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Gamma Interferon-Producing CD4$^+$ T Lymphocytes in the Lung Correlate with Resistance to Infection with *Mycobacterium tuberculosis*

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The human immune system efficiently limits the replication of *Mycobacterium tuberculosis* in most infected individuals. Only 5 to 10% of infected people develop clinical tuberculosis, a sign of the inability of the immune system to control the infection. We have studied the C3H/HeJ (C3H) and C57BL/6 (B6) inbred mouse strains, which differ in their susceptibility to tuberculosis, in order to ascertain the immunological determinants of a successful immune response against *M. tuberculosis* and to establish a system to identify genes that influence susceptibility to tuberculosis. We found that the resistant B6 mice were able to control infection in both the lung and spleen, while susceptible C3H mice were incapable of limiting bacteria growth, especially in the lung, and succumbed to infection within 4 weeks. We determined that the susceptibility of C3H mice was independent of the Toll-like receptor 4 (*tlr4*) genetic locus and allelic major histocompatibility complex differences. Although the splenic immune responses were similar in the two mouse strains, the local immune responses in the lungs of the infected mice differed greatly. The pulmonary immune response in resistant B6 mice was characterized by an early influx of both CD4$^+$ and CD8$^+$ lymphocytes that produced gamma interferon (IFN-γ). In contrast, the immune response of C3H mice in the lung was characterized by a delayed and decreased influx of lymphocytes, which produced little IFN-γ. These results suggest an important role for the early appearance of IFN-γ-producing lymphocytes in the lung in resistance to infection with *M. tuberculosis*.

It has been estimated that nearly a third of the world's population has been infected with the human pathogen *Mycobacterium tuberculosis*. The majority of infected adults acquire long-lasting immunity to the organisms; only 5 to 10% of infected individuals develop pulmonary disease. A compromised immune system caused by malnutrition, AIDS, or cancer is a risk factor for developing clinical tuberculosis; however, most individuals who develop reactivation disease later in life have none of these predisposing conditions. This susceptibility to *M. tuberculosis* is likely due to genetic differences in the population. This hypothesis is supported by the observations that pulmonary tuberculosis concordance was higher among monozygotic twins than dizygotic twins and the finding that the *nramp1* genotype correlated with disease among West Africans (5, 9, 20). Other polymorphic human genes have been identified, some of which affect the cellular immune response to *M. tuberculosis* and may influence the outcome of the host response to mycobacterial infection (4, 41). Similarly, differences have been observed in the survival of inbred mouse strains after inoculation with virulent *M. tuberculosis*, indicating that the genetic background can have a profound effect on the control of infection (32). These mice provide a valuable resource for the investigation of the genetic and immunological basis for susceptibility to tuberculosis.

Given the strong correlation between control of tuberculosis and cell-mediated immunity, the genetic differences between susceptible and resistant individuals are likely to involve the immune system. Several components of the immune system have been shown to be necessary for the development of protective immunity to tuberculosis. In addition to both CD4$^+$ and CD8$^+$ T cells, the infection of mice treated with cytokines or cytokine-blocking antibodies and, more recently, of knockout mice, has defined the roles of gamma interferon (IFN-γ), tumor necrosis factor alpha, interleukin-12 (IL-12), and IL-6 as essential for a protective immune response to *M. tuberculosis* (10–16, 24, 40). In contrast, the Th2 cytokines IL-4 and IL-10 do not appear to be involved in immunity (35). The discovery with mice that these cytokine pathways were critical led to the identification of defects in the genes encoding IL-12 and the receptors for IL-12 and IFN-γ in certain patients with severe atypical mycobacterial infections (1, 2, 22, 34). However, genetic defects have not been identified in the majority of patients and it is still not understood why some individuals develop immunity while others develop disease.

Studies using knockout mice have critically defined the role of certain cytokines in immunity to tuberculosis; however, an understanding of the role played by cytokines in an intact animal has not clearly emerged. In the present study, we investigated the immunological basis for the difference between susceptible C3H/HeJ (C3H) and resistant C57BL/6 (B6) mice after intravenous infection with virulent *M. tuberculosis*. We found a correlation between the appearance of IFN-γ-producing CD4$^+$ T cells in the lung and resistance to *M. tuberculosis*. Susceptibility in the C3H mice was manifested by an increased mycobacterial burden and reduced survival, which was associated with the delayed appearance of cytokine-producing pul-

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monary T cells. Although we cannot determine whether this difference represents a defect in lymphocyte recruitment or T-cell activation, these studies identify key immunological differences between resistant and susceptible inbred mouse strains.

MATERIALS AND METHODS

Mice. Six-week-old female C57BL/6 (B6), C3H/HeJ (C3H), C3H/HeOuJ, C3H.SW-H2(b)/SnJ, and (C57BL/6 × C3H/HeJ) F1 (B6C3F1/J) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). All 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 infections. Virulent *M. tuberculosis* (Erdman strain) was originally obtained from Barry Bloom (Albert Einstein College of Medicine, Bronx, N.Y.). The bacteria were passed through mice and subsequently grown once in Middlebrook 7H9 medium supplemented with oleic acid-albumin-dextrose complex (Difco, Detroit, Mich.) and stored at −80°C. Prior to injection into mice, an aliquot was thawed, sonicated twice for 10 s using a cup horn sonicator, and then diluted in 0.9% NaCl–0.02% Tween 80. Mice were infected intravenously via the lateral tail vein with 10^6 live mycobacteria. The size of the inoculum was confirmed by plating an aliquot onto 7H10 agar plates (Remel, Lenexa, Kans.).

Survival studies. Each experimental group consisted of 10 mice (range, 8 to 15), and after inoculation with *M. tuberculosis*, the mice were monitored for survival. The results were analyzed using the method of Kaplan and Meier, and the survival curves for each group were compared using the log rank test (Prism software package; GraphPad, San Diego, Calif.).

CFU determination. For each time point, three to five infected animals were used per experimental group. Lungs and spleens were aseptically removed from euthanatized animals. Prior to removal, lungs were perfused by injecting 5 to 10 ml of sterile phosphate-buffered saline (PBS) into the right ventricle of the heart after severing the inferior vena cava. The left lung and half of the spleen from each animal were homogenized in 0.9% NaCl–0.02% Tween 80 using sterile Teflon homogenizers (Fisher, Pittsburgh, Pa.). To quantitate viable mycobacteria, organ homogenates were serially diluted 10-fold and plated onto 7H10 agar plates (Remel). Colonies were counted after incubation for 3 weeks at 37°C.

Histology. Tissue was preserved in 10% buffered formalin, embedded in paraffin, sectioned, and stained either with hematoxylin and eosin, with trichrome, or for acid-fast bacilli (AFB) as previously described (3).

Preparation of mononuclear cells. The right lung and half the spleen from each infected or uninfected mouse were used to isolate mononuclear cells. Tissue was pooled from 3 to 5 infected or 5 to 10 uninfected mice. Spleens were ground between sterile glass slides, and the red blood cells were lysed in 0.15 M NaCl–1 mM $KHCO_3$–0.1 mM Na EDTA (pH 7.2 to 7.4). Splenocytes were then washed and counted. Pooled lungs were finely minced with razor blades and incubated at 37°C for 2 h with 125 to 150 U of type IV collagenase (Sigma, St. Louis, Mo.)/ml. Lung tissue was then pressed through a wire 60-mesh cell sieve (Sigma) and further filtered through a 70-μm nylon Falcon cell strainer (Fisher). Cells were resuspended in RPMI 1640-based media containing 10% fetal calf serum and supplemented with L-glutamine, nonessential amino acids, essential amino acids, sodium pyruvate, HEPES, 2-mercaptoethanol, and penicillin-streptomycin (complete media). The cell suspension was underlaid with Ficoll (density, 1.016). Density centrifugation was carried out for 20 min at 300 × *g* at room temperature. Lung mononuclear cells were obtained from the interface, washed, counted, and resuspended at 4×10^6 cells/ml in complete media.

Intracellular cytokine staining. Splenocytes and lung mononuclear cells were incubated at 37°C for 3.5 h with 10 μg of brefeldin A (Sigma)/ml with or without 10 ng of phorbol 12-myristate 13-acetate (PMA; Sigma)/ml and 1 μg of ionomycin (Sigma)/ml. Cells were then washed in fluorescence-activated cell sorter (FACS) buffer (5% fetal bovine serum and 0.02% NaN_3 in PBS) and blocked in FACS buffer containing 50 μg of anti-FcR [CD16/32] antibody (clone 2.4G2 from the American Type Culture Collection [ATCC])/ml. A total of 10^6 cells per condition were stained with antibodies to CD4 or CD8 (clones GK1.5 and 53-6.72, respectively, from ATCC) or a control immunoglobulin G2a (IgG2a; Pharmingen, San Diego, Calif.) for 20 min on ice. Cells were washed and fixed overnight at 4°C in 1% paraformaldehyde in PBS. Cells were subsequently washed and incubated with 2.5 μg of anti-cytokine antibodies (Pharmingen)/ml in Permeabilization Media B (Caltag, Burlingame, Calif.) for 20 min at room temperature. After two washings, the cells were analyzed using a FACSort (Becton Dickinson, San Jose, Calif.). The FlowJo software program (Tree Star, Inc., Stanford, Calif.) was used to analyze the data. Cell numbers were calculated as follows: (total number of cells isolated) × (% lymphoid cells) × (% $CD4^+$ or $CD8^+$ cells) × (% $CD4^+$ or $CD8^+$ cells making cytokine). Five mice were pooled for each time point, and the data have been normalized as cells per half organ. Experiments comparing the cytokine production by T cells from infected B6 and C3H mice were carried out four times. There were insufficient numbers of surviving C3H mice for analysis 4 weeks after infection in two of the four experiments, and consequently the data from the week 4 time point were excluded from the statistical analysis. A $\log_{10}$ transformation of the data was done to stabilize the variance. The data were analyzed by analysis of variance using the SAS general linear model (SAS Institute, Cary, N.C.).

RESULTS

Survival of C3H and B6 mice. After intravenous inoculation of 10^6 CFU of *M. tuberculosis* (Erdman), the survival of B6 and C3H mice was monitored. The mean survival time (MST) of B6 mice was greater than 210 days, indicating that B6 mice were relatively resistant to *M. tuberculosis* (Fig. 1A). In contrast, susceptible C3H mice died precipitously 4 weeks after infection (MST, 28 days), indicating that they were unable to carry out an effective immune response. Similar to the B6 parental strain, the (B6 × C3H) F1 progeny had a resistant phenotype, as has been observed by others (Fig. 1B) (32).

The major histocompatibility complex (MHC) locus was unlikely to play a role in determining susceptibility, as the survival of congenic C3H (*H-2*^{b}) mice that expressed the same MHC haplotype as B6 mice (*H-2*^{b}) was not significantly different from the survival of C3H (*H-2*^{k}) mice (Fig. 1B). C3H/HeJ mice are unique among the C3H substrains in that they carry a mutation in the *lps* locus that renders them unresponsive to lipopolysaccharide (LPS). The gene product of the *lps* locus has recently been identified to be the Toll-like receptor 4 (TLR4) (36). To determine whether the susceptibility of C3H/HeJ mice was a consequence of this mutation, the survival of C3H/HeOuJ mice, which do not carry the mutation, was compared to that of C3H/HeJ mice. No significant differences were found in the survival of these strains after intravenous inoculation with *M. tuberculosis*, indicating that the *tlr4* gene does not modify susceptibility in our model (Fig. 1C).

Control of infection by C3H and B6 mice. The ability of B6 and C3H mice to control the growth of *M. tuberculosis* in the lung and spleen was determined by measuring the mycobacterial burden in these target organs 1, 2, 3, and 4 weeks after infection. B6 mice, but not C3H mice, controlled mycobacterial replication in the lung and the spleen within 3 weeks of inoculation (Fig. 2). In the resistant B6 strain, the counts of viable *M. tuberculosis* in the lung and spleen increased during the first 14 days after infection to just greater than 10^6 CFU per half organ. Subsequently, B6 mice were able to control the infection as reflected by falling colony counts (Fig. 2). In contrast, the susceptible C3H mice were never able to significantly control the infection, especially in the lung, and peak colony counts approached 10^8 to 10^9 CFU per half lung by day 28. Therefore, the total mycobacterial burden in the lung of C3H mice exceeded that of B6 mice by nearly 200-fold.

Pathological changes in target organs. The lungs of both B6 and C3H mice contained numerous granulomas by 3 weeks after intravenous (i.v.) inoculation with *M. tuberculosis* (Fig. 3, panels 1 and 2). However, the pulmonary granulomas from the lungs of C3H mice were considerably larger. The histological appearance of the pulmonary granulomas from the lungs of resistant B6 mice had small focal areas of granulomatous inflammation dominated by macrophage/epithelioid and lym-

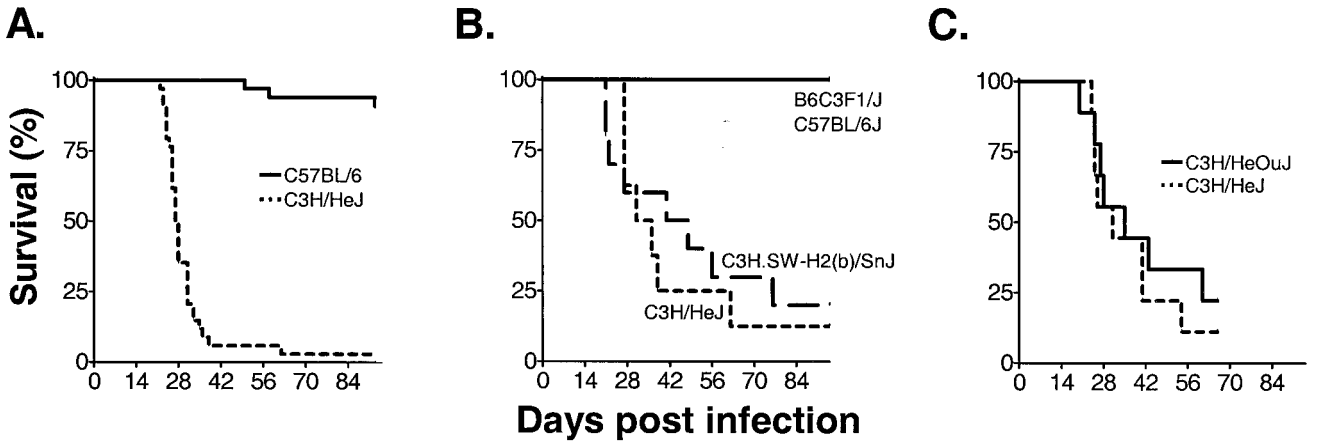

FIG. 1. Survival of C57BL/6J and C3H/HeJ mice following *M. tuberculosis* infection. (A) C57BL/6J (solid line) and C3H/HeJ (broken line) mice were inoculated i.v. with 10^6 CFU of *M. tuberculosis* (Erdman). The results were pooled from three independent experiments with a total of 33 or 34 mice in each group. The survival curves were generated using the Kaplan-Meier method, and the increased mortality of the C3H/HeJ mice was statistically significant ($P < 0.0001$ by the log rank test). C3H/HeJ MST = 28 days; C57BL/6 MST > 190 days. (B) Survival of C57BL/6, C3H/HeJ, C3H.SW-(*H-2*^{b})/SnJ, and B6C3F1 mice following *M. tuberculosis* infection. The survival of C3H/HeJ (*H-2*^{k}) and C3H.SW-(*H-2*^{b})/SnJ mice was similar following infection (*P*, not significant). In contrast, the prolonged survival of C57BL/6 and the F1 mice was statistically significant compared to the inbred C3H mouse strains ($P < 0.0001$). (C) Survival of C3H/HeJ and C3H/HeOuJ mice following *M. tuberculosis* infection. C3H/HeJ (dashed line) and C3H/HeOuJ (solid line) mice were infected as described above. Each group contained 10 mice, and the differences in survival between the two mouse strains following infection were not statistically significant by the log rank test. C3H/HeJ MST = 31 days; C3H/HeOuJ MST = 35 days.

phoid cell infiltrates without necrosis (Fig. 3, panel 3). In contrast, histological examination of lungs from susceptible C3H/HeJ mice 3 weeks after infection with *M. tuberculosis* revealed large focal areas of granulomatous inflammation with neutrophilic infiltrates and signs of early necrosis (Fig. 3, panel 4). By 4 weeks, the lungs of near-morbid C3H mice were nearly completely consolidated with extensive areas of necrosis and neutrophil infiltration (data not shown). While only scant collagen deposition was seen in the B6 inflammatory infiltrates, extensive fibrosis (as indicated by the blue-staining collagen fibers) was observed in the lungs of C3H mice (Fig. 3, panels 5 and 6).

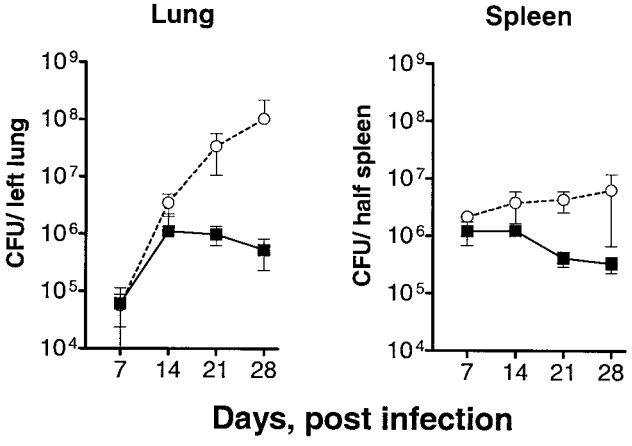

FIG. 2. Mycobacterial burden in the target organs from infected mice. C57BL/6J (solid line) and C3H/HeJ (dashed line) mice were inoculated i.v. with 10^6 CFU of *M. tuberculosis*. The number of CFU in the lung and spleen was determined weekly as described in Materials and Methods. Error bars represent the standard errors of the means, and the data are representative of four independent experiments.

Lastly, whereas only rare AFB could be detected in the lungs of B6 mice (Fig. 3, panel 7), the C3H lung tissue had abundant mycobacteria in large clumps consistent with unchecked bacterial replication (Fig. 3, panel 8). The extent of disease seen in C3H mice was reminiscent of that seen in IFN-γ knockout mice following *M. tuberculosis* infection (10, 14). Therefore, based on several criteria, the failure of C3H mice to control the infection early in its course had more severe pathological consequences, including lung consolidation, fibrosis, and necrosis, compared to B6 mice.

T cells in the spleen and the lung. As the immunological basis for the profound difference in the susceptibilities of the B6 and C3H mouse strains to tuberculosis is unknown, several parameters were studied to identify relevant host resistance factors. Mononuclear cells from lungs and spleens of C3H and B6 mice were isolated during the course of infection, and their phenotypes were analyzed by flow cytometry.

In the spleen, uninfected C3H and B6 mice had similar numbers of $CD4^+$ T cells ($\sim 5 \times 10^6$ per half organ) and $CD8^+$ T cells ($\sim 3 \times 10^6$ per half organ). Following infection, the $CD4^+$ subset, and particularly the $CD8^+$ subset, underwent greater expansion earlier in B6 mice than in C3H mice, although these differences were not statistically significant (Fig. 4).

While the lungs of uninfected C3H and B6 mice had similar numbers of resident lymphocytes ($\sim 25 \times 10^4$ cells per half lung), a dramatic difference in the number of pulmonary lymphocytes was observed after infection with *M. tuberculosis*. Compared to C3H mice, the lungs of B6 mice had an early and rapid appearance of large numbers of $CD4^+$ T cells. Two weeks after infection, B6 mice had nearly fivefold more pulmonary $CD4^+$ T cells than C3H mice. For example, in one experiment, B6 mice had 139×10^4 cells per half lung whereas

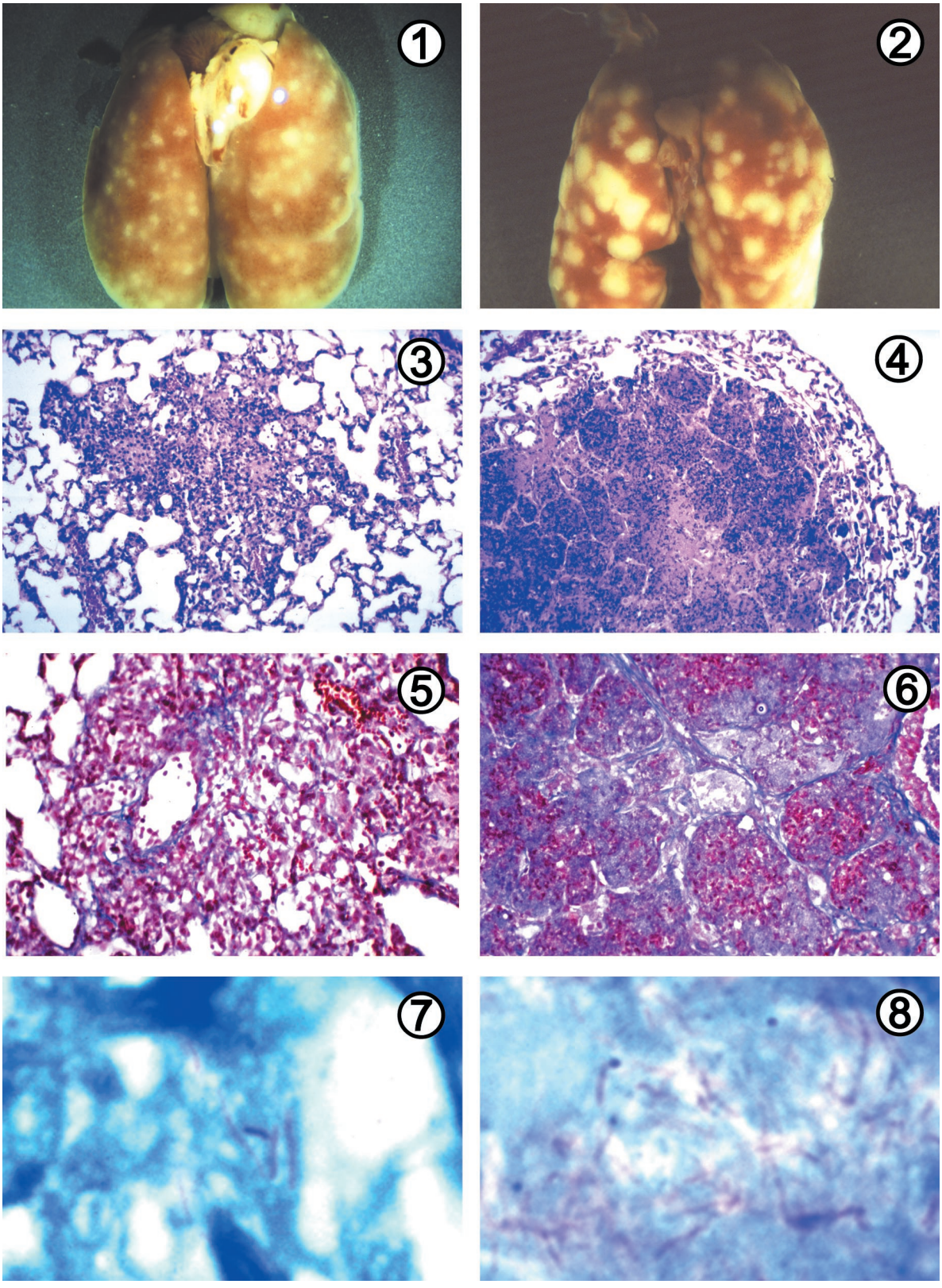

FIG. 3. Lung pathology from *M. tuberculosis*-infected mice. Lung tissue was obtained from C57BL/6J mice (panels 1, 3, 5, and 7) and from C3H/HeJ mice (panels 2, 4, 6, and 8) 21 days after intravenous inoculation with *M. tuberculosis*. The gross appearances of the lungs from *M. tuberculosis*-infected B6 mice (panel 1) and C3H mice (panel 2) are compared. Shown are representative sections of formalin-fixed paraffin-embedded lung tissue stained with hematoxylin and eosin (panels 3 and 4; magnification, ×25), with trichrome (which stains collagen fibers blue) (panels 5 and 6; magnification, ×50), or for AFB (panels 7 and 8; magnification, ×4,000). See text for details.

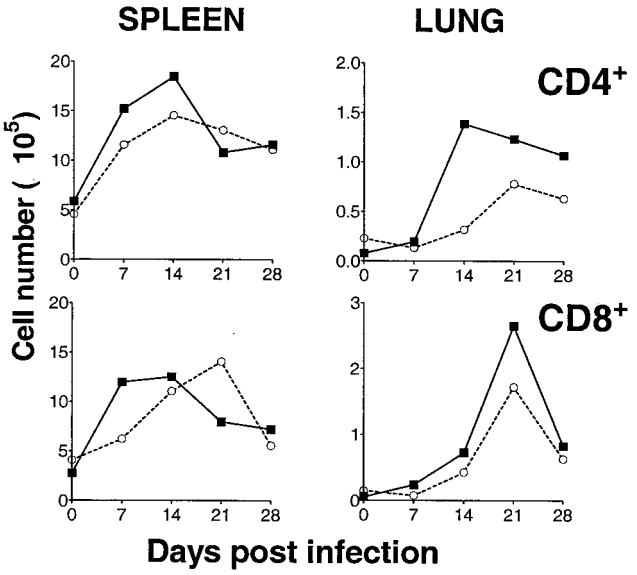

FIG. 4. The numbers of $CD4^+$ and $CD8^+$ cells in the lungs and spleens of C57BL/6J (solid line) and C3H/HeJ (dashed line) mice infected with *M. tuberculosis*. The data represent the number of cells per half organ. This figure is representative of four independent experiments, and the numbers have been normalized to cells per half organ. The data for uninfected mice (day 0) represent the mean of three independent determinations carried out using pooled tissue from 5 to 10 mice, and these numbers have also been normalized.

C3H mice had only 32×10^4 cells (Fig. 4). The number of $CD4^+$ T cells in the lungs of C3H mice began to increase by the third week; however, the appearance of these cells was delayed and their expansion was quite modest compared with the B6 response. Analysis of the four experiments indicated that this difference between B6 and C3H mice was statistically significant ($P = 0.0161$). Since C3H mice began to die 4 weeks after infection, this difference is likely to be biologically significant. The kinetics of $CD8^+$ T-cell recruitment or proliferation in the lungs of both mouse strains were similar and peaked at 3 weeks after infection. B6 mice consistently had more pulmonary $CD8^+$ T cells than did C3H mice during the course of infection, and this difference was statistically significant ($P = 0.0194$). The increased numbers of $CD4^+$ and $CD8^+$ T cells in B6 mice could reflect more efficient homing or recruitment of cells to the lung or increased T-cell proliferation within the lung.

Cytokine production in the spleen and lung. Since the early appearance of $CD4^+$ T cells in the lungs of C57BL/6 mice correlated with increased survival, cytokine production by these T cells was further studied. We used intracellular cytokine flow cytometry to determine cytokine production at the single-cell level. In order to enhance the sensitivity of cytokine detection, cells were briefly cultured with brefeldin A, which prevents cytokine secretion and leads to intracellular accumulation, and PMA and ionomycin, which increase cytokine production by previously committed T cells. Under these stimulation conditions, approximately 5 to 7% of splenic and lung mononuclear $CD4^+$ lymphocytes from uninfected mice produced IFN-γ (Fig. 5) and few, if any, unstimulated T cells produced the cytokine (Fig. 5). Three weeks after i.v. inoculation with *M. tuberculosis*, 9% of the splenic $CD4^+$ T cells produced IFN-γ (Fig. 5). A significant increase in IFN-γ^+ cells was observed in the lungs of infected mice, with intracellular IFN-γ detected in 69% of $CD4^+$ and 66% of $CD8^+$ T cells after stimulation (Fig. 5). In the absence of stimulation, little or no IFN-γ production was observed, even in infected mice (Fig. 5). We found no significant strain variation in the percentage of cytokine-producing cells from uninfected B6 and C3H mice (data not shown).

Splenocytes and lung mononuclear cells were isolated weekly from infected mice and were analyzed for the production of IFN-γ, IL-10, granulocyte-macrophage colony-stimulating factor, and IL-4. No IL-4 was detected during the first 4 weeks of infection in the spleen or lung. Although a small percentage of T cells produced granulocyte-macrophage colony-stimulating factor and IL-10, no significant differences between the B6 and C3H mice were appreciated (data not shown).

Cytokine production by T cells in the lungs of the two strains was dramatically different between the two strains throughout the entire course of infection. A significant increase in the number of IFN-γ-producing $CD4^+$ cells occurred by day 14 in the lungs of B6 mice and peaked at day 21, with 69% (85×10^4 cells) of the $CD4^+$ T cells producing IFN-γ (Fig. 6 and 7). In contrast, an increase in the number of IFN-γ-producing T cells in the lungs of C3H mice was delayed until day 21. At that time, only 40% (31×10^4 cells) of the $CD4^+$ T cells in the lungs of C3H mice produced IFN-γ, which was considerably lower than that seen in the lungs of B6 mice. In addition, appreciably more pulmonary $CD8^+$ T cells made IFN-γ in B6 mice than C3H mice. This difference was also greatest at day 21. Three weeks after infection, 40% (106×10^4 cells) of pulmonary $CD8^+$ cells from B6 mice made IFN-γ while only 27% (46×10^4 cells) of $CD8^+$ cells from C3H mice produced the cytokine (Fig. 7). Statistical analysis of the data from all four experiments revealed that the number of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in the lungs of B6 mice was significantly greater than that found in the lungs of C3H mice ($P = 0.0003$ and 0.0195, respectively).

Both $CD4^+$ and $CD8^+$ IFN-γ-producing T cells were detected in the spleens of B6 and C3H mice, and during the course of infection, an increase in the number of IFN-γ-producing splenic T cells was observed for both mouse strains (Fig. 7). In C3H mice, IFN-γ-producing T cells gradually increased in numbers during the course of infection and peaked by day 21. In contrast, B6 mice had an early increase in IFN-γ-producing T cells and these cells were already evident by day 7 (Fig. 7). This difference in the kinetics may explain the improved control of mycobacterial replication in the spleens of B6 mice compared to C3H mice (Fig. 2). However, we did not observe a statistically significant difference in the number of IFN-γ-producing $CD4^+$ or $CD8^+$ T cells in the spleens of B6 mice compared to C3H mice.

The rapid appearance of $CD4^+$ and $CD8^+$ IFN-γ-producing T cells in the lungs of B6 mice correlates with immunity to tuberculosis as measured by control of mycobacterial replication. C3H mice, in contrast, succumb to infection in part because the infection escapes control by the immune system during the delay in recruitment of IFN-γ-producing T cells to the lung (Fig. 7).

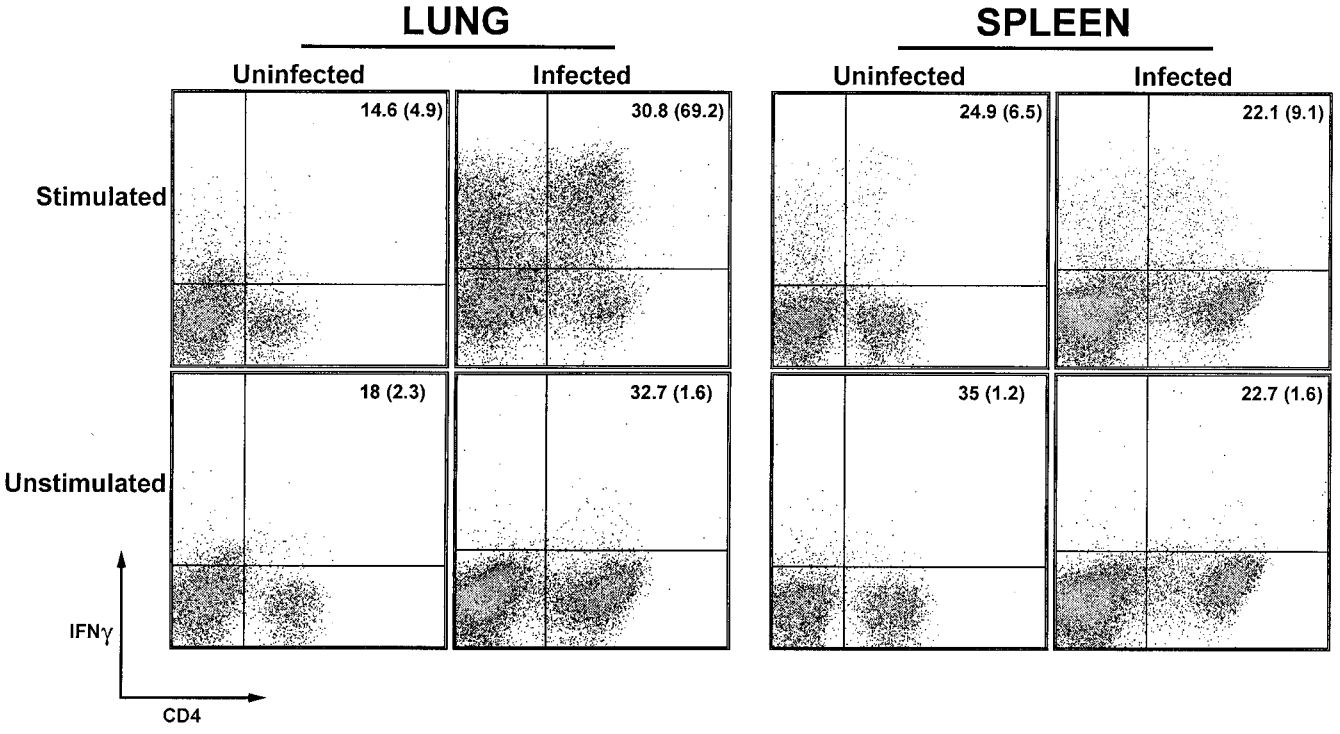

FIG. 5. IFN-γ production by lymphocytes from the spleens and the lungs of uninfected and infected C57BL/6J mice. Splenocytes and lung mononuclear cells were pooled from uninfected or infected mice 3 weeks after i.v. inoculation with *M. tuberculosis*. Cells were cultured in vitro with brefeldin A for 3.5 h, either in the presence (stimulated condition; upper row) or absence (unstimulated condition; lower row) of PMA and ionomycin. Flow cytometry detected IFN-γ production by $CD4^+$ lymphocytes as described in Materials and Methods. The numbers in the upper right quadrant of each dot plot are the percentages of lymphoid cells that were $CD4^+$ and (in parentheses) the percentages of $CD4^+$ cells that produced IFN-γ. These figures are representative of four independent experiments.

DISCUSSION

Tuberculosis is a chronic disease of the lung in murine models, regardless of whether the mice are inoculated intravenously or via the respiratory route. Despite this, little is known about the local pulmonary immune response since the attention of immunologists has only recently shifted from the spleen to the lung. This distinction is highlighted by our own studies in which we have begun to systematically study the immunological differences between intact inbred mouse strains that differ in their susceptibility to tuberculosis.

After inoculation with virulent *M. tuberculosis*, resistant C57BL/6 mice were able to generate a protective immune response in the lung. Mycobacterial replication in the lung continued for approximately 2 weeks following inoculation and was subsequently controlled, as manifested by a falling mycobacterial burden. The control of the infection correlated with the appearance in the lung of $CD4^+$ and $CD8^+$ T cells, a majority of which produced IFN-γ. Although the infection was not entirely cleared from the lungs, leading to the development of a persistent bronchopneumonia with granulomatous inflammation, C57BL/6 mice had prolonged survival.

In contrast to the effective immune response generated by C57BL/6 mice, protective local immunity to *M. tuberculosis* was not elicited in susceptible C3H/HeJ mice. Following infection, mycobacterial replication continued without evidence of significant control, resulting in a 10,000-fold increase in the number of mycobacteria found in the lung within 4 weeks. Although cytokine-producing T cells ultimately appeared in the lungs 3 weeks after infection, the response was ineffectual and the mice abruptly died at 4 weeks postinfection. The lungs of these mice were remarkable for massive consolidation and necrosis, reminiscent of the pathological findings seen in IFN-γ knockout mice (14). We believe that this similarity may be a consequence of the delayed appearance of IFN-γ-producing T cells in the lungs of C3H mice.

Given these results, we were surprised to observe that in the spleen mycobacterial replication was controlled in both mouse strains. In contrast to our findings in the lung, significant differences in the numbers of cytokine-producing cells in the spleen were not observed throughout most of the infection. We did observe an early burst of IFN-γ-producing cells in the spleens of B6 mice but not in C3H mice in all three experiments performed. This difference may account for better control of infection in the B6 spleen and, ultimately, a more efficient immune response in the B6 lung.

The differences in survival and immune response to *M. tuberculosis* infection are likely to be the result of allelic differences between the two strains (23). The known genetic differences between C3H/HeJ mice and C57BL/6 mice that affect immune function probably do not play a major role in determining resistance. One of the major allelic differences between the strains is the MHC ($H\text{-}2^k$ [C3H] versus $H\text{-}2^b$ [B6]). The MHC haplotype modifies the susceptibility of mice and humans to tuberculosis. Experiments using congenic strains of

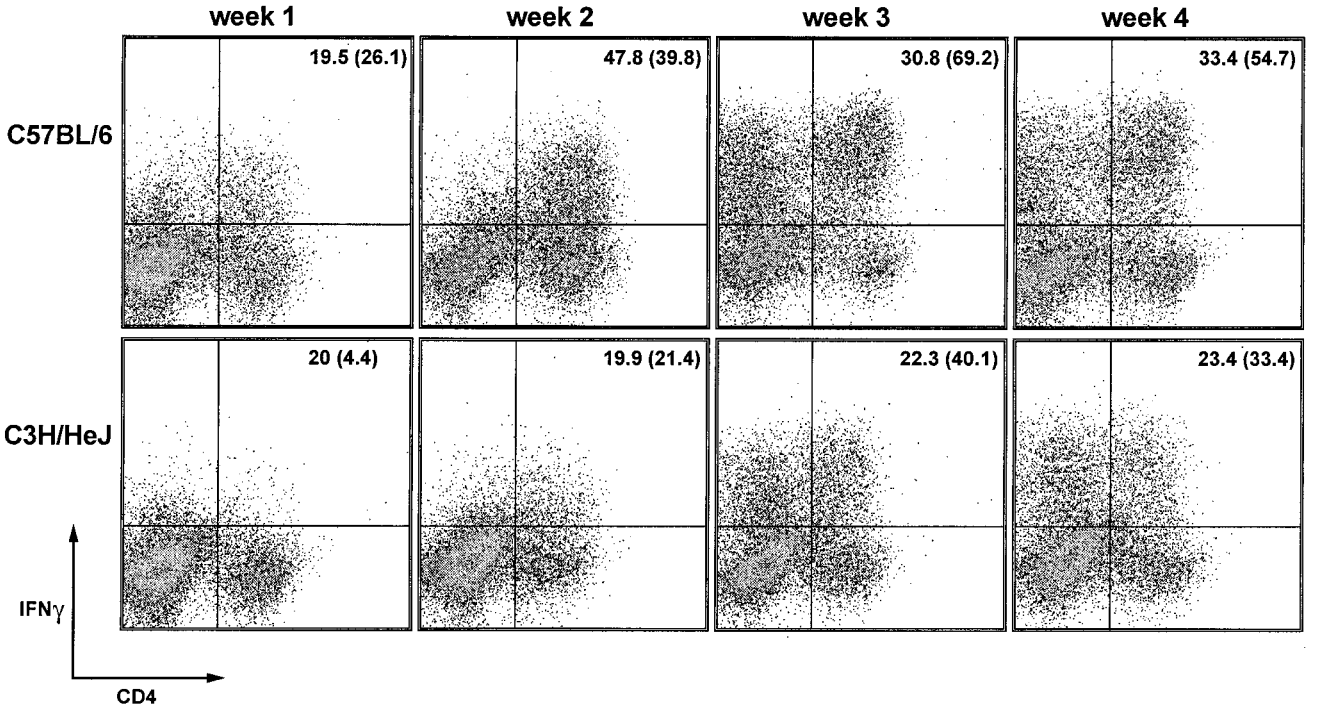

FIG. 6. IFN-γ production by lymphocytes from the lungs of C57BL/6J (upper row) and C3H/HeJ (lower row) mice after infection with *M. tuberculosis*. At weekly time points after i.v. inoculation, lung mononuclear cells were cultured with brefeldin A, PMA, and ionomycin for 3.5 h. Size-gated lymphoid cells were analyzed by flow cytometry to determine IFN-γ production. The numbers in the upper right quadrant of each dot plot are the percentages of lymphoid cells that were $CD4^+$ and (in parentheses) the percentages of $CD4^+$ cells that produced IFN-γ. These figures are representative of four independent experiments.

mice have shown that the *H-2*^{k} haplotype may variably modify susceptibility to tuberculosis (6, 7, 32). In our system, congenic C3H mice that carried the *H-2*^{b} haplotype were as susceptible to infection as their *H-2*^{k} counterparts.

Another important genetic difference is that C3H mice express the *bcg*^{r} allele whereas C57BL/6 mice express the *bcg*^{s} allele. Positional cloning of the *bcg* locus led to the identification of *nramp1*, a gene encoding an integral membrane phosphoglycoprotein expressed specifically in macrophages. The susceptible *bcg*^{s} allele has a single glycine to aspartic acid substitution at residue 169 in the Nramp1 protein which leads to degradation of the protein (17, 19, 39). Although the *bcg*^{r}/*Nramp1*^{Gly169} allele correlates with resistance to intracellular pathogens, including *Leishmania donovani, Salmonella enterica* serovar Typhimurium, *Mycobacterium bovis* BCG (BCG), and some strains of *Mycobacterium avium* (21, 39), it is not associated with resistance to *M. tuberculosis* (29–33). Thus, C3H/HeJ mice do not have a generalized defect in their ability to resist infections. In fact, they are less susceptible than C57BL/6 mice to infection by several bacteria, including *Salmonella, Leishmania, M. avium*, and BCG, perhaps because of their *nramp1* genotype (17).

Finally, C3H/HeJ mice are unique among all other C3H substrains because they harbor a mutation in the *lps* locus which makes them resistant to the effects of LPS. The *tlr4* gene, a member of the *toll* family of receptors, has recently been mapped to the *lps* locus. Since C3H/HeJ mice have a defective *tlr4* gene, LPS fails to induce an inflammatory response when administered parenterally or when cultured with C3H/HeJ macrophages. Mycobacterium cell walls do not contain LPS but instead the related glycolipid lipoarabinomannan, and recent studies indicate that macrophages from C3H/HeJ mice are activated by lipoarabinomannan despite their hyporesponsiveness to LPS (27). Our studies showing that there is no survival difference between the C3H/HeJ substrain and other substrains of C3H (C3H/SnJ and C3H/HeOuJ) that do not harbor the *tlr4* mutation support the emerging paradigm that TLR4 is an important mediator of macrophage activation during the LPS response, while activation of macrophages by mycobacteria, fungi, and gram-positive bacteria is mediated by TLR2 (8, 26–28, 37, 38). Further evidence that the susceptibility to tuberculosis is a feature of the C3H strain and is independent of the *lps* locus comes from the independent observation that the C3H/HeOuJ strain is resistant to BCG Montreal but susceptible to *M. tuberculosis* (H37Rv) (25).

Our findings suggest that susceptible C3H mice die because of an insufficient immune response in the lung that leaves the host unable to control mycobacterial growth and replication. This phenotype may result from a defect in lymphocyte activation, either from inefficient antigen presentation or subsequently during lymphocyte proliferation. Alternate explanations include an abnormality in lymphocyte trafficking or homing to the lung, defective effector function (either by the lymphocyte or by the macrophage), or an inability to sustain the immune response.

Since we consistently saw an early peak of IFN-γ-producing

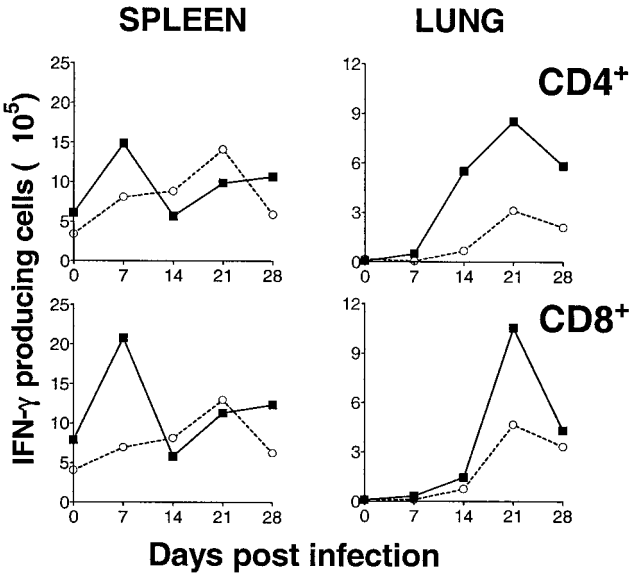

FIG. 7. IFN-γ production by $CD4^+$ or $CD8^+$ lymphocytes from the spleens and the lungs of C57BL/6 (solid line) and C3H/HeJ (dashed line) mice. At weekly time points after infection, splenocytes or lung mononuclear cells were cultured with brefeldin A for 3.5 h, either in the presence or absence of PMA and ionomycin. Cells were analyzed by flow cytometry as described in Materials and Methods. The data represent the number of cells per half organ. These figures are representative of four independent experiments. The data for uninfected mice (day 0) are the averages of three independent determinations carried out using pooled tissue from 5 to 10 mice and have been normalized to cells per half organ.

$CD4^+$ and $CD8^+$ cells in the spleens of B6 mice and not C3H mice, it is likely that the defect in C3H mice occurs during the initiation phase of the immune response. Despite their inability to control bacterial growth in the lung, C3H mice were able to limit mycobacterial replication in the spleen, albeit not as well as B6 mice. This dichotomy may be explained by the fact that aerobic *M. tuberculosis* organisms thrive in the local environment of the lung much better than in the spleen. In addition, the spleen, as a secondary lymphoid organ, is much better able to control infection than the lung. Thus, the weaker immune response initiated in C3H mice may be sufficient to curb bacterial growth in the spleen, while in the lung it is ineffectual.

The cytokine IL-12 plays an important role in immune initiation during *M. tuberculosis* infection. Infected macrophages produce IL-12, which induces T cells to produce IFN-γ. Thus, the impaired C3H response may result from defective or polymorphic genes that are critical in the IL-12 pathway. It would be of interest to determine whether C3H mice have a defect similar to that of BALB/c mice with respect to their ability to sustain a response to IL-12 because of a putative allelic variation in the *tpm1* locus (18). This possibility is particularly intriguing since both BALB/c mice and C3H mice are susceptible to intravenous infection with *M. tuberculosis* and, as with BALB/c mice, administration of exogenous IL-12 increases the resistance of C3H mice (S. Behar, unpublished observation).

Although several genes critical to the host immune response to *M. tuberculosis* have been identified using knockout mice, little is known about which genes may be important in determining relative susceptibility to tuberculosis in genetically intact and outbred populations. The existence of inbred mouse strains that differ in their susceptibility to tuberculosis indicates that host resistance is under genetic control. Our results indicate that allelic differences in genes that govern the immune response to tuberculosis could be the basis for the difference in susceptibilities of individuals in outbred populations. Although our study does not directly address which loci and genes underlie the susceptible phenotype of C3H/He mice, other investigators are mapping the relevant loci (3, 23) and our approach may provide functional data that will be useful in the interpretation of such genetic experiments. Furthermore, the identification of immunological parameters that correlate with susceptibility or resistance to tuberculosis will be useful in the assessment of new vaccines and pharmacological treatments for *M. tuberculosis*.

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