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Dissemination of *Mycobacterium tuberculosis* Is Influenced by Host Factors and Precedes the Initiation of T-Cell Immunity

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We report that dissemination of *Mycobacterium tuberculosis* in the mouse is under host control and precedes the initiation of T-cell immunity. Nine to eleven days after aerosol inoculation, *M. tuberculosis* disseminates to the pulmonary lymph nodes (LN), where *M. tuberculosis*-specific T cells are detected 2 to 3 days thereafter. This indicates that the initial spread of bacteria occurs via lymphatic drainage and that the acquired T-cell immune response is generated in the draining LN. Dissemination to peripheral sites, such as the spleen and the liver, occurs 11 to 14 days postinfection and is followed by the appearance of *M. tuberculosis*-specific T cells in the lung and the spleen. In all cases studied, dissemination to the LN or the spleen preceded activation of *M. tuberculosis*-specific T cells in that organ. Interestingly, bacteria disseminate earlier from the lungs of resistant C57BL/6 mice than from the lungs of susceptible C3H mice, and consequently, C57BL/6 mice generate an immune response to *M. tuberculosis* sooner than C3H mice generate an immune response. Thus, instead of spreading infection, early dissemination of *M. tuberculosis* may aid in the initiation of an appropriate and timely immune response. We hypothesize that this early initiation of immunity following inoculation with *M. tuberculosis* may contribute to the superior resistance of C57BL/6 mice.

The ability of the host to recognize the presence of pathogens is crucial for the development of the adaptive immune response to an infection. As most organs are unable to generate an adaptive immune response in situ, the current model of T-cell-mediated immunity suggests that microbial antigens must be delivered to the draining lymph nodes (LN) before a T-cell response can be generated. For example, following cutaneous infection with *Leishmania major*, the trafficking of infected Langerhans cells to the draining LN is critical in the development of T-cell immunity. Although the dissemination of certain enteric and pulmonary pathogens from the initial site of infection occurs within days, other pathogens, such as *L. major*, can remain undetected for weeks, which significantly delays the onset of immunity (4, 9, 12, 21, 30, 39). If the presence of microbial antigens in the LN is important in activating antigen-specific T cells, then the timing of dissemination to the draining LN may be critical in determining whether the infectious agent or the host immune system ultimately triumphs.

Mycobacterium tuberculosis is a human pulmonary pathogen that poses a unique challenge for the host immune system. In addition to being a facultatively intracellular organism, it has special tropism for macrophages and has developed strategies for survival in these cells, such as inhibiting the fusion of the phagosome with the lysosome (38). This tactic may impair the ability of the immune system to recognize that an infection has taken place, since the processing and expression of bacterial antigens on the surface of infected cells are delayed. Little is known about the early events that follow infection of alveolar macrophages by *M. tuberculosis* which lead to the initiation of immunity. For example, it is not known whether *M. tuberculosis* disseminates from the lung as free bacteria or if the trafficking from the lung of infected myeloid cells leads to the spread of *M. tuberculosis*, as is now appreciated for other pathogens (20, 27, 35). The route of spread, whether hematogenous or via the lymphatics, also remains unknown. Finally, it is not clear what factors influence the movement of bacteria and their antigens from the initial site of infection to the draining LN.

Despite the fact that *M. tuberculosis* has developed strategies to avoid immune detection, a protective anti-*M. tuberculosis* response is generated in the majority of infected people. In fact, only 5 to 10% of infected individuals develop clinical disease despite the fact that *M. tuberculosis* establishes a latent infection, even in asymptomatic people (29). Clinically, it has been observed that impairment of cell-mediated immunity leads to an increased risk of reactivation tuberculous disease. However, tuberculosis also develops in individuals with no obvious impairment of the immune system, and it is currently thought that such individuals may have genetic polymorphisms that alter their susceptibility to disease (1, 8, 11). Proving this in humans can be difficult. However, the use of animal models has clearly established that the host genetic background modulates the generation of protective immunity. For example, differences exist in the survival of inbred mouse strains following inoculation with virulent *M. tuberculosis* (16, 25). It has been shown that susceptible and resistant mouse strains also differ in their histopathological and immune responses (7, 14, 15, 17, 33, 37). In addition, host genes that influence these survival differences, such as the allelic *sst1* locus which in part determines resistance or susceptibility of C57BL/6 (B6) and

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C3H mice to tuberculosis, are beginning to be identified (10, 19).

It has previously been observed that B6 mice have prolonged survival following aerosol or intravenous inoculation compared to the survival of susceptible C3H mice (17). In the present study, we sought to determine whether the initiation of the immune response to *M. tuberculosis* differs in these strains. We used an aerosol delivery system to experimentally inoculate mice with *M. tuberculosis* by the natural route of infection. This infection model allowed us to examine the influence of host factors on the early dissemination of *M. tuberculosis* from the lung, both locally to the pulmonary lymph node (PLN) and systemically to the liver and the spleen. We found that dissemination initially proceeded via the lymphatics to the draining PLN, where subsequently an antigen-specific response was first detected. Antigen-specific immunity was observed in the LN or the spleen only after bacteria had disseminated to that organ. Our analysis revealed that host genetic factors do influence the rate of initial bacterial dissemination from the lung. Surprisingly, bacteria disseminate from the lungs of resistant B6 mice earlier than from the lungs of susceptible C3H mice. Since this seems to promote earlier initiation of immunity and correlates with enhanced long-term survival, we suggest that effective host immunity requires early development of adaptive immunity.

MATERIALS AND METHODS

Mice. Female B6 and C3H/HeJ mice that were 6 weeks old were purchased from Jackson Laboratories (Bar Harbor, Maine). B6-SJL-PTprc{a}BoCrTac-Rag2^{tm1} N10 (B6 RAG$^{-/-}$), C3H/HeNTac-Rag2^{tm1} N12 (C3H/HeN RAG$^{-/-}$), and control C57BL/6Ntac and C3H/HeNTac-MTV (C3H/HeN) mice were originally purchased from Taconic (Germantown, N.Y.) and bred by us. Splenectomized and sham-splenectomized B6 mice were purchased from Taconic. For each experiment, experimental groups of mice were matched for both age (within 1 to 2 weeks) and gender. Mice were housed in a biosafety level 3 facility under specific-pathogen-free conditions at the Animal Biohazard Containment Suite (Dana Farber Cancer Institute, Boston, Mass.) and were used in a protocol approved by the institution.

Bacteria and aerosol infections. Virulent *M. tuberculosis* (Erdman strain) was originally obtained from Barry Bloom (Harvard School of Public Health, Boston, Mass.). The bacteria were passaged through mice, grown once in Middlebrook 7H9 medium supplemented with oleic acid-albumin-dextrose complex (Difco, Detroit, Mich.), and stored at −80°C as described previously (7). Mice were inoculated by the aerosol route by using a nose-only exposure unit (Intox Products, Albuquerque, N.M.). For each experiment, an aliquot was thawed, sonicated, and diluted to a concentration of approximately 4×10^6 CFU per ml in 0.9% NaCl–0.02% Tween 80. Preliminary experiments established that this concentration of *M. tuberculosis* allowed delivery of 350 ± 200 cells per mouse (mean ± standard deviation), an inoculum which we chose based on its ability to cause progressive lung pathology (28). A 10-ml suspension of *M. tuberculosis* was loaded into a nebulizer (MiniHEART nebulizer; VORTRAN Medical Technologies, Sacramento, Calif.), and animals were exposed to the bacterial aerosol for 20 min. The titer of the *M. tuberculosis* suspension was confirmed by plating serial dilutions onto 7H10 agar plates (Remel, Lenexa, Kans.).

CFU determination. The left lung, the spleen (in some experiments, one-half of the spleen), the left liver lobe, and the PLN were aseptically removed from euthanized animals. Blood was purged from the lungs by perfusing RPMI 1640 through the right ventricle of the heart after the inferior vena cava was severed. The tissue was homogenized in 0.9% NaCl–0.02% Tween 80 with a Mini-Bead Beater-8 (BioSpec Products, Bartlesville, Okla.). Viable mycobacteria were quantitated by plating 10-fold serial dilutions of organ homogenates onto 7H11 Mitchison agar plates (Remel). Colonies were counted after 2 to 3 weeks of incubation at 37°C.

Preparation of cells. Spleens and LN were dissociated by grinding the tissue between sterile frosted-glass slides. Red blood cells were lysed by treating the cells for 5 min with lysis buffer (0.15 M NaCl, 1 mM $KHCO_3$, 0.1 mM Na EDTA; pH 7.3). Lung tissue removed as described above was minced and digested for 1.5 h at 37°C with 150 U of collagenase type IV (Sigma, St. Louis, Mo.) per ml. The tissue was then pressed through a 60-mesh cell sieve (Sigma) and then a 10-μm-pore-size nylon Falcon cell strainer (Fisher, Houston, Tex.) to remove debris. Red blood cells were lysed as described above. All cells were resuspended in complete medium (RPMI, 10% fetal calf serum, 2% HEPES, 10% L-glutamine, 10% penicillin-streptomycin, 10% essential amino acids, 10% nonessential amino acids, 10% sodium pyruvate, 0.1% β-mercaptoethanol, 0.2% NaOH) for use in in vitro restimulation assays (7).

In vitro restimulation assays. Splenocytes, lung cells, or LN cells (2.5×10^6 cells/ml) in a volume of 2 ml (splenocytes and lung mononuclear cells) or 0.2 ml (PLN cells) were incubated in complete medium containing 1 μg of concanavalin A per ml, in *M. tuberculosis* H37Ra sonicate (3) (diluted 1:1,000, 1:5,000, or 1:25,000 in complete medium), or in medium alone for 48 h at 37°C. Culture supernatants were assayed for cytokines by an enzyme-linked immunosorbent assay by using antibody pairs and cytokines from Pharmingen (San Diego, Calif.) (2). At the antigen concentrations used, a clear dose response was observed for both B6 and C3H mononuclear cells. For clarity, only the gamma interferon (IFN-γ) production in response to stimulation with the middle antigen concentration (1:5,000) is presented below. The antigen-dependent IFN-γ production observed in this assay was blocked by antibodies specific for class II major histocompatibility complex or CD4, and no IFN-γ was produced when splenocytes from infected RAG$^{-/-}$ (recombinase-activating gene knockout) mice were used. These observations suggest that cytokine production by CD4$^+$ T cells in response to class II major histocompatibility complex-presented antigens is measured by this assay. In the absence of antigen, no IFN-γ was detected, and all cell populations produced IFN-γ in response to concanavalin A at all time points tested (data not shown).

Histological analysis. Blood was purged and the lungs were fixed by perfusion with Z-fix (Anatech Ltd., Battle Creek, Mich.) via the right heart ventricle, followed by injection of Z-fix into the lungs via the trachea. Paraffin-embedded 5-μm-thick sections were stained with hematoxylin and eosin. Lung sections stained with hematoxylin and eosin were scanned with a Polaroid SprintScan (Polaroid Corporation, Boston, Mass.), and morphometric analysis was carried out by using the NIH Image software (Scion Inc., Frederick, Md.). Random lung sections from each mouse in a group were analyzed by using a threshold lesion size of 20 pixels^2. The percentage of infiltrated lung tissue was calculated for each lung section, and groups of mice were compared by using an unpaired *t* test.

Statistics. Two-way analysis of variance (ANOVA) with Bonferroni post tests was used to compare the bacterial burdens in the organs of B6 and C3H mice after log transformation of the individual CFU values. For samples that contained no colonies, the values were transformed by using $y = \log(y + 1)$. In some experiments, an unpaired *t* test was used. Statistical analysis of B6, B6 RAG$^{-/-}$, C3H, and C3H/HeN RAG$^{-/-}$ mouse strains was carried out by using a one-way ANOVA with a Tukey post test for multiple comparisons. All analyses were done by using the Prism software program (GraphPad, San Diego, Calif.).

RESULTS

Dissemination of *M. tuberculosis* is modulated by the host genetic background. To determine how host genetic background influences dissemination of *M. tuberculosis* from the lung, B6 and C3H/HeJ mice were infected aerogenically with approximately 350 CFU of virulent *M. tuberculosis* by using a nose-only aerosol exposure system. Under these conditions, the C3H/HeJ mice succumbed to the infection early after aerosol infection (median survival time, 65 ± 36 days [mean ± standard deviation; six experiments]), while B6 mice survived more than 220 days (three experiments). At various times after infection, four to six mice per strain were sacrificed, and the viable bacteria in different organs were quantitated. The bacterial burden in the lungs of C3H/HeJ mice ultimately surpassed that of B6 mice (data not shown). However, during the first 3 weeks following infection, the numbers of CFU in the lungs recovered from both strains of mice were nearly identical in all experiments performed, indicating that bacterial replication was similar for the two strains in the early phase of infection (Fig. 1A and see Fig. 6C; also data not shown). Extrapul-

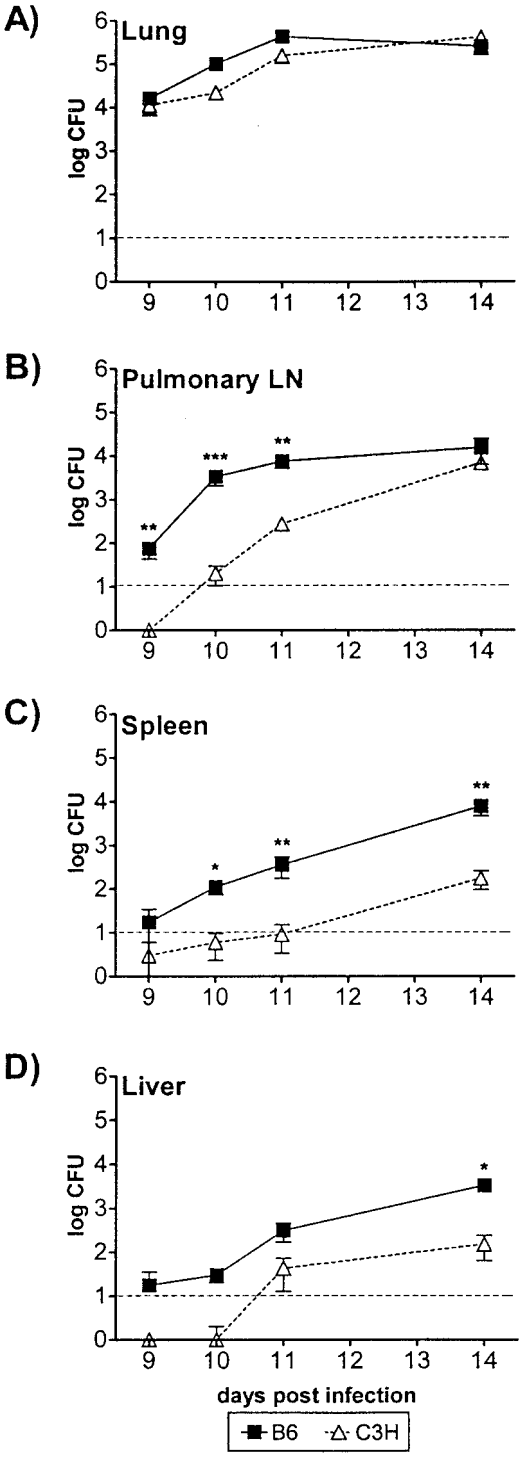

FIG. 1. Dissemination of *M. tuberculosis* following aerosol inoculation. B6 and C3H/HeJ mice were sacrificed at various times after infection, and the numbers of *M. tuberculosis* CFU in the lungs (A), PLN (B), spleens (C), and livers (D) were determined. The symbols indicate means, and the error bars indicate the standard deviations based on four to six mice per time point per group. The dashed line indicates the limit of detection of *M. tuberculosis* (10 CFU). A two-way ANOVA with Bonferroni post tests was used to test for statistically significant differences between the groups of mice, and the *P* values are indicated as follows: one asterisk, $P < 0.05$; two asterisks, $P < 0.01$; and three asterisks, $P < 0.001$. The data are representative of the data obtained in three to five separate experiments.

monary bacteria appeared in the draining PLN by day 9 postinfection in B6 mice and by day 11 in the majority of C3H/HeJ mice (Fig. 1B). Thus, viable *M. tuberculosis* emigrated from the lungs earlier in resistant animals. Dissemination to the local PLN was followed in 1 to 3 days by systemic dissemination to other organs. Again, this dissemination occurred earlier in B6 mice than in C3H/HeJ mice (Fig. 1C and D). For example, *M. tuberculosis* was detected by day 10 in the spleens of B6 mice, but it was not detected until day 14 in the spleens of C3H/HeJ mice (Fig. 1C and D). Fourteen days following infection, 10- to 100-fold more bacteria were typically found in the spleens of B6 mice than in the spleens of C3H/HeJ mice. *M. tuberculosis* was not detected in the axillary LN, which do not drain the lungs, until systemic dissemination had occurred (data not shown). These observations indicate that bacteria disseminate via the lymphatics to the draining PLN and then spread hematogenously to other solid organs, such as the spleen and the liver. Dissemination, both local and systemic, occurred earlier in B6 mice, suggesting that there is a delay in emigration of bacteria from the lungs in C3H/HeJ mice.

Dissemination is independent of B and T lymphocytes but is dependent on host factors. *M. tuberculosis* is an intracellular pathogen that infects macrophages, and under certain conditions it is able to survive by evading detection by the immune system. We entertained the hypothesis that the killing of infected macrophages by T cells could induce extracellular release of bacteria and lead to bacterial dissemination. Furthermore, the formation of inflammatory lesions would provide the bacteria with access to lymphatics and blood vessels. Alternatively, the adaptive immune response may play no role in inducing dissemination of bacteria. To test whether dissemination of *M. tuberculosis* from the lung was T and B cell independent, we inoculated B6, C3H/HeN, B6 $RAG^{-/-}$, and C3H/HeN $RAG^{-/-}$ mice by the aerosol route and measured the bacterial load in the spleen 14 days postinfection. We found that B6 and B6 $RAG^{-/-}$ mice had similar numbers of CFU in their spleens (Fig. 2A). C3H/HeN and C3H/HeN $RAG^{-/-}$ mice also had comparable numbers of CFU in their spleens. As usual, all mice with the C3H/HeN background had reduced numbers of CFU in their spleens compared with the numbers in animals with the B6 background. Similar results were obtained for the liver (data not shown). All mice had similar numbers of CFU in their lungs at this time point (Fig. 2B). Therefore, the timing of dissemination of bacteria from the lungs is determined by innate host factors and occurs independent of B and T cells.

Dissemination precedes the acquired immune response. The surprising finding that *M. tuberculosis* disseminates from the lungs of B6 mice earlier than from the lungs of C3H mice was difficult to reconcile with the superior resistance of B6 mice, unless dissemination has a previously unrecognized benefit to the host. We hypothesized that early bacterial dissemination may be beneficial if it leads to more rapid initiation of the adaptive immune response. To test this hypothesis, we studied the relationship between the dissemination of *M. tuberculosis* to the spleen and the development of a splenic immune response. At various times following infection, spleens were removed and divided in half. One half was used to determine the bacterial load, and the other half was used to

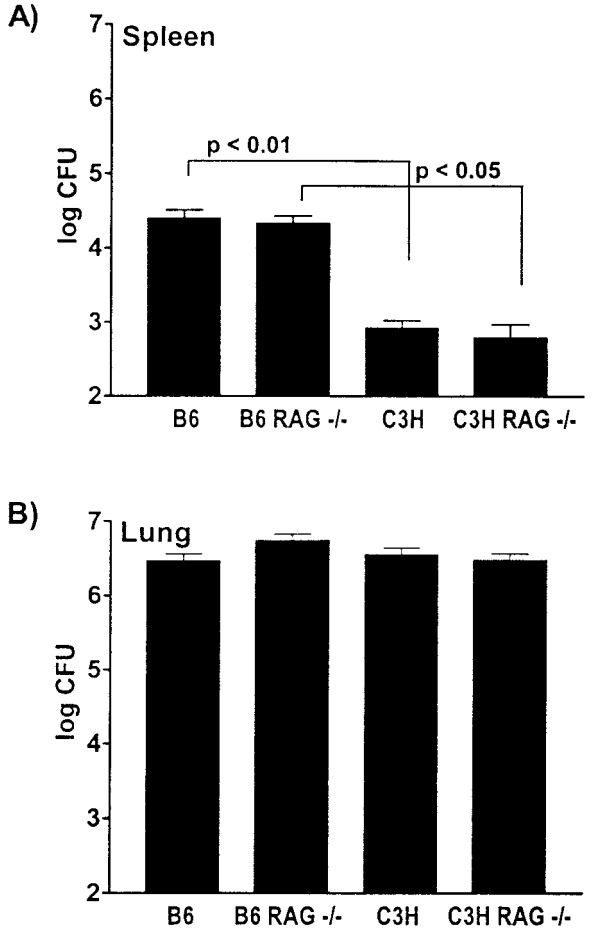

FIG. 2. Dissemination is similar in $RAG^{+/+}$ and $RAG^{-/-}$ mice. B6, B6 $RAG^{-/-}$, C3H/HeN, and C3H/HeN $RAG^{-/-}$ mice were sacrificed at 14 days postinfection. The numbers of *M. tuberculosis* CFU in the spleens (A) and lungs (B) were determined. The bars and error bars indicate the means and standard deviations, respectively, based on six mice per group. The number of lung CFU did not vary significantly among the four different strains of mice. The number of splenic CFU did not differ significantly between the B6 and B6 $RAG^{-/-}$ mice or between the C3H/HeN and C3H/HeN $RAG^{-/-}$ mice. Significant differences were detected between B6 and C3H/HeN mice and between B6 $RAG^{-/-}$ and C3H/HeN $RAG^{-/-}$ mice, as indicated. All analyses were carried out by using the one-way ANOVA with the Tukey posttest for multiple comparisons. The data are representative of the data obtained in four experiments.

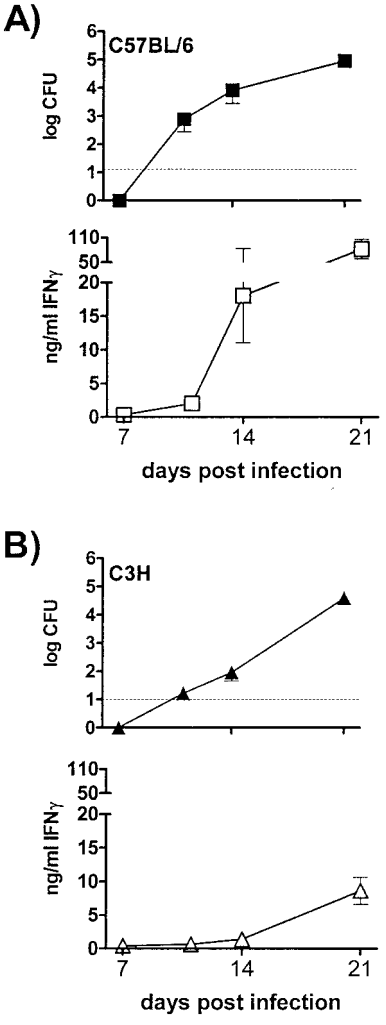

FIG. 3. Dissemination of *M. tuberculosis* precedes development of an acquired immune response. Spleens were removed from B6 mice (A) and C3H/HeJ mice (B) at 11, 14, and 21 days after infection. One half of each spleen was used to determine the number of bacterial CFU (solid symbols), and the other half was used in in vitro restimulation assays to determine IFN-γ production in response to *M. tuberculosis* antigen (open symbols). The dashed line indicates the limit of CFU detection (10 CFU). The amounts of IFN-γ produced after 48 h of stimulation with a 1:5,000 dilution of *M. tuberculosis* sonicate are shown. The error bars indicate the standard deviations based on six mice per group.

prepare splenocytes for an in vitro restimulation assay. It was not possible to carry out these assays in parallel for the PLN because of their small size.

We found that a splenic immune response was never detected in the absence of splenic CFU in more than 250 mice tested for both B6 and C3H mice (data not shown). *M. tuberculosis* began to appear in the spleens of B6 mice 11 days postinfection; however, an antigen-specific immune response by B6 splenocytes was not detected until 14 days postinfection (Fig. 3A). In contrast, dissemination to the spleens of C3H/HeJ mice did not occur until day 14, and a splenic antigen-specific immune response was not detected until 19 to 21 days postinfection (Fig. 3B). Interestingly, B6 splenocytes produced 10-fold more IFN-γ than C3H/HeJ splenocytes at 21 days postinfection. B6 splenocytes also made more interleukin-12 (IL-12) and tumor necrosis factor alpha than C3H/HeJ splenocytes (data not shown). Splenocytes from the two strains made similar amounts of IL-10 and did not produce IL-2 or IL-4 (data not shown). Thus, the early bacterial dissemination that was observed in B6 mice correlated with the early initiation of systemic antimycobacterial immunity, while the delay in dissemination in C3H mice was coupled with delayed activation of antigen-specific T cells.

The adaptive immune response is initiated in the PLN. Although development of the splenic immune response is a reasonable surrogate for systemic immunity, T-cell immunity is thought to be initiated in the draining LN. Since mycobacteria

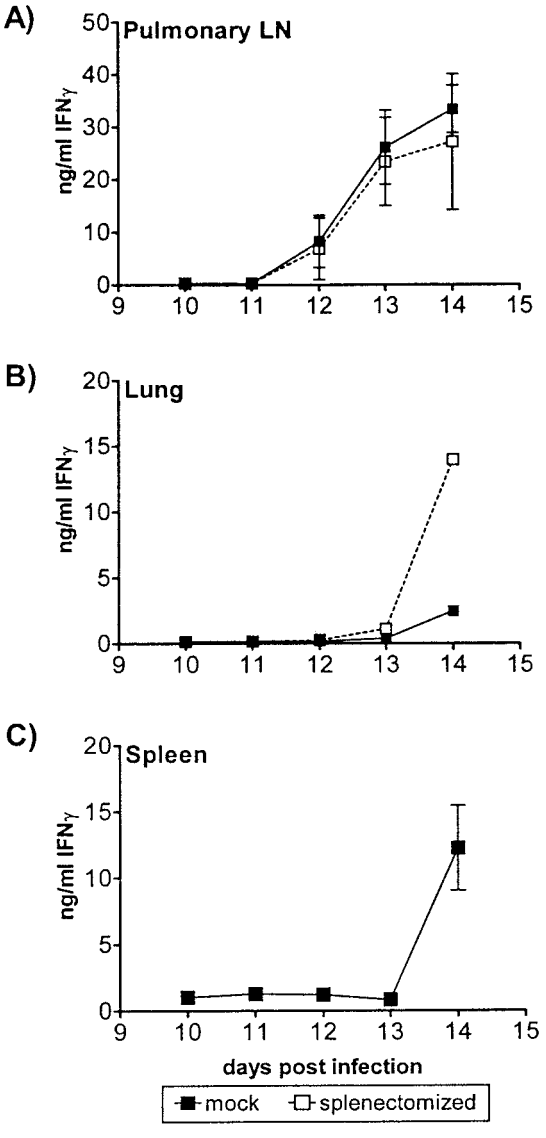

FIG. 4. Recall response to *M. tuberculosis* antigen by splenectomized or mock-splenectomized mice. At various times after infection, cell suspensions were prepared from the PLN (A), lungs (B), or spleens (C) of B6 mice which had been splenectomized or mock splenectomized prior to inoculation. The amounts of IFN-γ produced after 48 h of stimulation with a 1:5,000 dilution of *M. tuberculosis* sonicate are shown. The error bars indicate the standard deviations based on three to six mice per group. The spleens and LN were assayed individually. The lung mononuclear cells were pooled; thus, there are no error bars in panel B. The data are representative of the data obtained in two experiments.

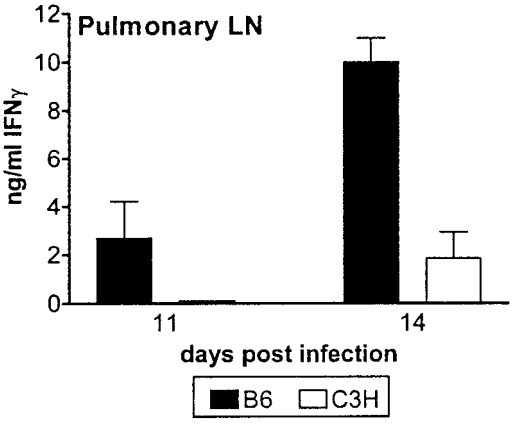

FIG. 5. An antigen-specific response can be detected earlier in B6 PLN than in C3H/HeJ PLN following infection with *M. tuberculosis*. PLN cells were prepared from B6 and C3H/HeJ mice 11 and 14 days postinfection and stimulated in vitro with *M. tuberculosis* antigen. The IFN-γ levels shown are the mean responses of cells to a 1:5,000 dilution of H37Ra sonicate, and the error bars indicate the standard deviations based on four mice per group. The groups of mice were compared to each other by using an unpaired *t* test, and $P = 0.003$ at day 14. The data are representative of the data obtained in two experiments.

first disseminate to the PLN, we sought to establish whether generation of the immune response follows the same pattern in the PLN as it does in the spleen. A recall response to *M. tuberculosis* antigen was first detected in the B6 PLN beginning on day 12, 3 to 4 days after the appearance of bacteria in these nodes (Fig. 4A). In contrast, the immune response in the spleen and the lung was not detected until day 14 (Fig. 4B and C). Interestingly, the antigen-induced IFN-γ responses of both B6 lung mononuclear cells and splenocytes occurred simultaneously. No antigen-specific T cells were detected in the peripheral axillary LN (data not shown). These findings strongly suggest that initiation of the immune response occurs in the draining PLN.

We examined the role of the spleen in generating an antigen-specific immune response to *M. tuberculosis*. Splenectomized and mock-splenectomized B6 mice were infected, and at various times the recall responses of lung, PLN, and spleen mononuclear cells (in mice that had spleens) to *M. tuberculosis* antigen were measured. The kinetics of the IFN-γ response in the PLN were similar in splenectomized and intact mice, and the responses were detected by day 12, 2 days earlier than the responses in the lungs and spleens were detected (Fig. 4). Interestingly, although the kinetics of antigen-induced IFN-γ production by lung mononuclear cells from splenectomized mice was similar to that in the sham group, the splenectomized group made more IFN-γ in response to *M. tuberculosis* sonicate (Fig. 4B). This finding is consistent with immune cells from the PLN entering the vascular circulation before homing to the lung. The spleen may trap antigen-specific lymphocytes, possibly due to the presence of antigen or infected cells. In the absence of the spleen, perhaps more antigen-specific T cells are able to home directly to the lung parenchymal tissue.

The delay in detection of systemic antimycobacterial immunity observed for C3H mice was most likely secondary to a delay in initiation of the immune response in the PLN. However, other possibilities included a delay in the emigration of T cells from the LN or an intrinsic defect in the initiation of the adaptive immune response. To distinguish these possibilities, the PLN recall responses in B6 and C3H/HeJ mice were compared. PLN cells from B6 mice made IFN-γ in response to exogenously added *M. tuberculosis* antigen both earlier and to a greater extent than cells from C3H/HeJ PLN (Fig. 5). Thus, as in the spleen, the IFN-γ response in the PLN is delayed in C3H/HeJ mice compared to the IFN-γ response in the PLN of B6 mice, and this delay is likely to be a result of slower dis-

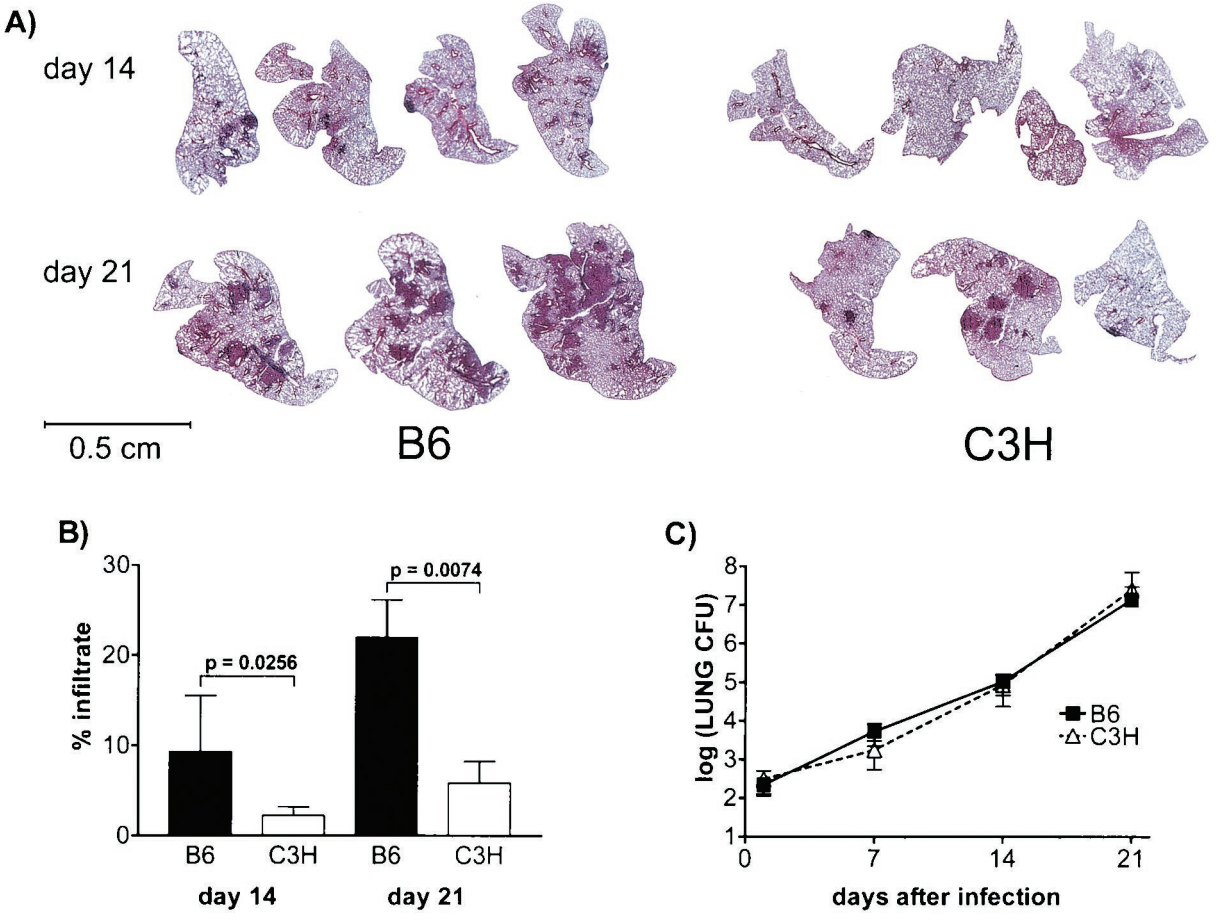

FIG. 6. Gross pathology of the lung and image analysis. (A) Lung sections from B6 and C3H/HeJ mice stained with hematoxylin and eosin. Three or four representative lung sections per time point are shown. (B) Percentages of infiltrated lung determined as described in Materials and Methods. Six mice were analyzed per strain per time point, and the data for the B6 and C3H/HeJ mouse strains were compared by using an unpaired *t* test. (C) *M. tuberculosis* CFU from the left lung of each mouse in panel A were quantitated. Two-way ANOVA with Bonferroni post tests did not reveal a statistically significant difference in the number of lung CFU between the two strains.

semination of live *M. tuberculosis* from the lung to the LN and not an intrinsic defect in initiation of the immune response.

Dissemination correlates with the appearance of pulmonary lesions. The cellular mechanisms of dissemination are unknown. Even the basic question of whether the bacteria disseminate in an intracellular or extracellular form remains unanswered. Our data suggest that bacteria initially emigrate from the lungs via the lymphatics, since bacteria were detected in the draining PLN sooner than in other organs. Lung tissue was examined at various times after aerosol inoculation to ascertain pathological correlates of dissemination. No lesions were identified by light microscopy in the lungs of B6 and C3H/HeJ mice 7 days following infection, and at this time little or no dissemination was observed (Fig. 1; also data not shown). By day 14, when B6 lung cells were just beginning to be able to make antigen-induced IFN-γ, the lungs in both strains of mice looked relatively healthy, with few lesions (Fig. 6A). The pulmonary lesions that were present contained predominantly macrophages and neutrophils and few lymphocytes (data not shown). Morphometric analysis revealed that the lung lesions in B6 mice were larger than those in C3H/HeJ mice (Fig. 6B). Interestingly, the pulmonary lesions in the lungs of infected congenic $RAG^{-/-}$ mice at this time looked quite similar to those in the lungs of $RAG^{+/+}$ mice (data not shown). The difference in lesion size was more apparent by day 21 (Fig. 6A). The pathological differences reflect variation between the B6 and C3H/HeJ host responses since the numbers of mycobacteria present in the lungs were virtually identical in B6 and C3H/HeJ mice at these times (Fig. 1A and 6C). Ultimately, the lesions in B6 mice evolved into granulomas containing lymphocytic infiltrates, whereas the premorbid lesions in C3H/HeJ mice consisted of areas of granulomatous inflammation punctuated by necrosis (data not shown). Although we cannot discriminate whether the formation of inflammatory lesions leads to dissemination or is a consequence of dissemination, the two processes are temporally linked and are a dramatic example of how allelic differences influence the host response to pathogens.

DISCUSSION

In human tuberculosis, little is known about dissemination of *M. tuberculosis* from the lung or the initiation of antimycobacterial immunity following infection. It is likely that *M. tu-*

berculosis disseminates from the lung parenchyma to the draining LN via the lymphatics based on the observation that the Gohn complex, a calcified granuloma in the lung periphery that is associated with similar lesions in the hilar LN, occurs in both symptomatic and asymptomatic people. However, the Gohn complex is a pathological finding, and when it develops during the course of infection is uncertain. To better understand the early events following *M. tuberculosis* infection in humans, we used a mouse model of *M. tuberculosis* infection to study bacterial dissemination and its role in the development of immunity in resistant B6 and susceptible C3H mice following experimental inoculation with virulent *M. tuberculosis* by the aerosol route. Two counterintuitive observations were made. First, bacteria disseminate from the lungs earlier in the resistant B6 mice than in the susceptible C3H mice. Second, pulmonary lesions develop more rapidly in B6 mice than in C3H mice. We were surprised that early dissemination and lesion formation are associated with resistance instead of progression of disease since at later stages of infection we have observed more bacteria and more tissue damage in the lungs of the susceptible mice. However, our study of the kinetics of antituberculous immunity following infection reconciled this discrepancy. We found that initiation of T-cell-mediated immunity occurs only after dissemination of live *M. tuberculosis* to the pulmonary LN has taken place. The earlier initiation of the adaptive immune response to *M. tuberculosis* may ultimately enable B6 mice to better contain the spread of bacteria within the lungs and other organs.

Although such results have not been previously obtained for mice, similar observations were made in the 1950s and 1960s by Lurie, who used rabbit strains bred for susceptibility or resistance to tuberculosis (13). Lurie noted that dissemination from the lungs to the draining LN was greater in resistant rabbits than in sensitive rabbits. The resistant rabbits also had earlier "allergic sensitization and antibody formation" and developed earlier pulmonary inflammatory lesions than the susceptible rabbits (13). Lurie attributed these findings to retention of more bacteria within the lungs of resistant rabbits upon infection and believed that resistant rabbits had a greater innate phagocytic capacity than susceptible rabbits. We do not believe that this is the case in our system because B6 and C3H mice have identical bacterial loads in the lungs at the time that dissemination takes place.

It is not known how and in what context *M. tuberculosis* moves from the lung to the local LN. Since lymphatics are absent from the alveolar walls, we thought that the appearance of inflammatory lung lesions would correlate with dissemination of bacteria from the lung. However, no lesions were observed at day 7, and few lesions were observed at day 14, although bacteria were detected as early as day 9 in B6 PLN. These observations argue that dissemination is not a consequence of tissue damage. Presumably, infected macrophages or dendritic cells could carry viable bacteria via the lymphatics to the draining LN, although *M. tuberculosis* has been reported to cross the bronchial epithelium via M cells under certain circumstances (31). The mycobacterial protein heparin binding hemagglutinin A (HbhA), which mediates the attachment of *M. tuberculosis* to epithelial cells and macrophages, is important in facilitating dissemination to the spleen (18, 22, 24). The absence of B cells has been shown to delay the dissemination of CDC1551 (a clinical isolate of *M. tuberculosis*) to the spleen and the liver in experiments performed with mice that lack mature B cells (BKO), although it has been reported by other workers using strain Erdman that the ultimate outcome of infection is not altered in these mice (5, 32). As in our studies, the delay in dissemination in BKO mice was coupled with a delay in the development of pulmonary lesions. Interestingly, our observations with the $RAG^{-/-}$ mice, which do not have B or T cells, differ, as we saw no effect of the RAG gene on early dissemination or granuloma formation in both B6 and C3H mice. Thus, we argue that components of the acquired immune response, such as mature B and T cells, do not play a role in early dissemination. Although it is difficult to reconcile our results with the results of the BKO mouse study, there may be differences in mice that have both B and T cells missing rather than mature B cells alone. In addition, different strains of *M. tuberculosis* were used in the two studies, and this may have affected the outcome. The observed association of the timing of dissemination with pulmonary inflammation, however, is in agreement with our findings.

Resistance to tuberculosis is multifactorial and is controlled by both environmental and genetic factors, many of which ultimately affect the efficiency of the host immune response to infection. Allelic differences between B6 and C3H mice have been identified in the *nramp1* gene and the supersusceptibility 1 (*sst1*) locus. Both are important in modulating susceptibility to mycobacterial infections; however, the mechanisms are unknown (10, 36). Our preliminary data suggest that neither the *sst1* locus nor the *nramp1* gene controls dissemination (unpublished observations). Our results with $RAG^{-/-}$ mice suggest that control of dissemination lies in the interaction of *M. tuberculosis* with innate components of the immune system. The toll-like receptors are a family of proteins that have a role in innate immunity (26, 34). Although the C3H/HeJ substrain has a defective *tlr4* gene, we did not find that this gene affected the survival of C3H mice in an intravenous inoculation model of tuberculosis (7). Similarly, the *tlr4* gene does not appear to affect dissemination (compare Fig. 1 and 2, which show results obtained with C3H/HeJ and C3H/HeN mice, respectively). Thus, although the specific genes remain to be identified, it is evident that the host genetic background modulates dissemination, possibly as a consequence of differences in cell trafficking, infectibility of host cells, host cell control of bacteria, or host cell survival.

Our data suggest how dissemination, initiation of T-cell-mediated immunity, and trafficking of immune lymphocytes are interrelated (Fig. 7). Following aerosol inoculation and initial infection of the lung (1), *M. tuberculosis* disseminates via lymphatic drainage to the local PLN by day 9 in B6 mice and by day 11 in C3H mice (2). Two to three days after dissemination to the PLN, antigen-specific T cells can be detected there (3). During this time, *M. tuberculosis* can be detected in the spleen and the liver by day 10 in B6 mice and by day 14 in C3H mice (4). Whether the bacteria that are hematogenously spread originate from the lung or the LN is unknown. It is possible that increased vascular permeability and tissue damage within pulmonary inflammatory lesions allow *M. tuberculosis* or *M. tuberculosis*-infected cells to penetrate blood vessels and enter the systemic circulation. Alternatively, *M. tuberculosis* could enter the circulatory system either through the effer-

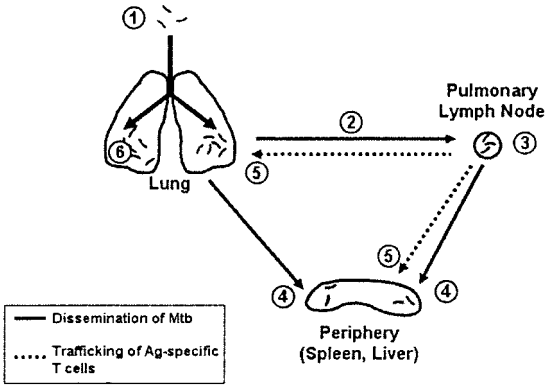

FIG. 7. Model of extrapulmonary dissemination and initiation of T-cell immunity. Mtb, *M. tuberculosis*; Ag, antigen.

ent lymphatics or through venous drainage of the PLN. By day 14 in B6 mice and by day 19 in C3H mice, antigen-specific T cells are detected in the spleen and the lung (5). It is possible that these T cells are activated in the PLN and traffic to the lungs and the spleen. In B6 mice, small pulmonary inflammatory lesions start to develop around this time, and by day 21 they are quite pronounced (6).

Although the timing of this process is delayed in C3H mice compared to B6 mice, dissemination always precedes the development of the T-cell response in both strains of mice. Thus, we believe that dissemination of *M. tuberculosis* from the lung is essential in the generation of T-cell-mediated immunity. Although intravenous infection does not convert C3H mice to a resistant phenotype, there is evidence from other studies that an earlier immune response reduces the severity of disease. When the same number of bacteria is seeded into the lung, infection by the aerosol route is more lethal than intravenous infection (23). This effect has been attributed to the more rapid induction of protective immunity in the intravenously infected animals (6). In the context of these two studies, our observations suggest that it is the delay in dissemination following aerosol inoculation that leads to the observed difference in mortality between intravenously and aerogenically infected animals.

We do not yet know whether the earlier dissemination and initiation of immunity observed in B6 mice contributes to their superior resistance. However, the comparison of B6 and C3H mice illustrates how host genes can affect dissemination and, in turn, how dissemination strongly correlates with the development of the immune response. Based on these results, we hypothesize that early bacterial dissemination may enhance host resistance, as it leads to earlier induction of the adaptive immune response.

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