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Old Mice Are Able To Control Low-Dose Aerogenic Infections with *Mycobacterium tuberculosis*

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Previous work in this laboratory has led to the development of the hypothesis that the increased susceptibility of old mice to tuberculosis infection reflects a limited ability by immune CD4 mediator cells to accumulate at sites of bacterial implantation. To test this hypothesis with very low dose infections, the present study documented the course of a low-dose aerogenic infection with virulent *Mycobacterium tuberculosis* Erdman against time in the target organs of young (3-month-old) and old (24-month-old) B6D2F$_1$ hybrid mice. The results of the study indicated that the infection was controlled by the two groups of mice at similar rates, although the bacterial load in the old mice was eventually somewhat higher. Despite these similarities, some subtle differences between the young and old mice were also evident and included evidence of increased hematogenous spread of the infection from the lungs to other organs in the old mice. Interestingly, very poor expression of the cytokine interleukin-12 was observed in the lungs of infected old mice, leading to the hypothesis that the poor CD4 response in such animals could be partially attributed to the lack of this Th1-type, CD4 T-cell-enhancing cytokine. In this regard, treatment of old mice with exogenous interleukin-12 increased resistance and promoted gamma interferon secretion by CD4 T cells from these mice, although the effects were generally modest. These data suggest that old mice possess CD4-independent compensatory mechanisms by which to deal with low-dose pulmonary tuberculosis infections, although such mechanisms are less efficient than those seen in young animals.

It is a widely held belief that immune responsiveness gradually decays with age, rendering the animal more susceptible to infectious diseases (16). These diseases include tuberculosis, which remains relatively common in elderly humans (13).

Earlier studies in this laboratory showed that at a time when T-cell-mediated immunity in mice to intravenous infection with virulent *Mycobacterium tuberculosis* was strongly expressed in young mice, only very low levels of immunity could be detected in mice 24 to 28 months of age (10). As a result, the old mice had difficulty in controlling and containing the organism. This led us to hypothesize that the age-related increase in susceptibility to the tuberculosis infection reflected the inability of these mice to generate protective T cells against these bacilli.

We were able to disprove this hypothesis, however, in subsequent studies in which the course of the infection in young and old mice was monitored against time in terms of the capacity of T cells from such animals to secrete gamma interferon (IFN-γ) in response to mycobacterial antigens (12). These studies revealed that while IFN-γ secretion by CD4 T cells from 24-month-old mice was initially minimal, secretion of this cytokine by cells harvested on day 30 of the infection was equivalent to that of cells harvested earlier during the infection from younger animals. This result suggested that in old mice some sort of delay occurred in adequately focusing mediator T cells in infected tissues, and this notion was further strengthened by the observation (12) that CD4 T cells from old mice had substantially reduced expression of homing molecules such as L-selectin and CD11a, which are important in allowing lymphocytes to cross inflamed endothelial surfaces (7, 17).

These data suggested that immune CD4 T cells in old mice could indeed be adequately generated by the animal but were less capable of undergoing transendothelial migration and accumulation at sites of infection where immunity needed to be expressed. Further, because the initial inoculum of injected bacteria was substantial (10^5 intravenously), it was apparent that the bacterial load could easily reach fatal levels in the old mice prior to the occurrence of immunity.

If this was indeed the case, we reasoned that with a considerably lower infectious challenge, effective host resistance in the old mouse most likely could be mobilized in time. To test this idea, we compared the courses of tuberculosis infection in young and old mice exposed aerogenically to very low dose *M. tuberculosis*. The results of these studies confirm our notion to some extent by showing that the courses of infection in the lungs of these animals were similar. Despite this, however, a number of differences between young and old mice were still evident, including dissemination of the infection from the lungs and the cytokine and cellular responses in the lungs of these animals. In the latter case, mRNA for the cytokine interleukin-12 (IL-12) was poorly expressed in the lungs of the old mice, perhaps explaining the observed lack of CD4 cell response in this tissue. Administration of exogenous cytokine resulted in an increase in the expression of homing receptors on CD4 cells, an increase in CD4 message in the lungs, increased antigen-specific IFN-γ production, and a reduction in bacterial numbers. Together, these data suggest that old mice rely on compensatory mechanisms to contain tuberculosis infection in the lungs when the CD4 response is of reduced effectiveness.

MATERIALS AND METHODS

Experimental infections. B6D2F$_1$ hybrid mice of 2 and 24 months of age were purchased from the Trudeau Institute, Saranac Lake, N.Y., or from the National Institute of Aging colony at Charles River Inc., Wilmington, Del. For airborne

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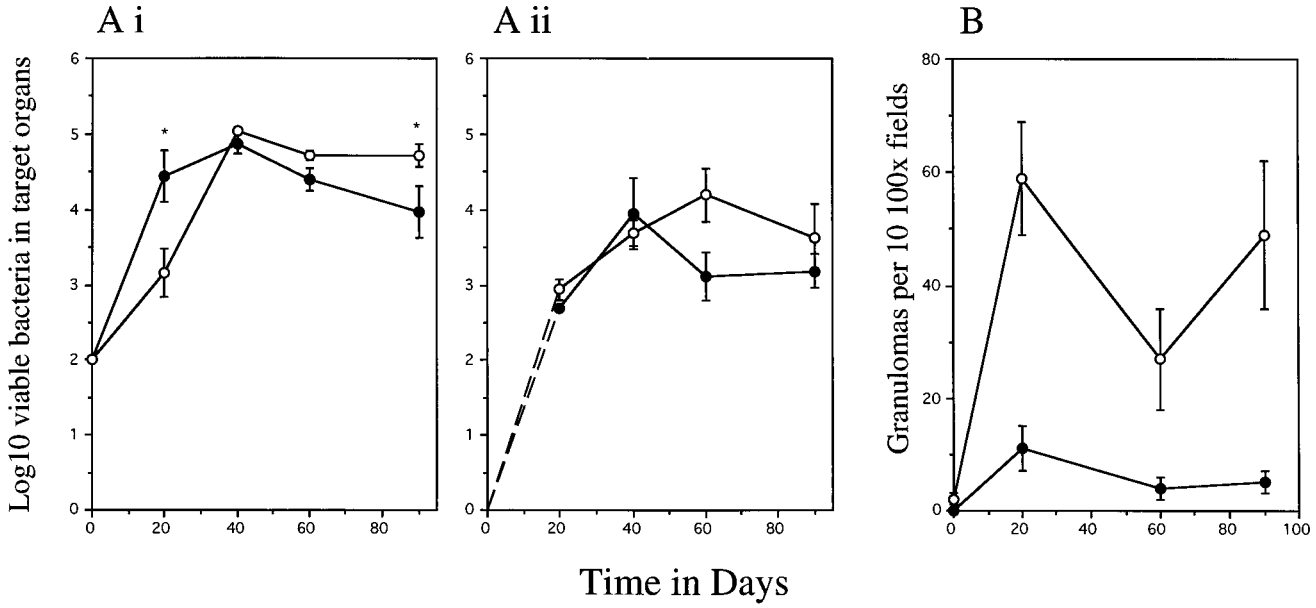

FIG. 1. Course of infection in young (●) and old (○) mice given 50 *M. tuberculosis* bacteria aerogenically. (A) Data shown are the mean numbers of bacteria in the lung (i) and spleen (ii) ± standard errors ($n = 4$). The rate of bacterial growth was significantly slower in old mice over the early phase of the infection (*, $P < 0.001$). (B) Livers of young and old mice were examined microscopically, and the number of granulomas in 10 low-power fields was measured. Values shown are the means ± standard errors ($n = 4$).

infections, mice were placed in the exposure chamber of a Glas-col Airborne Infection Apparatus (Glas-col Inc., Terre Haute, Ind.). The nebulizer compartment was filled with 10 ml of a suspension of *M. tuberculosis* at a concentration previously calculated to provide an uptake of approximately 50 viable bacilli within the lungs over a 30-min exposure. Harvested old mice were carefully examined following euthanasia for evidence of any unexpected pathology and discarded from the study if so identified. The course of bacterial infection was monitored against time by plating serial dilutions of individual whole-organ homogenates on nutrient Middlebrook 7H11 agar and counting bacterial colony formation after 21 days of incubation at 37°C in humidified air.

Analysis of levels of mRNA in infected tissues. Relative amounts of mRNA for IL-12 p35 chain, IL-12 p40 chain, tumor necrosis factor alpha (TNF-α), IFN-γ, IL-2, CD3, CD4, CD8, γδ T-cell receptor, macrophage inflammatory peptide 1α (MIP-1α), MIP-1β, interferon-inducible protein-10 (IP-10), macrophage chemotractant protein-1 (MCP-1), and inducible nitric oxide synthase (iNOS) were determined by a quantitative reverse transcriptase PCR protocol (18). Primers and probes for IL-12 p35 (5), IL-2, IFN-γ, hypoxanthine phosphoribosyltransferase, TNF-α, iNOS, CD8, CD4 (4), IL-12 p40 (19), MIP-1α, MIP-1β, and IP-10 (14) were as published. CD3 γ-chain primers were GCCATCTCCAAGGA AACCAAC and CTCTCTACTGGGCTCTCTCC, and the probe was GGTGT CCCCCCTTCTATCCA. JE primers were AGAGAGCCAGACGGAGGAAG and GTCACACTGGTCACTCCTAC, and the probe was CCAGATGCAGT TAACGCCCC. Briefly, total RNA was extracted from lung tissue with cold RNAzol (Cinna/Biotecx, Friendswood, Tex.). One milligram of RNA was then reverse transcribed, using a murine Moloney leukemia virus-derived reverse transcriptase (Gibco BRL, Grand Island, N.Y.), and amplified with *Taq* polymerase (*Thermus aquaticus* polymerase from Promega, Madison, Wis.). RNA was analyzed for hypoxanthine-guanine phosphoribosyltransferase mRNA (a housekeeping gene) as a measure of total readable mRNA in each sample. After amplification, the products were electrophoresed, transferred to Hybond N+ (Amersham, Arlington Heights, Ill.), and hybridized with the appropriate oligo probe labelled by an enhanced chemiluminescence system (ECL; Amersham). Light output was measured by determination of the pixel value detected on Hyperfilm-ECL (Amersham) analyzed on a Microtek Scanmaker IIxe and adjusted relative to the corresponding hypoxanthine-guanine phosphoribosyltransferase signal. The statistical significance of the difference between the mean signals from control versus experimental tissues was determined by the Student's *t* test, and the increase in signal was expressed as the fold increase over the control signal.

Administration of exogenous IL-12. IL-12 (a kind gift of Stan Wolf, Genetics Institute, Cambridge, Mass.) (15) was reconstituted in saline and administered at a rate of 0.4 μg of IL-12 intraperitoneally at the time of infection and every other day thereafter. The bioactivity of the IL-12 was 5×10^6 U/mg, and the preparation contained less than 1.2 endotoxin units per mg. Control animals received saline delivered by the same method.

Secretion of IFN-γ in vitro. Antigen-specific CD4 T cells were purified from the spleens of infected mice exposed to IL-12 in vivo. The T cells were then overlaid (10^6 per well) on cultured bone marrow-derived macrophages pulsed 24 h earlier with mycobacterial culture filtrate protein antigens as described previously (12). In addition, immune CD4 cells from non-IL-12-exposed mice were cultured in the same manner but with increasing concentrations of murine recombinant IL-12 added directly to the culture medium. Supernatants were harvested from both types of cultures 72 h later and tested for IFN-γ by two-site sandwich enzyme-linked immunosorbent assay, using antibodies R4.6A2 and XMG1.2, as previously described (12).

Flow cytometric analysis. Spleen cell suspensions harvested from infected mice at the indicated times were enriched for T cells and then stained and analyzed as described previously (12). Briefly, cells were stained with fluorescein-conjugated anti-CD4 (clone RM-4-5) and either anti-CD44 (clone IM7) and anti-CD45 (clone 16A) or biotin-labeled anti-CD11a (clone 2D7). The biotin-labeled antibody was detected with avidin-phycoerythrin (background labeling was approximately 1 U of fluorescence). All reagents were obtained from PharMingen (San Diego, Calif.).

Histology. Tissues from four mice per experimental group were perfused with fresh 10% formaldehyde in phosphate-buffered saline. Sections were made from paraffin blocks and stained with hematoxylin and eosin.

RESULTS

Course of aerogenic infections in young and old mice. The courses of virulent *M. tuberculosis* infection in the lungs (Ai) and spleens (Aii) of young and old $B6D2F_1$ mice are shown in Fig. 1. It was found that the bacterial infection grew to similar levels in both sets of mice. Interestingly, the initial rate of growth over the first 20 days of the infection was appreciably slower in the old animals, perhaps indicating better expression of innate immunity in these animals. By day 40, the growth of infection was halted in both sets of animals, although a better rate of clearance was seen in the young mice. Further, dissemination of the infection from the lungs to the spleen was seen in both groups.

In this regard, differences in the degree of control of disseminating bacilli was observed in the old mice. This control occurs regardless of the age of the animals, but in the present study we consistently observed much larger numbers of granulomas forming in the livers of the 24-month-old mice. In the young animals, approximately one granuloma could be seen

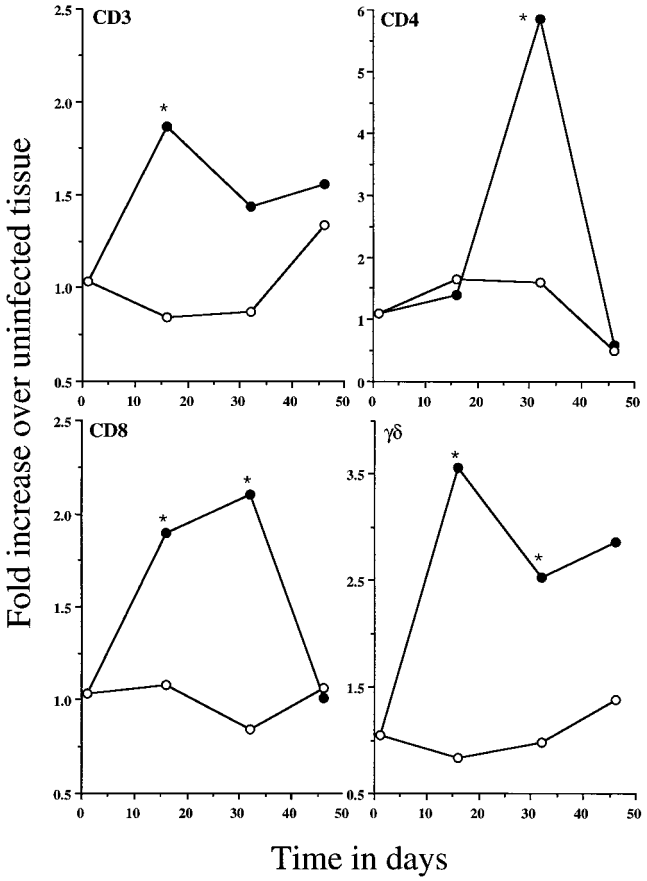

FIG. 2. Lung tissue from aerogenically infected young (●) and old (○) mice was analyzed for mRNA sequences specific for lymphocyte markers. Amplification was performed with cytokine-specific primers, and the product was blotted onto a nylon membrane. The DNA was quantitated with a fluorescein-labelled specific probe which was detected by horseradish peroxidase-conjugated antibody. The amount of antibody bound is directly related to the signal induced in a chemiluminescent substrate, which was detected on film. Datum points indicate the fold increase in signal in infected over control animals (n = 3 to 8). *, significant difference between the means of control and experimental signals as determined by Student's t test ($P < 0.05$).

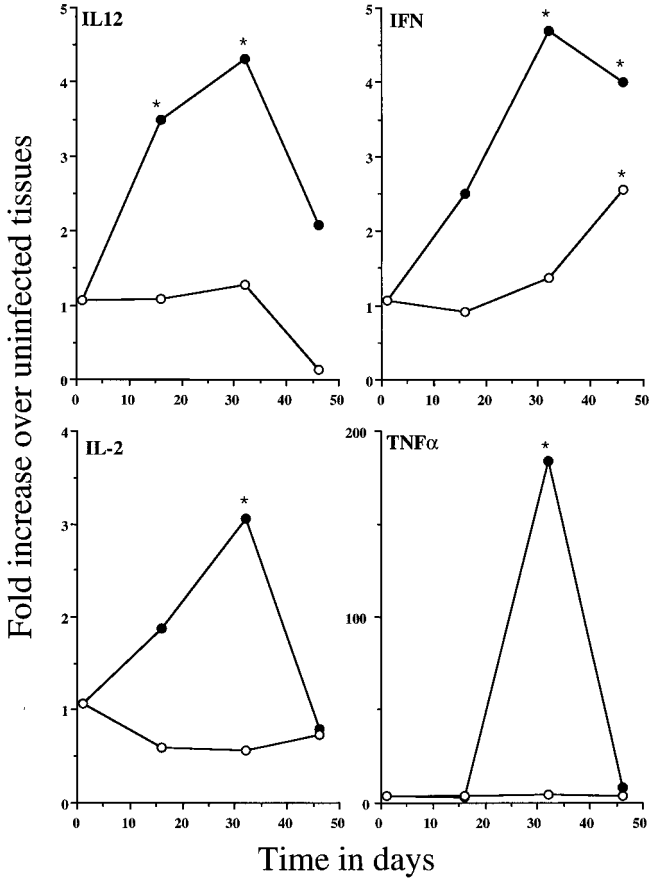

FIG. 3. Lung tissue was processed and analyzed for cytokine-specific sequences of mRNA as described in the legend to Fig. 2. *, $P < 0.05$.

per low-power field (n = four mice) in the livers on day 20 of the infection, whereas a mean of 5.8 granulomas was seen in equivalent fields in the livers of old mice (Fig. 1B). Given the early stage of the infection, these data indicate that hematogenous spread of the infection from the lungs occurred to a greater level in the old mice.

Young and old mice differ in expression of cytokine mRNA in response to aerogenic infection with *M. tuberculosis*. Although this study was unable to directly determine cellularity patterns in the lungs of infected mice, we were able to provide an indirect insight into immunological events in this organ by monitoring the expression of both key lymphocyte markers and cytokines against time. In Fig. 2, the absence of a specific lymphocyte response in the lungs of old mice is suggested by a complete lack of increase in mRNA for the cell markers CD3, CD4, CD8, and γδ T-cell receptor. In contrast, young mice demonstrate a significant increase in lymphocyte markers by day 15 of infection.

The absence of lymphocytes in old mice is mirrored by an apparent lack of cytokine response (Fig. 3). Only the mRNA for IFN-γ increases during infection in the old mice, and this increase occurs at the time of bacterial growth control. Other cytokines involved in the immune response, namely, IL-12 p40, IL-2, and TNF-α, are stimulated in young mouse lungs in response to infection but not in the lungs of old mice.

IL-12 is a heterodimer, composed of a constitutively expressed p35 chain and an inducible p40 chain (15), which has been demonstrated to strongly promote IFN-γ production in CD4 cells (1). mRNA for both chains was reduced in the old mice, with very little constitutive expression of p35 observed (Fig. 4).

Treatment of old mice with exogenous IL-12 increases their immunity to *M. tuberculosis*. The reduced expression of IL-12 mRNA in old mice was consistent with our previous hypothesis indicating a reduction in the effectiveness of the Th1 CD4 pathway in elderly mice. In view of this, we conducted experiments to determine whether exogenous treatment of old mice with IL-12 might increase their resistance to tuberculosis infection. The results of this experiment are shown in Fig. 5. Subsequent to an intravenous infection, bacterial growth in control 24-month-old animals was contained in the liver (Fig. 5Ai) and spleen (Fig. 5Aii); however, in the lungs (Fig. 5Aiii), the infection grew progressively over the 30-day experimental period. Injection of mice with 400 ng of IL-12 every other day reduced the bacterial load in each of the target organs, although the effect was modest. In vitro examination of immune CD4 T cells from IL-12-treated, infected old mice showed an increase in IFN-γ secretion in response to mycobacterial antigens compared with the same cells from saline-treated mice (Fig. 6), and the exogenous addition of IL-12 to ex vivo cul-

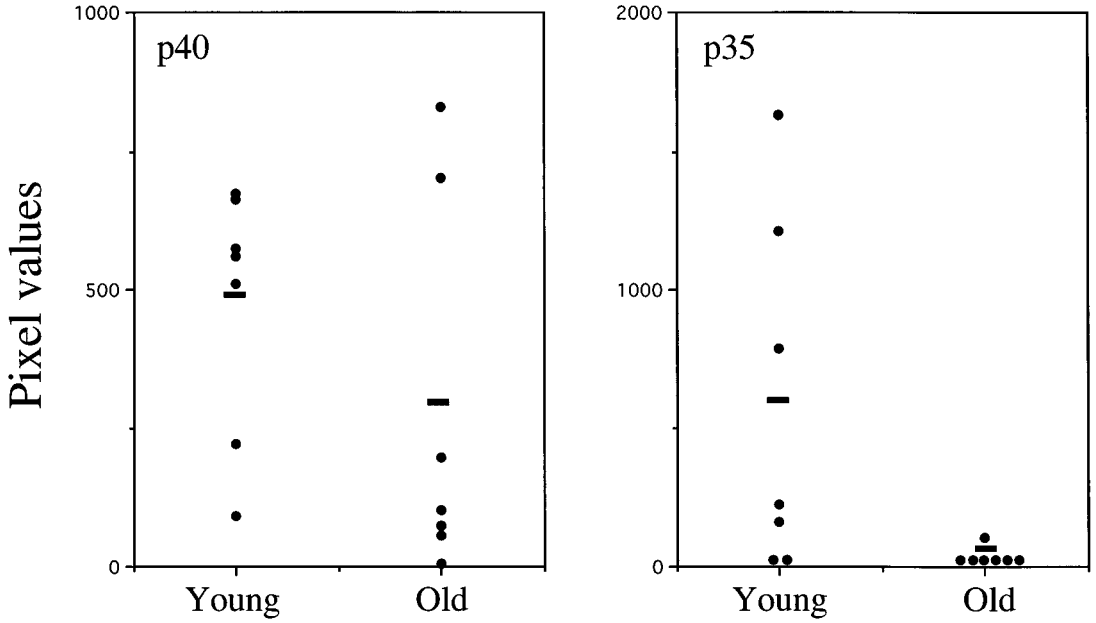

FIG. 4. Lung tissue from uninfected young and old mice was processed as described in the legend to Fig. 2. Data represent the pixel values taken from the film exposed to a Southern blot; this is related to the amount of mRNA, as described in Materials and Methods. The mean values are not statistically significant ($P = 0.09$); however, this pattern of reduced p35 and p40 mRNA expression in old mice was seen in three separate experiments.

tures of CD4 cells from infected old mice also increased their ability to produce IFN-γ; however, both effects were modest.

The protective effect of IL-12 was more pronounced in the lungs of mice infected by the aerogenic route (Fig. 5B), with a 2-log reduction in bacterial numbers. Thus, when the initial inoculum is low, the IL-12 is better able to augment immunity.

In further experiments, the effects of exogenous IL-12 treatment of old infected mice on the expression of T-cell activation markers were also examined. Spleen cells from old mice were harvested either just prior to or 18 days into the infection and analyzed by flow cytometry. Stained cells were gated for CD4 cells, and second-color analysis was performed on expression of the CD44, CD45RB, and CD11a markers. As shown in Table 1, treatment of mice with IL-12 increased the number of CD4 cells converting to the $CD44^{hi}$ phenotype and also increased the number of cells expressing the $CD45RB^{lo}$ phenotype. In addition, IL-12 treatment was further associated with an increased shift from the $CD11^{lo}$ to the $CD11^{hi}$ phenotype in the CD4 population.

Lymphocytes could not be isolated easily from the lungs of the aerogenically infected mice; however, comparisons of the mRNA from IL-12-treated and that from control mice were

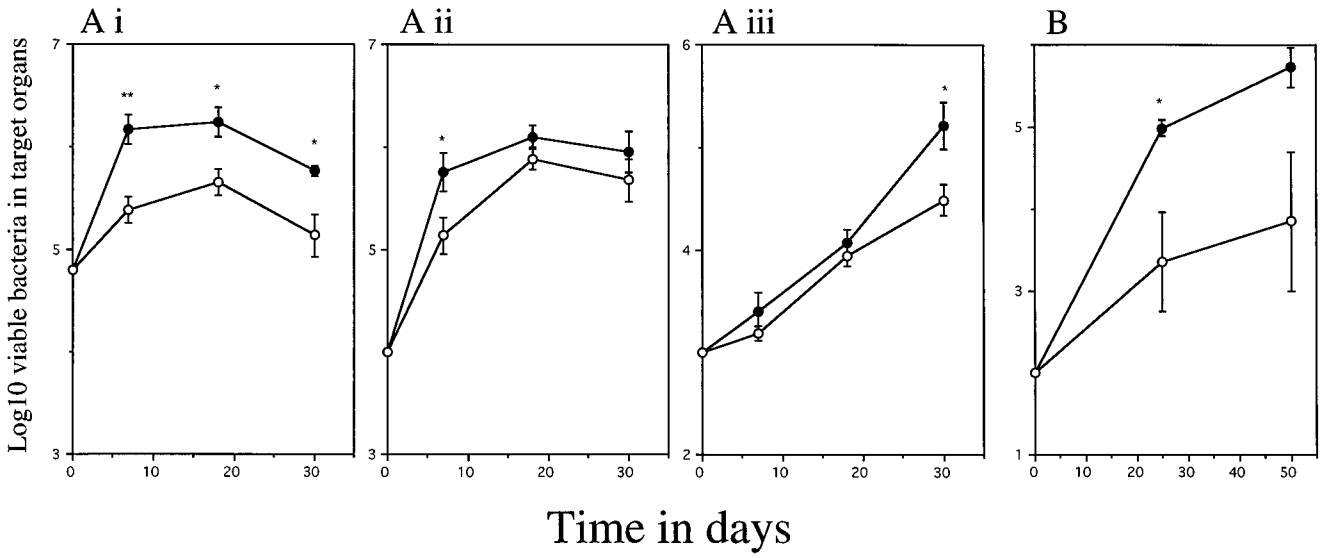

FIG. 5. Twenty-four-month-old mice were infected either intravenously (A) with 10^5 or aerogenically (B) with 100 *M. tuberculosis* Erdman bacteria and treated at the time of infection and every other day thereafter with 400 ng of recombinant IL-12 (○) or saline (●). Datum points are the means of four mice per time point (± standard errors). IL-12 was protective in the liver (**, $P < 0.001$), spleen, and lung (*, $P < 0.05$) in the systemic challenge (Ai, -ii, and -iii) and more strikingly in the lung in the aerosol model ($P < 0.05$) (B).

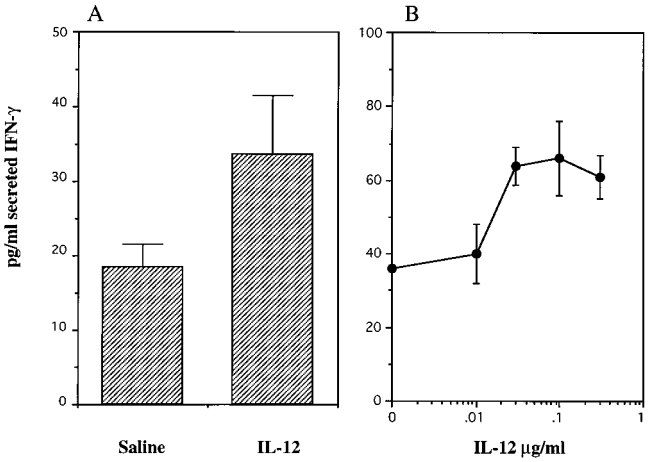

FIG. 6. (A) Treatment of *M. tuberculosis*-infected mice with IL-12 (400 ng every other day) increases the amount of IFN-γ secreted by CD4 T cells purified from the spleens of mice and cultured on antigen-pulsed macrophages. Data are expressed as the means (± standard errors) of triplicate wells ($P < 0.05$). (B) IL-12 enhances the release of IFN-γ by antigen-specific CD4 T cells. At day 30 of the *M. tuberculosis* infection, CD4 cells were harvested from the spleens of mice and cultured in vitro with antigen-pulsed macrophages and increasing doses of IL-12. Data are expressed as the means (± standard errors) of triplicate wells.

TABLE 2. Increase, induced by IL-12, in mRNA in the lungs of old mice aerogenically exposed to tuberculosis

Molecule	Day 25		Day 50	
	Fold increase[a]	P^b	Fold increase	P
IL-12 p35	5.99	0.002	0.91	0.878
IL-12 p40	4.64	0.002	0.72	0.167
MIP-1α	4.65	0.002	0.76	0.606
MIP-1β	8.57	0.002	0.51	0.331
IL-10	1.49	0.043	0.76	0.250
JE	1.69	0.100	0.43	0.279
IFN-γ	0.95	0.890	0.66	0.574
TNF-α	2.03	0.001	0.62	0.193
iNOS	1.89	0.001	0.71	0.193
CD3	1.13	0.113	1.02	0.811
CD4	2.14	0.001	0.75	0.018
CD8	2.74	0.001	0.75	0.022

[a] Increase over signal from saline-treated mice.
[b] P value for difference between means of the signals from saline- versus IL-12-treated mice.

possible. Exogenous IL-12 upregulated its own expression in vivo in addition to increasing message for the chemokines MIP-1α, MIP-1β, and, to a lesser extent, IP-10. The level of TNF-α, which augments the inflammatory process, was also increased in IL-12-treated lungs. Not surprisingly, increased message for the lymphocyte markers CD4 and CD8 was seen in the treated mice. The effect was not maintained in the absence of IL-12 treatment (see day 50, Table 2).

Histological analysis of infected lung tissue at day 25 revealed mononuclear cell inflammatory infiltrates in tissues treated with IL-12; these infiltrates were absent in saline-treated lung tissue. Similarly, liver sections from IL-12-treated mice demonstrate increased histiocytic and lymphocytic infiltration compared with untreated liver tissue (Fig. 7).

DISCUSSION

The central hypothesis under test in these experiments was that old mice are more susceptible than young mice to moderately high intravenous inocula of virulent *M. tuberculosis* because they have dysfunctional CD4 T cells that accumulate poorly at sites of infection, thus allowing the bacterial numbers to reach lethal levels. Under conditions in which very low infectious doses are used, we hypothesized that the longer time course of the progressive early phase of the infection might allow the CD4 cell response enough time to respond and halt the disease process.

In general, the results of this study do not tend to support this hypothesis in that they fail to show any direct evidence of an increased CD4 response to the low-dose infection. Thus, although the results clearly show that old mice are able to contain and control the aerosol infection at levels similar to those observed in young mice, a number of subtle but clear differences observed in the lungs of the old mice seem to suggest that the containment process may be due to compensatory mechanisms that the animal is able to generate in response to the low-dose infection.

These mechanisms appear to be at both the innate and acquired levels. In terms of the former, we consistently observed a noticeable slowing of the growth of the infection over the first few weeks of the infection. In this regard, macrophage populations in old mice seem to behave normally (11) and in fact may be able to express increased nonspecific resistance to intracellular bacterial parasites compared with that in young animals (6).

An important consequence of this adaptive response may have been the increased dissemination of the infection seen in the old mice. This was particularly evident in the liver, which suggests that leakage of bacilli from the lungs into the bloodstream is higher and perhaps more sustained in the older mice.

TABLE 1. Exogenous IL-12 alters the expression of adhesion and activation markers on CD4-positive T cells

T cells	Level of fluorescence	Gate boundary (channel no.)	Day 0				Day 18			
			Saline-treated mice		IL-12-treated mice		Saline-treated mice		IL-12-treated mice	
			% Cells within gate	Mean channel no.	% Cells within gate	Mean channel no.	% Cells within gate	Mean channel no.	% Cells within gate	Mean channel no.
CD44	Low	1–161	53	132	36	126	35	126	23	127
	High	161–256	48	187	65	187	66	188	77	196
CD45	Low	1–146	49	94	62	86	64	77	67	88
	High	146–256	52	171	39	175	37	169	34	173
CD11a	Low	1–114	45	82	26	85	51	75	47	76
	High	114–256	57	149	75	154	51	139	54	149

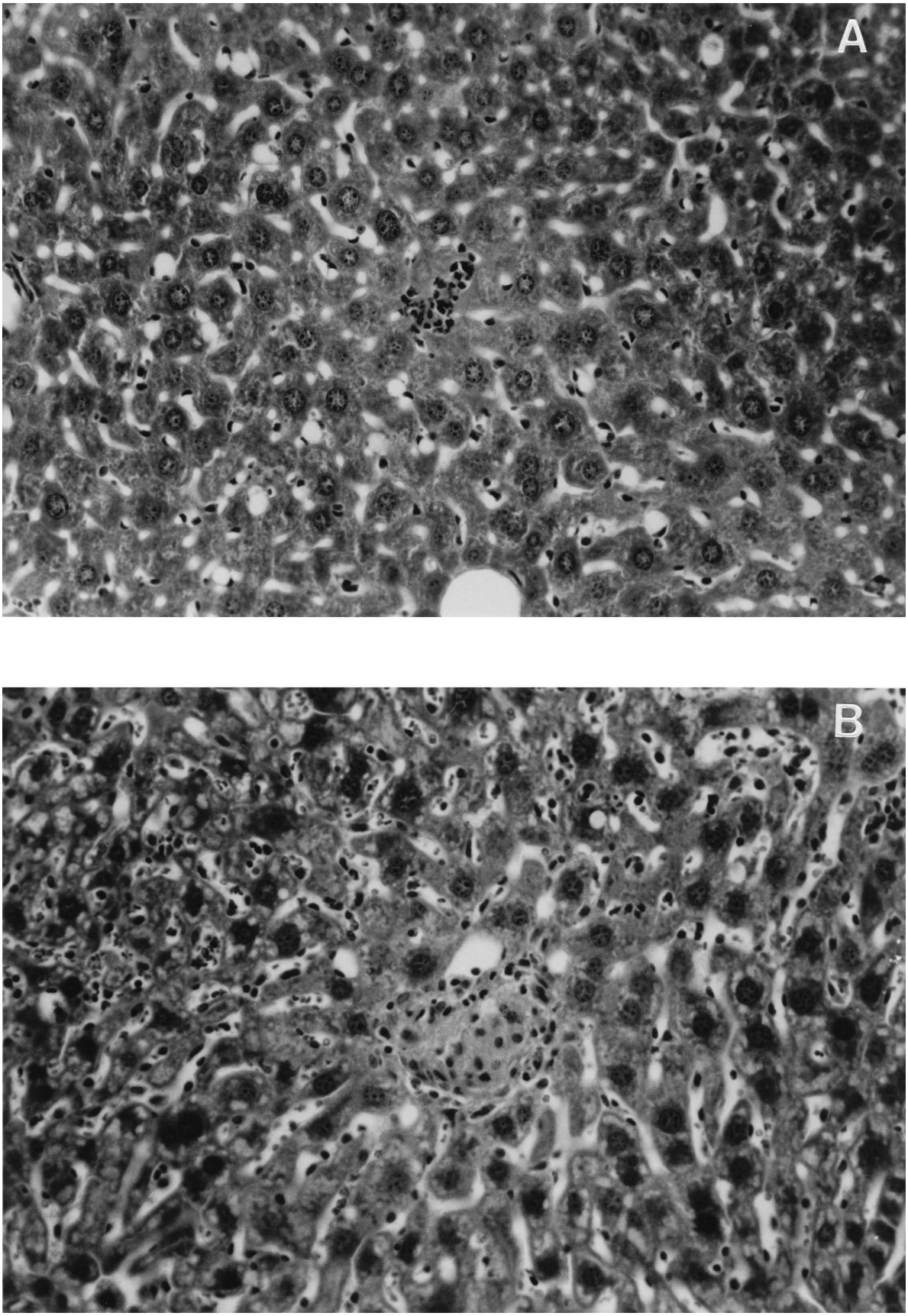

FIG. 7. Liver sections from saline (A)- and IL-12 (B)-treated mice infected 25 days earlier by aerosol. Less mononuclear cell accumulation can be seen in the saline-treated liver than in the IL-12-treated liver. Magnification, ×190.

This may in turn explain the high incidence of recrudescent tuberculosis in this animal model (10) and is in keeping with the clinical observation that tuberculosis in elderly humans often presents in a miliary form (8). Similarly, disseminated tuberculosis seen in human immunodeficiency virus-positive individuals also seems to point to the important role of the CD4 T cell in the containment of the infection (2).

Within the infected lung tissues, clear differences could be seen in terms of the expression of message for the cytokines. Unlike in young mice, in which the p35 chain of the IL-12 heterodimer is constitutively expressed, in old mice reduced expression of this molecule was detected prior to the aerosol exposure. In a study reported elsewhere (3), we have shown that IL-12 enhances the resistance of young mice to *M. tuberculosis* infection but that it does not appear to be essential, because although the resistance of these animals is lessened if neutralizing anti-IL-12 monoclonal antibodies are given in vivo, the infection is still slowly contained. In the present scenario, therefore, it appears that the dysfunctional nature of the CD4 population (in terms of poor expression of molecules that facilitate transendothelial migration) is further compounded by the lack of expression of the IL-12 molecule, which in turn fails to drive the expansion of the Th1-like, IFN-γ-secreting CD4 differentiation pathway.

Another observation from the study cited above was that granuloma integrity in young mice given anti-IL-12 monoclonal antibodies was diminished, which may help explain the increased "leakiness" of lung granulomas that is apparently occurring in the old mice. As shown in the present study, treatment of old mice with IL-12 increased resistance, stimulated IFN-γ secretion by CD4 T cells, upregulated expression of adhesion molecules, upregulated the local chemokine response, and resulted in more rapid accumulation of mononuclear cells. These effects were rather modest, however, and any potential therapy with IL-12 is further complicated by previous observations of the substantial toxicity of this molecule in vivo (3).

An obvious paradox in this study was the clear dysfunction in expression in old mice of the type of immune response seen in young mice but a similar ability to control the growth of bacteria in infected lungs in both young and old mice. Another paradox is that, in our experience, old mice appear to die when the lung bacterial load is in the region of 10^7 to 10^8 (10), while young severe combined immunodeficiency mice do not usually die until the bacterial load is around $10^{9.7}$ to 10^{10} (3). Thus, although old mice can control bacterial growth, the immune response induced may be more lethal than the actual bacterial load.

Having said that, the results of the present study do not confirm those of North (9), who observed progressive growth of a low-dose intravenous infection with *M. tuberculosis*, with bacterial loads in both young and old mice reaching levels in target organs well in excess of 10^{11}, and yet virtually no fatalities in these animals. We have never recorded such data in over a decade of experience with this model and are at a loss to explain his results.

In summary, the results of the present study further confirm the dysfunctional nature of the T-cell response in aging mice and show that one element of this diminished response may be related to a defect in production of the Th1 CD4-enhancing cytokine IL-12. In this low-infectious-dose model, other cellular responses, such as increased macrophage activation, may act as compensatory mechanisms, although such mechanisms appear to be less efficient in containing bacteria at the initial sites of infection.

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