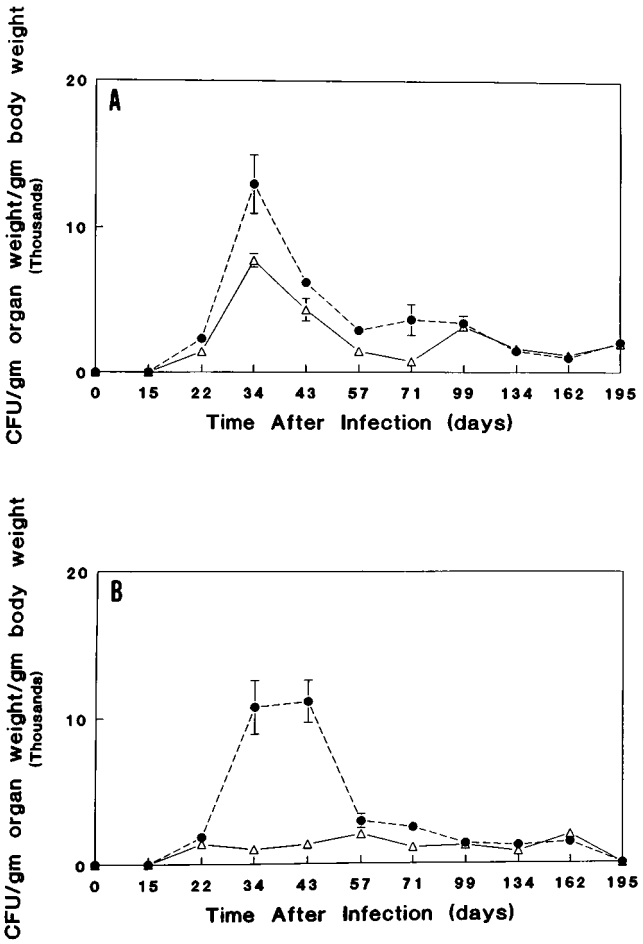


FIG. 2. Growth of *M. tuberculosis* in the spleens and lungs of BCG-resistant (△) and -susceptible (●) mice following injection of 100 CFU. The data shown are from Fig. 1.

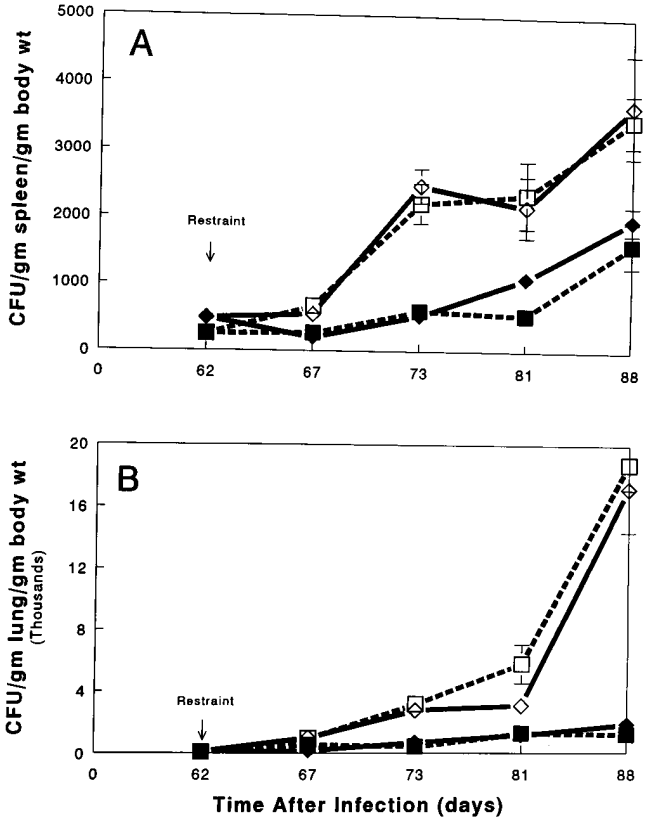


FIG. 4. Activation of the HPA axis results in reactivation of *M. tuberculosis* growth in BCG-resistant and -susceptible BALB/c mice. Mice were infected with $10^2$ CFU of *M. tuberculosis*. After 60 days, when a steady-state infection was established, the mice were restrained for five 15-h cycles, rested for 2 days, and restrained again for five 15-h cycles as described in reference 3. *M. tuberculosis* growth in the spleens (A) and lungs (B) was monitored by plate counting at the designated times after infection. The data represent the mean ± the standard deviation of five mice for each time point for each treatment. Symbols: ◆, control *Bcg*$^r$; ■, control *Bcg*$^s$; ◇, restraint *Bcg*$^r$; □, restraint *Bcg*$^s$. wt, weight.

## DISCUSSION

The results of this investigation show for the first time that *Bcg* (candidate *Nramp*) controls the growth of *M. tuberculosis*. The difference in *M. tuberculosis* growth prior to the development of specific immunity is similar to that observed for other mycobacterial species (9, 12, 13, 19, 20, 22–24). The control of mycobacterial growth appeared to be manifested in both the lungs and the spleen. However, the effect of *Bcg* was more apparent in the lungs then in the spleen. Our observation that *Bcg* controls the growth of *M. tuberculosis* is different from that reported by Musa et al. (16) following infection of mice by aerosol. However, the results of their experiments, which used several different BCG-resistant or -susceptible inbred strains of mice, were more variable; some experiments indicated a difference in the growth of *M. tuberculosis*, while others found no difference. Differences in the major histocompatibility complex of the strains of inbred mice or in cytokine production could account for the variability of their observations and the differences between our observations. An important difference in our studies is that we used histocompatible *H-2*$^d$ mice, as well as congenic *Bcg*$^r$ and *Bcg*$^s$ mice, to confirm the role of *Bcg*.

Orme and Collins have reported that *Bcg* is manifested in the spleen but not in the lungs or the liver (19). In contrast, Goto et al. have found that *Bcg*-mediated resistance is apparent in both the spleens and lungs of mice (10). The differences and similarities in our finding may be explained by the observation that *Bcg* appears to be manifested differently depending on the organ in which the microorganism primarily replicates (27). *M. tuberculosis* replicates in lung macrophages, and *Bcg*

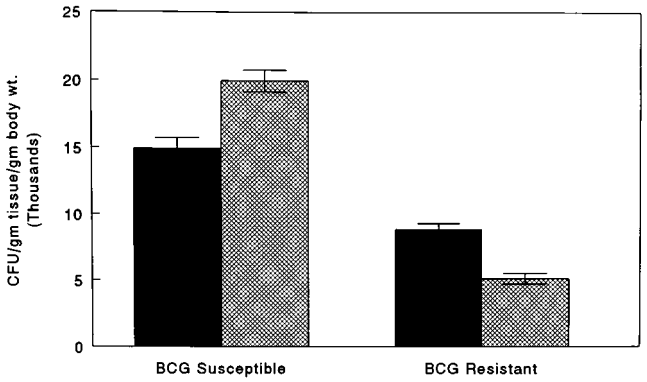


FIG. 3. Differential growth of *M. tuberculosis* in the spleen (■) and lungs (▨) of congenic BALB/c.*Bcg*$^r$ and BALB/c.*Bcg*$^s$ mice. Mice were inoculated with 100 CFU, and the numbers of microorganisms in their spleens and lungs were determined by plate counting at 35 days after infection. The data represent the mean ± the standard deviation of five mice for each treatment. wt., weight.

appears to be more effective in the lungs then in the spleen. Similarly, thioglycolate-elicited peritoneal macrophages do not support mycobacterial growth, and the growth of *Leishmania donovani* appears to be limited to some resident macrophage populations. Neither resident peritoneal nor bone marrow-derived macrophages supported the replication of the parasite. The observation that splenic adherent cells from resistant mice do not control *Leishmania* growth is in marked contrast to their effect on the growth of BCG and salmonellae (7, 27). Thus, *Bcg* appears to be expressed differentially, depending on the ability of macrophages from different anatomical locations to support mycobacterial growth.

Our results reinforce the observation of Gros et al. concerning the effect of *Bcg* on mycobacterial growth (12). Their results showed that the *Bcg* gene effect was manifest by 21 days after inoculation of $10^4$ CFU of *M. bovis* BCG. After that time, an immune response developed that further affected the growth of the mycobacteria. We also observed that the development of a specific immune response resulted in a marked decrease in the number of microorganisms in the spleens and lungs of $Bcg^s$ mice. However, the development of immunity was not apparent until after 35 days of infection with $10^2$ CFU of *M. tuberculosis*. The differences in the time course of *Bcg*-mediated effects can be accounted for by the differences in inoculum size. This interpretation is reinforced by the data in Fig. 1 following inoculation of $10^3$ CFU. The *Bcg*-mediated differences were apparent 22 days after infection in both spleens and lungs. It appears, therefore, that the inoculum size determined when the *Bcg*-mediated differences became apparent.

Inoculation of small doses of *M. tuberculosis* results in the establishment of latent disease. No differences were observed in the ability of either $Bcg^r$ or $Bcg^s$ mice to establish latency, which was probably the result of the development of specific immunity. Kramnik et al. (15) found that growth of BCG in resistant and susceptible mice did not result in differences in the pattern of cytokine production. Additionally, Cox et al. (6) found that injection of mice with anti-CD4 antibody resulted in reactivation of the growth of *M. tuberculosis*, indicating a role T cells in latency maintenance. A recent study by Daynes et al. (8) has shown that increased levels of corticosterone can result in a shift from the Th1 to the Th2 type of CD4 T cells, resulting in a decrease in macrophage-activating cytokines. Decreases in activating stimuli may result in the reinitiation of *M. tuberculosis* growth that we observed.

The establishment of latency provided us with the opportunity to determine if suppression of immunity could result in reactivation of *M. tuberculosis* growth. We used restraint to activate the HPA axis. This resulted in an increase in corticosterone and ACTH, as well as splenic epinephrine and norepinephrine (data not shown), and reinitiation of mycobacterial growth. This observation is similar to that reported by Cox et al. (6), who found that injection of mice with pharmacologic doses of corticosteroids resulted in reactivation of *M. bovis* BCG growth. The magnitude of the reactivation was greater in innately resistant CBA/Ca mice then in susceptible C57BL/6 mice. We found that reactivation of *M. tuberculosis* growth was equivalent in $Bcg^s$ and $Bcg^r$ DBA.2 mice. Our observation that *M. tuberculosis* growth was reactivated in both BCG-resistant and -susceptible mice was somewhat surprising given our previous observations that *M. avium* growth was not affected by HPA axis activation in $Bcg^r$ mice (3) and that *M. avium* growth in macrophages from $Bcg^r$ mice was not affected by corticosterone (4). We interpret this observation as indicating that reactivation of *M. tuberculosis* growth in $Bcg^r$ and $Bcg^s$ mice was probably the result of suppression of specific T-cell-mediated responses. While it is tempting to conclude that the *Bcg* gene plays little part in HPA axis-induced reactivation of *M. tuberculosis* growth, studies in our laboratory have indicated that *Bcg* gene expression by macrophages from $Bcg^r$ and $Bcg^s$ mice is differentially affected by corticosterone (unpublished data). The cellular and hormonal basis of the HPA axis-mediated effects on reactivation of the growth of tubercule bacilli is currently under investigation.

Establishment of latent tuberculosis infections provides us with the opportunity to study the events that are important for establishment of latency and for reactivation of mycobacterial growth in a mouse model. During the course of natural infection in humans, patients present with active disease. Diagnosis of infection in individuals without active disease usually occurs following skin testing of those who have been exposed to others with active disease (14). Therefore, humans have either latent or active disease. It is not possible, therefore, to monitor their immunological status during the transition from latency to active disease. This model provides us with the opportunity to study the role of T cells and macrophages during the course of disease from the time of initial infection to the establishment of latency and the progression during reactivation to active tuberculosis.

## ACKNOWLEDGMENTS

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