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Role of Gamma Interferon and Tumor Necrosis Factor Alpha during T-Cell-Independent and -Dependent Phases of *Mycobacterium avium* Infection

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To design an effective immunotherapy for *Mycobacterium avium* infections, the protective host response to the infection must be known. Here we analyzed the role of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) in the innate and acquired responses to *M. avium* infections in mice. T-cell depletion studies showed that CD4$^+$ T cells were required for control of the infection. CD4$^+$-depleted mice showed enhanced bacterial proliferation and at the same time showed a reduction in the level of expression of both IFN-γ and TNF-α mRNAs in spleen cells. In contrast, *M. bovis* BCG immunization restricted *M. avium* proliferation and at the same time promoted expression of the mRNAs for the two cytokines. In vivo depletion studies using specific monoclonal antibodies showed that both IFN-γ and TNF-α are involved in an early protection possibly involving NK cells, and furthermore, IFN-γ is involved in the later T-cell-protective response to infection. In vivo neutralization of IFN-γ during *M. avium* infection also blocked the priming for enhanced TNF-α secretion triggered by endotoxin. Both cytokines were found to be involved in the resistance expressed in BCG-immunized animals and exhibited additive bacteriostatic effects in vitro on bone marrow-derived macrophages infected with different strains of *M. avium*. These data suggest that both cytokines act in an additive or synergistic fashion in the induction of bacteriostasis and that IFN-γ is also involved in priming TNF-α secretion.

Secondary *Mycobacterium avium* infections are very frequent in AIDS patients with CD4$^+$ T-cell counts below 100/mm^3 (22) and increase the morbidity and shorten the survival time of these patients (19). Management of antimicrobial chemotherapy of *M. avium* infections is difficult and is still the subject of clinical trials (18). Thus, it is highly important to understand the mechanisms involved in the control of this mycobacterial infection in healthy individuals so as to devise new therapeutic approaches for the treatment of *M. avium* infections, such as immunotherapy.

In the mouse model, it has been shown that CD4$^+$ T cells play a major role in the control of infection by atypical mycobacteria, such as *M. avium* (23) and *M. kansasii* (15). It is also apparent that other cell populations, such as natural killer (NK) cells, may also be involved in early protection against these infections (6, 17). Protection by both CD4$^+$ T cells and NK cells is thought to be mediated by cytokines produced by these cells in response to infection. In this regard, it has been demonstrated that tumor necrosis factor alpha (TNF-α) is protective both in vitro and in vivo (7, 8, 11). The role of gamma interferon (IFN-γ) is less clear, since it may have protective, as well as growth-promoting, effects in vitro (2, 10, 12, 14, 25). Moreover, Denis (11) was not able to show any protective effect of this cytokine in vivo. In addition, immunotherapy of AIDS patients with recombinant IFN-γ has had limited success (24). To assess the role played by both IFN-γ and TNF-α in the protection of mice from *M. avium* infection in vivo and to understand the mechanisms of protection, we tested the effects of the administration of neutralizing monoclonal antibodies (MAb) to these two cytokines during *M. avium* infection in naturally susceptible mice. We found that both IFN-γ and TNF-α cooperate in the induction of protection against *M. avium* at early and later time points of infection.

MATERIALS AND METHODS

Animals. Female C57BL/6 and BALB/c mice were purchased from the Gulbenkian Institute (Oeiras, Portugal). T-cell-depleted C57BL/6 mice were obtained by the following protocol. Mice were thymectomized at 4 weeks of age by suctioning the thymus gland through an incision made in the upper anterior part of the chest; 2 weeks later, the mice received an intravenous dose of either phosphate-buffered saline (PBS) (thymectomized controls) or 0.2 mg of anti-CD4 and/or anti-CD8 antibodies diluted in 0.25 ml of PBS. Two days later, the animals were given an intraperitoneal (i.p.) dose of 0.2 mg of the same antibodies or PBS. Animals were infected on the next day, and antibodies were then administered i.p. every 10 days at the same dose. C.B-17.*scid* (SCID) mice were purchased from Bommice (Ry, Denmark) and screened for the leaky phenotype.

Bacterial infections. *M. avium* ATCC 25291 (from the American Type Culture Collection), 2447 (an AIDS isolate obtained from F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium), 2-151 (both smooth, transparent and smooth, domed morphotypes isolated from an AIDS patient and obtained from John Belisle, Colorado State University), and 101 (another AIDS isolate obtained from L. Young,

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Kuzell Institute, San Francisco, Calif.) and *M. bovis* BCG, Pasteur substrain (TMCC 1011), were grown in Middlebrook 7H9 medium (Difco, Detroit, Mich.) until the mid-log phase, centrifuged, and resuspended in saline with 0.04% Tween 80 and frozen at −70°C until use. Mice were inoculated intravenously by injection of 10^6 CFU of *M. avium* through a lateral tail vein. At different time points, mice were sacrificed by cervical dislocation and the organs were collected under aseptic conditions. The organs were ground in tissue homogenizers, serially diluted in a 0.04% Tween 80 solution in distilled water, and plated onto 7H10 agar medium. The plates were incubated for 2 weeks at 37°C, and the numbers of colonies were counted. In some experiments, mice were immunized with BCG prior to the challenge with *M. avium*. For that purpose, mice were inoculated subcutaneously with 10^6 CFU of BCG and the infection was treated 1 month later with isoniazid (100 mg/liter of drinking water) for another 1 month. Mice were challenged 3 days later with *M. avium*. Controls consisted of age-matched animals that were also treated with isoniazid. The chemotherapy had been shown to be effective in clearing the BCG inoculum. In each experiment, four mice were used per time point.

Reagents and antibodies. Mycobacterial growth media were purchased from Difco (Detroit, Mich.). Cell culture media were from GIBCO (Paisley, Scotland). Isonicotinic acid hydrazide (isoniazid), Tween 80, saponin, and incomplete Freund's adjuvant were from Sigma (St. Louis, Mo.). Recombinant mouse IFN-γ was supplied by Genentech, and TNF-α was purchased from Genzyme (Cambridge, Mass.). Anti-T-cell subset MAb were obtained from the hybridomas GK1.5 (anti-CD4, TIB 207 cell line from the American Type Culture Collection) and 2.43 (anti-CD8, TIB 210 cell line from the American Type Culture Collection) growing in ascites in HSD nude mice primed i.p. with incomplete Freund's adjuvant. Antibodies were purified by using an Econo-Pac Serum immunoglobulin G (IgG) purification affinity chromatography column (Bio-Rad, Richmond, Calif.). Cytokine-neutralizing MAb were obtained from hybridomas XMG1.2 (anti-IFN-γ IgG1), MP6-XT22 (anti-TNF-α IgG1), 11-B-11 (anti-interleukin 4 [IL-4] IgG1), and MP1-22E9 (anti-granulocyte-macrophage colony-stimulating factor [GM-CSF] IgG2a) kindly supplied by DNAX (P. Vieira and R. Coffman). Hybridomas were grown either in ascites in HSD nude mice primed with incomplete Freund's adjuvant or in serum-free culture medium. Antibodies were purified by affinity chromatography or simply by 50% ammonium sulfate precipitation. No differences in activity between antibody preparations obtained with the two different protocols were found.

Anti-cytokine treatments in vivo. Mice were infected and given 2 mg of purified cytokine-specific neutralizing MAb by i.p. injection at the chosen time points. Controls received the same amount of purified anti-β-galactosidase MAb of the same isotype (GL113 as IgG1 and GL117.41 as IgG2a).

Flow cytometry. Spleen cells from anti-T-cell antibody-treated or control animals both before and after infection were prepared by teasing a portion of the spleen in medium. Cells were stained with fluorescein isothiocyanate-conjugated rat anti-mouse CD4 or CD8 and/or R-phycoerythrin-conjugated

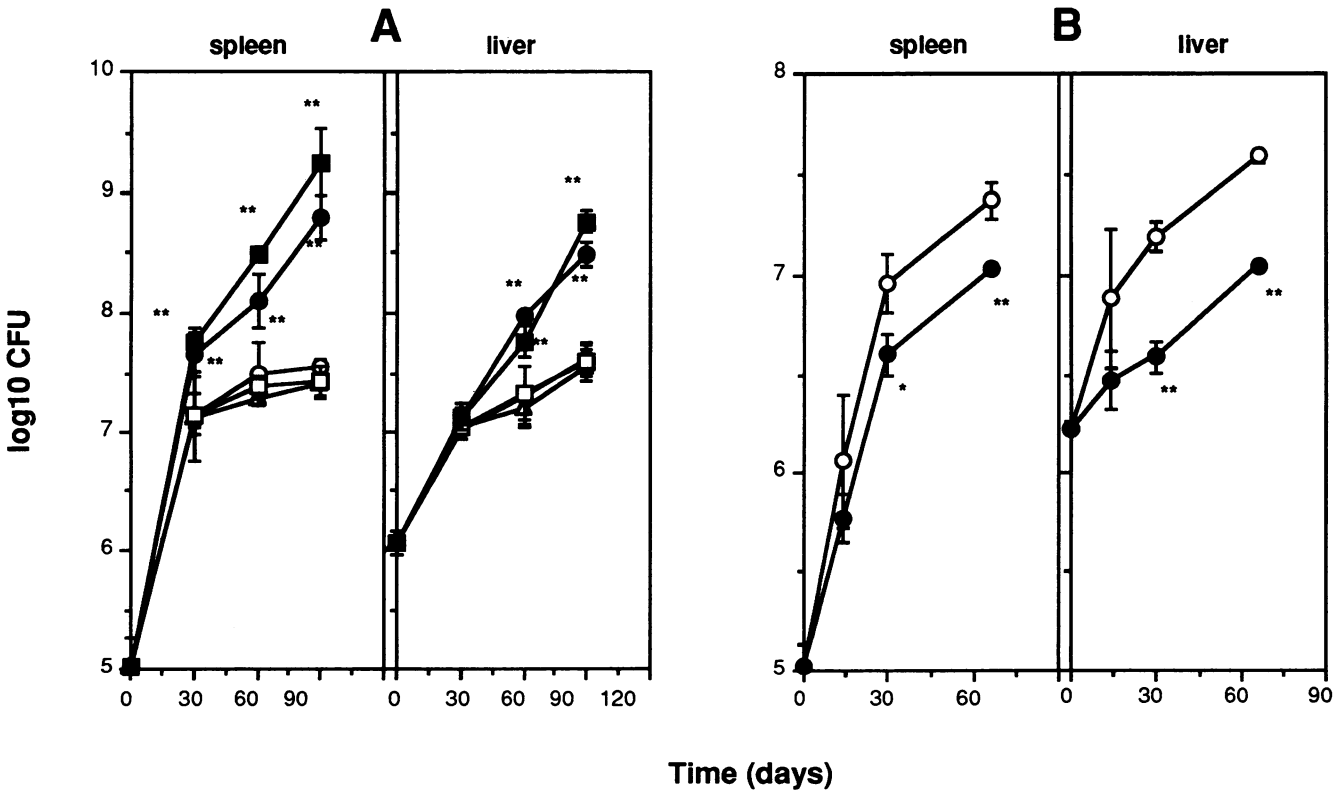

FIG. 1. Proliferation of *M. avium* 2447 in the spleens and livers of control C57BL/6 mice, T-cell subset-depleted mice, and immune animals. (A) Growth was analyzed in normal mice given PBS i.p. every 10 days (△; control population) and in thymectomized mice given either PBS (○), anti-CD4 (●), anti-CD8 (□), or both anti-CD4 and anti-CD8 (■) MAb i.p. every 10 days. (B) Growth was monitored in normal controls (○) and BCG-immune animals (●). Statistical analysis was done by comparing the treated groups with the controls (*, $P < 0.05$; **, $P < 0.01$). Each point represents the mean value for four mice, and the bars represent the standard deviation of the mean.

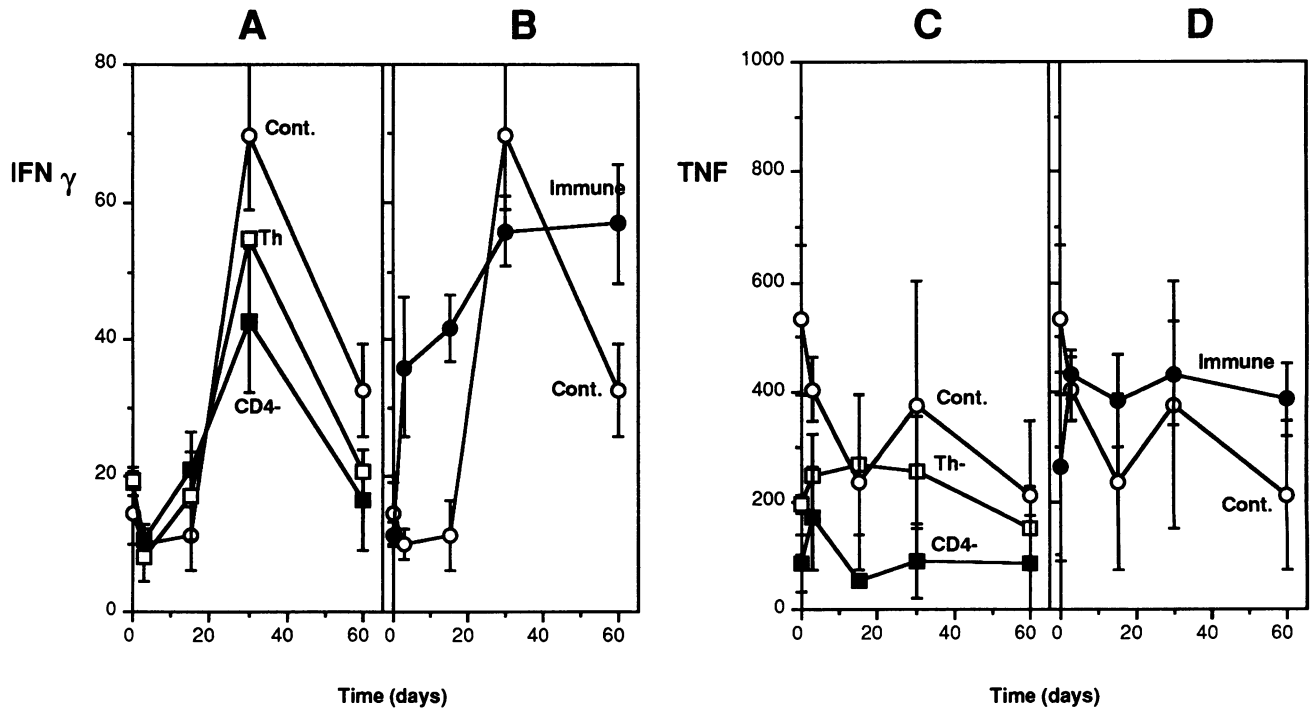

FIG. 2. Semiquantitative analysis of cytokine gene expression during *M. avium* infection in C57BL/6 mice with reverse transcription-PCR. Data are presented as arbitrary units corresponding to picograms of input RNA from standard Th1 cells giving the same dot blot hybridization signal after standardization for HPRT gene expression. Control infected mice (Cont.) were compared with thymectomized (Th) and CD4-depleted (CD4−) animals (A and C) and immunized animals (B and D) for expression of IFN-γ (A and B) and TNF-α (C and D). Each point represents the mean value for three mice, and the bars represent the standard deviation of the mean.

hamster anti-mouse CD3-ε MAb (Pharmingen, San Diego, Calif.) and analyzed in a FACScan apparatus (Becton Dickinson). With the administration of depleting antibodies every 10 days of infection, the depletion of the different T-cell subsets was maintained throughout the whole experimental period. The percentage of $CD4^+$ T cells in CD4-depleted animals was less than 1.4% of the spleen cells analyzed 10 days after the last in vivo antibody administration. Likewise, the percentage of $CD8^+$ T cells was less than 0.2% in the CD8-depleted animals. Depletion was observed when different MAb were used for the in vivo depletion and flow cytometric analysis.

Semiquantitative reverse transcription-PCR. Total spleen cell RNA from individual mice and total RNA from the HDK1 Th1 clone were extracted after lysis in guanidinium isothiocyanate buffer and reverse transcribed as previously described (21). cDNAs (0.5-μl volumes from the samples and 1:2 dilutions from the standard Th1 cell clone) were concomitantly amplified by PCR with hypoxanthine phosphoribosyltransferase (HPRT)-specific primers and a thermal cycler (GeneAmp 9600 PCR System; Perkin-Elmer Cetus) in the presence of thermalase DNA polymerase (one cycle of 2 min at 92°C, 30 cycles of 10 s at 91°C, 25 s at 59°C, and 25 s at 72°C). Dot blots of the products were hybridized with specific [γ-^{32}P]ATP-labeled probes internal to the amplified HPRT gene product. Autoradiographs were quantitated in a Masterscan (Bionis-CSPI, Richebourg, France), and samples were adjusted to similar levels of HPRT mRNA in accordance with a standard curve derived from known dilutions of the HDK1 cDNA samples. After adjustments for HPRT levels, standards and experimental cDNA samples were amplified for IFN-γ or TNF-α sequences with primers synthesized at the Pasteur Institute, spanning intervening sequences in the gene as previously described (21). The resulting PCR products were dot blotted and hybridized with lymphokine-specific [γ-^{32}P]ATP-labeled probes, in parallel with a titration of the standard HDK1 products run in each membrane for every experiment. Units of cytokine gene expression in experimental samples relative to picograms of input HDK1 RNA were then calculated after quantitation of these final dot blots from the linear part of the standard curves.

In vitro macrophage cultures. Bone marrow macrophages were obtained as previously described (2), by culturing bone marrow cells with L929 cell line-conditioned medium. Macrophages were infected for 4 h with *M. avium* bacilli, extensively washed, and cultured in 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, and no antibiotics. No L-cell-conditioned medium was added during the 7-day period of infection. Cytokines were added to the medium every day for up to 7 days without changing the medium. The macrophages remained attached to the plastic and looked healthy during this period without medium changes, and there was no apparent loss of cells. To determine the number of viable bacteria, macrophage monolayers were lysed with 0.1% (final concentration) saponin and the suspensions were serially diluted and plated onto 7H10 agar medium. The results are expressed as $\log_{10}$ growth indexes calculated by subtracting the $\log_{10}$ CFU at time zero of infection from the $\log_{10}$ CFU at day 7 (2). The procedure used did not involve washing the macrophage monolayers prior to CFU counting to avoid removing nonadherent or loosely adherent macrophages. In some cases, cytokine treatments caused some rounding of the macrophages and consequent detachment from the plastic surface. However, since macrophages were

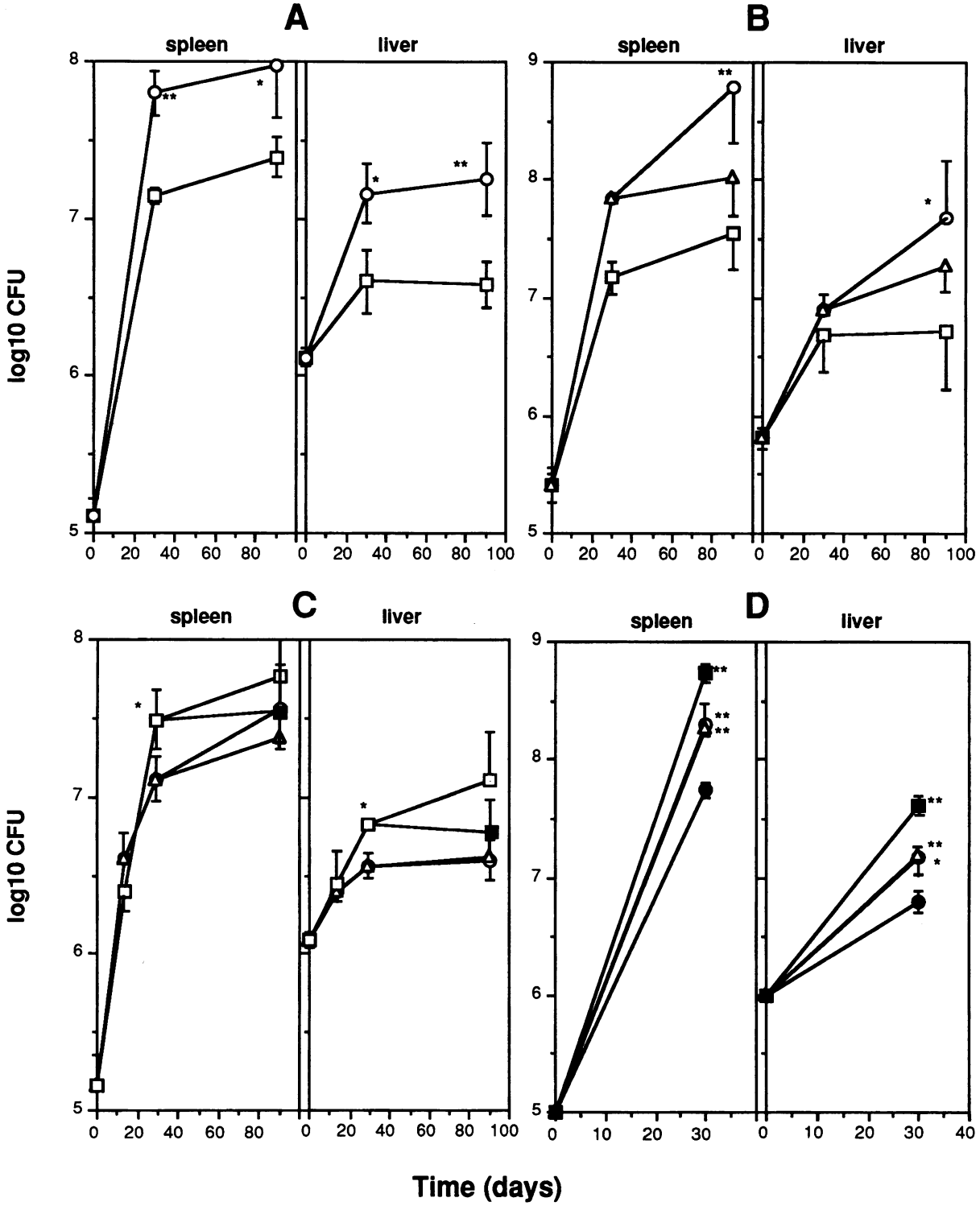

FIG. 3. Effect of administration of anti-IFN-γ, anti-TNF-α, or isotype control MAb (2 mg of XMG1.2, MP6-XT22, or GL113 [i.p.] per animal per dose) on the growth of *M. avium* 2447 in spleens and livers of BALB/c mice. (A) Effect of anti-IFN. BALB/c mice were infected with *M. avium* and given one dose of antibody on day zero of infection (□, control GL113; ○, anti-IFN). (B) Effect of anti-IFN. BALB/c mice were infected with *M. avium* and given GL113 every 2 weeks (□, control mice) or anti-IFN on days 0 and 14 of infection (△) or anti-IFN every 2 weeks from the beginning of the infection (○). (C) Effect of anti-TNF. Mice were infected with *M. avium* and given GL113 every 2 weeks (△) or anti-TNF on days 0 and 14 of infection (■) or anti-TNF every 2 weeks from the beginning of the infection (□) or anti-TNF every 2 weeks from day 30 to day 90 (○). (D) Additive effects of IFN and TNF. Growth of *M. avium* 2447 was monitored in BALB/c mice treated with GL113 (●), anti-TNF (△), anti-IFN (○), or anti-TNF plus anti-IFN (■) (2 mg of each antibody on days 0 and 15 of infection). Statistical analysis was done by comparing the treated groups with the controls (*, $P < 0.05$; **, $P < 0.01$). Each point represents the mean value for four mice, and the bars represent the standard deviation of the mean.

lysed in the culture medium, there was no loss of macrophages and cell-associated bacteria. No extracellular bacterial growth was observed during the 7-day infection period.

In vivo TNF-α secretion. Priming for TNF-α secretion in vivo was evaluated by injecting infected mice i.p. with 50 μg of *Escherichia coli* serotype O26:B6 endotoxin. Two hours later, mice were anesthetized with ether, blood was collected and allowed to clot for 1 h at 37°C, and serum was obtained by centrifugation. The TNF-α activity in the serum was determined by using the L929 cytotoxicity bioassay (20).

Statistical analysis. Data are shown as means. Where appropriate, the standard deviation was plotted. Data were compared by using Student's *t* test.

RESULTS

Kinetics of infection in T-cell-depleted and immunized animals. Untreated animals, thymectomized controls, and T-cell-depleted mice were infected with 10^6 viable *M. avium* bacilli. The progression of the infection was monitored for 3 months by determining the number of viable bacteria in the spleens and livers of infected animals. As previously described (3), strain 2447 stopped proliferating in the spleens and livers of naturally susceptible mice after the first month of infection because of the activity of T cells (Fig. 1A). Depletion of either $CD4^+$ or $CD4^+$ plus $CD8^+$ T cells abrogated the ability to arrest the proliferation of *M. avium* 2447 (Fig. 1A). Differences in bacterial counts between controls and CD4-depleted animals were already statistically significant at day 30 of infection in the spleen and after that time point in the liver. Neither removal of the thymus nor depletion of $CD8^+$ T cells alone had any significant effect on the extent of bacterial proliferation. On the other hand, previous immunization of mice with a subcutaneous inoculation of BCG led to the ability to control the *M. avium* infection sooner (Fig. 1B). The differences in bacterial loads between immunized and nonimmunized groups of mice were statistically significant at day 30 of infection and onwards (Fig. 1B).

Cytokine gene expression in vivo. Spleen cells from infected and control animals were collected, and their RNAs were extracted, reverse transcribed, and amplified by PCR with specific primers for the mRNA transcripts of the HPRT housekeeping enzyme. Samples were adjusted so that equivalent amounts of the products could be compared for expression of IFN-γ and TNF-α genes by using a semiquantitative method (see Materials and Methods). Results were expressed graphically after calculating the relative cytokine expression for uninfected (time zero in the graphs) and infected animals. C57BL/6 mice presented enhanced expression of the IFN-γ mRNA in their spleens after the second week of infection, with a peak on day 30 during the primary response to *M. avium* infection and a decrease after acquisition of the ability to control bacterial growth (Fig. 2A and B). Differences between infected and uninfected control mice were statistically significant on day 30 of infection ($P < 0.05$). The kinetics of TNF-α expression showed minor overall variations throughout the infection, although from day 15 onwards, the expression was parallel to that observed for IFN-γ in the same period (Fig. 2C and D). Unexpectedly, the basal levels of expression in uninfected mice were high and decreased after infection until day 15 and then increased in parallel with the IFN-γ levels, showing the same relative differences between the controls and CD4-depleted groups. BALB/c mice did not show such initial high levels of TNF expression, and the message increased after infection similar to what was observed after day 15 in C57BL/6 mice (data not shown). BCG-immunized C57BL/6 animals,

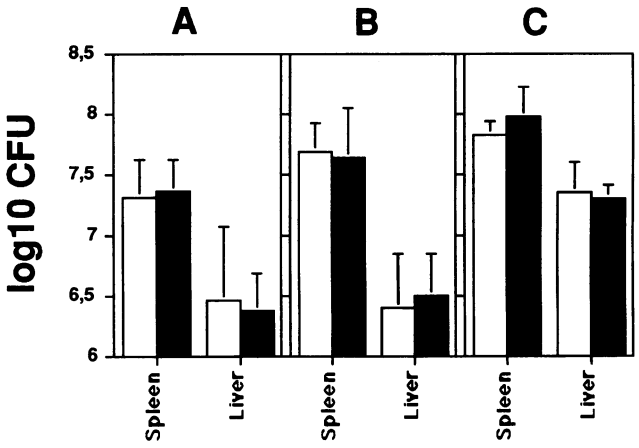

FIG. 4. Numbers of *M. avium* bacteria in the spleens and livers of BALB/c mice infected for 3 months with 10^6 CFU after neutralization of IL-4 or GM-CSF. The results of three experiments are shown for mice treated with isotype control antibody (GL113 in panels A and B and GL117 in panel C; open columns), anti-IL-4 (2 mg per dose on days 0, 15, and 30 [A] or 5 mg per dose on days 30, 45, 60, and 75 [B]), or anti-GM-CSF (2 mg per dose on days 0, 15, 30, 45, and 60 [C]; filled columns). No statistically significant differences between treated and control groups were detected. Differences between experiments were due to the use of different bacterial preparations and experimental variations. Each value represents the geometric mean and standard deviation of the mean number of CFU from four mice.

which controlled the infection sooner than nonimmunized controls (Fig. 1), produced higher levels of mRNA of both cytokines as early as 3 days after challenge, maintaining elevated expression throughout the period analyzed (Fig. 2B and D). Differences between infected and uninfected control mice were statistically significant ($P < 0.05$) on days 3, 15, and 60. Expression of IFN-γ and TNF-α during *M. avium* infection was regulated by T cells, as the expression of these cytokines was reduced after adult thymectomy and particularly after $CD4^+$ T-cell depletion (Fig. 2A and C). CD4-depleted mice had significantly lower IFN-γ expression at day 30 of infection than did infected controls ($P < 0.05$). Baseline expression of TNF-α was also affected in the same way by thymectomy or $CD4^+$ T-cell depletion (Fig. 2C).

The results shown above were similar to those obtained with BALB/c mice, showing the same $CD4^+$-mediated protection and similar cytokine expression profiles. To confirm the in vivo relevance of the above-described cytokines to protection against mycobacterial infection, we treated infected mice with cytokine-specific neutralizing antibodies to evaluate their effect on the bacterial proliferation and resistance to infection.

In vivo effects of anti-IFN-γ and anti-TNF-α antibody administration. In a first set of experiments, BALB/c mice were infected with 10^6 CFU of *M. avium* and a single dose of anti-IFN-γ antibody was administered at the same time. Growth of the bacteria was monitored for 3 months. The anti-IFN-γ antibody was shown to enhance the growth of the mycobacterium during the first month of infection compared with that in mice treated with an isotype control (Fig. 3A). The acquisition of bacteriostasis was, however, not inhibited, although it occurred at higher bacterial loads relative to control mice (Fig. 3A). In a subsequent experiment, the same antibody was administered either on days 0 and 14 or every 2 weeks throughout the infection. Administration of the antibody at early time points had the same enhancing effect as described previously, whereas the continuous neutralization of IFN-γ led

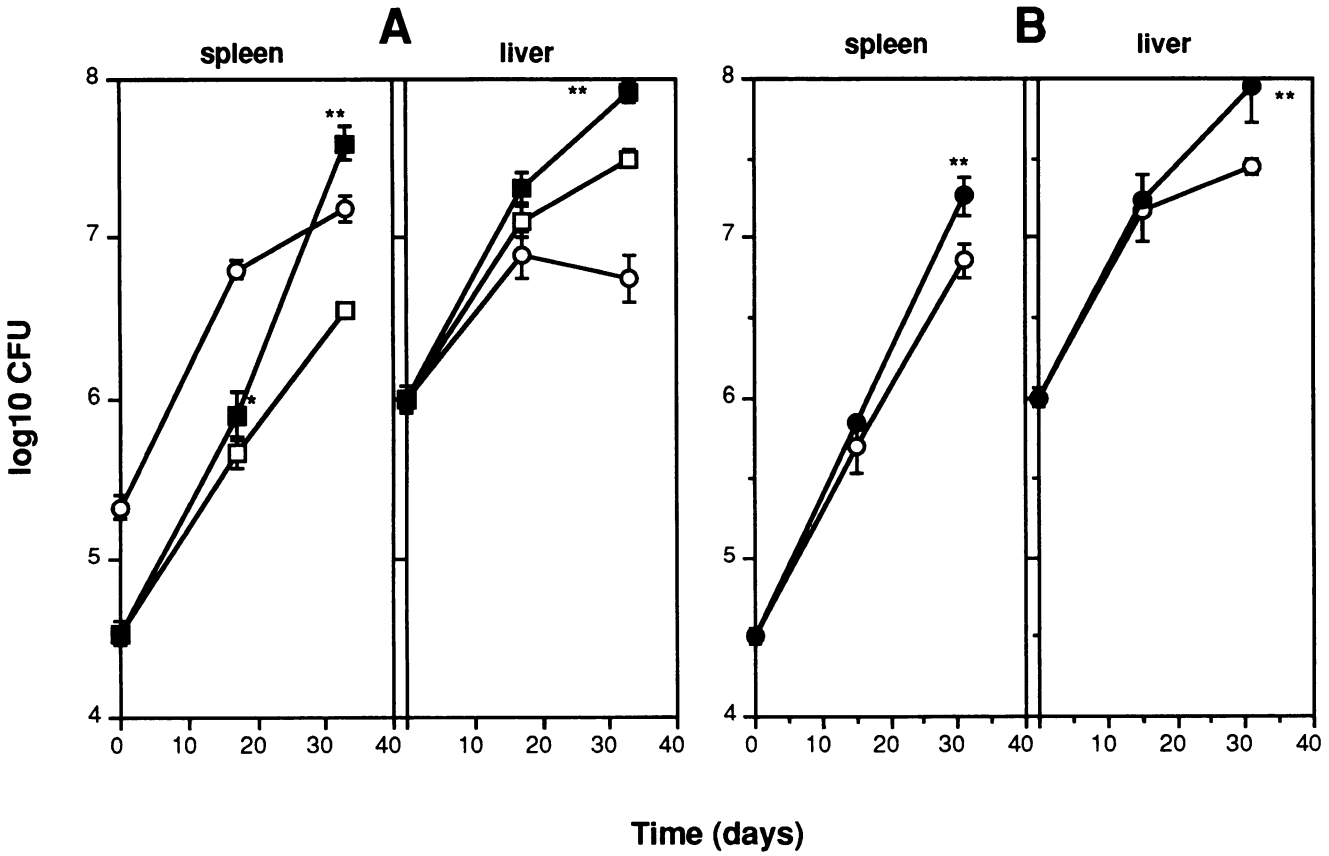

FIG. 5. Effects of IFN-γ or TNF-α neutralization in early resistance to infection in SCID mice. (A) Growth of *M. avium* in BALB/c mice (○) or in SCID mice treated with GL113 (□; control mice) or anti-IFN (■) on days 0 and 14 of infection. (B) Growth of *M. avium* 2447 in SCID mice treated with GL113 (○) or anti-TNF (●) (2 mg on days 0 and 15 of infection). Statistical analysis was done as described in the legends to the previous figures. Each point represents the mean value for four mice, and the bars represent the standard deviation of the mean.

to progressive bacterial growth in both the spleens and livers of infected animals compared with the acquisition of bacteriostasis in isotype control-treated mice (Fig. 3B). Late administration of the neutralizing antibody (i.e., at day 30 and every 2 weeks from then onwards) did not significantly affect the proliferation of *M. avium* (data not shown). An analysis of the role played by TNF-α in the resistance to *M. avium* was done in the same way as described for IFN-γ. The early administration of anti-TNF-α antibodies led to enhanced bacterial loads detected in the spleens and livers of infected mice at 1 month postinfection, but even with continued antibody administration every 2 weeks throughout the infection, there was no significant effect on the acquisition of bacteriostasis (Fig. 3C).

Simultaneous administration of anti-IFN-γ and anti-TNF-α antibodies showed additive effects, leading to more pronounced loss of the ability to slow the infection (Fig. 3D).

We found no effects on the proliferation of strain 2447 in BALB/c mice treated with an anti-GM-CSF MAb (2 mg per animal every 2 weeks for up to 3 months of infection) or when anti-IL-4 was administered either at the beginning of the infection (2 mg at days 0, 15, and 30) or during the acquired phase of immunity (5 mg every 2 weeks from day 30 to day 90) (Fig. 4).

The results presented so far show that immunity to *M. avium* may be divided into two phases, the second one depending on $CD4^+$ T cells. Both IFN-γ and TNF-α seem to play a role in protection. Thus, we analyzed in more detail the participation of these cytokines in an early, T-cell-independent phase and in the late, T-cell-dependent immune response. For the former case, we used T-cell-deficient severe combined immunodeficiency (SCID) mice, and for the latter we used immunized animals.

Effects of neutralizing antibody administration to SCID mice. The results described above show that T-cell-mediated protection becomes detectable in terms of differences between bacterial loads only after the first month of infection, and yet we already found a growth-enhancing effect of anti-IFN-γ or anti-TNF-α during the first 30 days of infection. To assess the role of innate mechanisms compared with T-cell-acquired resistance pathways in the early cytokine-dependent protection against *M. avium* infection, we infected SCID mice and treated them with anti-IFN-γ and anti-TNF-α antibodies. As can be seen in Fig. 5A, the spleens of SCID mice retained fewer bacteria after inoculation than did the spleens of control BALB/c mice because of their smaller size. However, the growth curve slopes were similar in the spleens of SCID and BALB/c animals during the initial 2 weeks of infection. SCID mice treated with the XMG1.2 antibody were rendered more susceptible to *M. avium* infection at 33 days of infection than were SCID mice that received the isotype control antibody (Fig. 5A). SCID mice failed to acquire the bacteriostatic activity evidenced by BALB/c mice by a downward trend in the slope of the growth curve of the mycobacteria already evident at day 33 of infection (Fig. 5A). Likewise, administration of

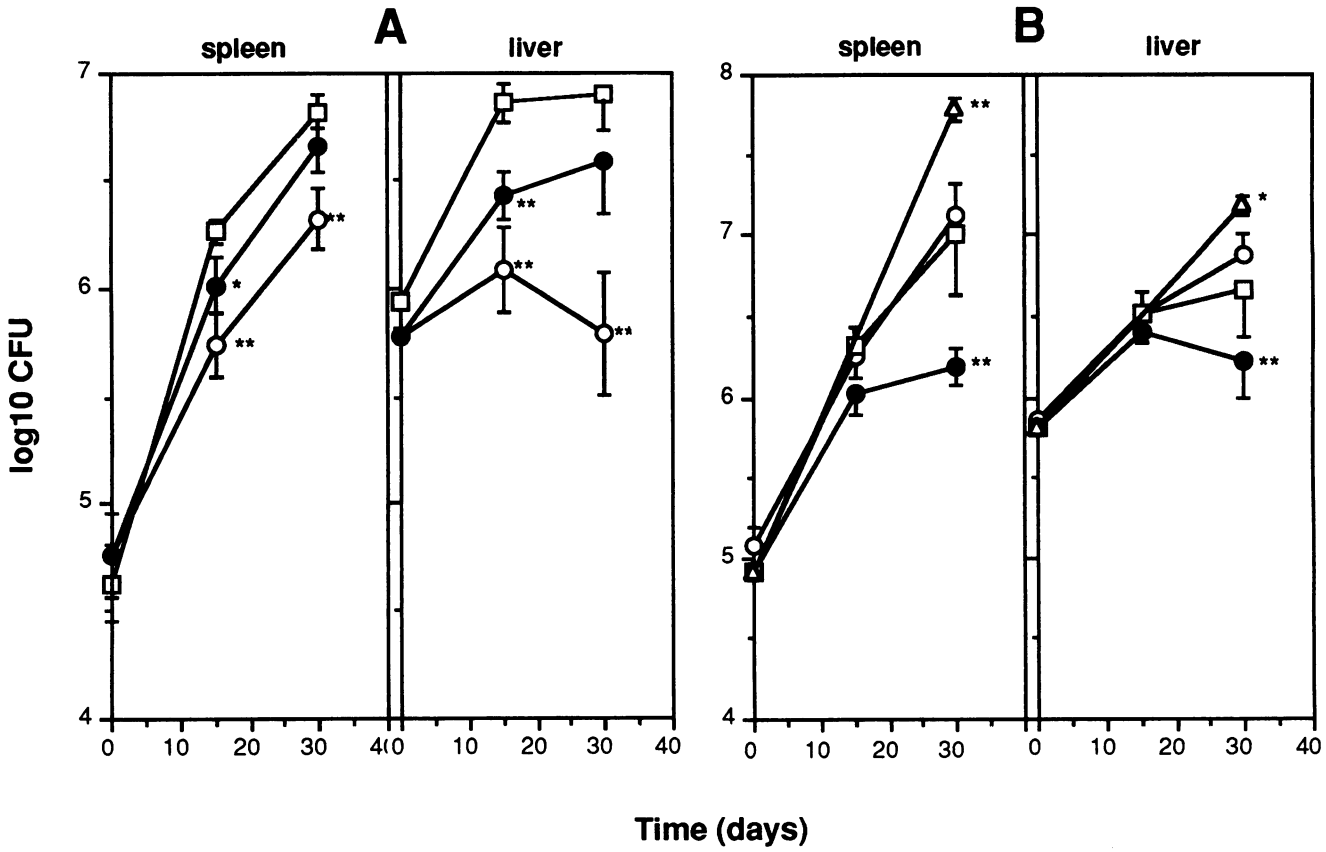

FIG. 6. Analysis of involvement of IFN-γ and TNF-α in the anamnestic response to *M. avium* infection. (A) Growth of *M. avium* in normal controls (□) or BCG-immune BALB/c mice treated every 2 weeks after challenge with GL113 (○) or anti-IFN (○). (B) Growth of *M. avium* in normal (○) or BCG-immune BALB/c mice treated every 2 weeks after challenge with GL113 (○), anti-TNF (□), or anti-TNF plus anti-IFN (△). Each point represents the mean value for four mice, and the bars represent the standard deviation of the mean.

anti-TNF-α antibodies enhanced the number of mycobacteria detected in the organs of *M. avium*-infected SCID mice (Fig. 5B).

Effects of antibody administration to immune animals. To analyze the participation of IFN-γ and TNF-α in the acquired immunity in a short-term experiment, BCG-immune mice were challenged with *M. avium* and given neutralizing antibodies at the time of *M. avium* challenge. Anti-IFN-γ partially blocked the protective effect of the BCG immunization (Fig. 6A). Anti-TNF-α antibodies were also able to reverse the protective effects of BCG immunization, and the combination of both antibodies showed additive effects, completely abrogating the ability to control *M. avium* proliferation during challenge of the immune mice (Fig. 6B).

Cooperation between IFN-γ and TNF-α in anti-*M. avium* activity. Since both IFN-γ and TNF-α have been shown to induce antimycobacterial activity in macrophages in vitro (2), we assessed whether these two cytokines act together on macrophages or whether IFN-γ is only involved in priming macrophages for TNF-α release. Macrophages differentiated from bone marrow precursors in the presence of macrophage colony-stimulating factor-containing L929 cell-conditioned medium were able to sustain *M. avium* growth in vitro and remained viable throughout the period of infection studied, without further addition of the conditioned medium during the infection period (Fig. 7). Both cytokines were able to reduce the proliferation of different *M. avium* strains, in contrast to macrophages cultivated in medium alone (Fig. 7). Thus, as can be seen in Fig. 7A, the treatment of the macrophage cultures with increasing amounts of recombinant IFN-γ was paralleled by a decrease in *M. avium* proliferation. This bacteriostasis-inducing effect was potentiated by addition of TNF-α (Fig. 7A). The differences between cultures treated or not treated with TNF-α were statistically significant in the absence of IFN-γ ($P < 0.05$) or in the presence of IFN-γ ($P < 0.01$ for all three concentrations of IFN-γ). The same effects were observed when different strains of *M. avium* were used (Fig. 7B). Although the results presented refer to experiments in which the cytokines were added after phagocytosis of the mycobacteria, we found that the effects of these two cytokines were similar when the macrophages had been treated prior to and during infection (results not shown).

Uninfected animals treated for 2 h with lipopolysaccharide did not show detectable levels of TNF in serum (the sensitivity limit was 100 U). During *M. avium* infection, the animals became primed to produce high levels of TNF following a 2-h challenge with endotoxin (Fig. 8). The priming for TNF-α release during *M. avium* infection was almost completely abrogated by in vivo treatment of infected mice with anti-IFN-γ antibodies (2 mg of antibody every 2 weeks) (Fig. 8). Infected mice treated with the antibody showed 1- to 1.5-log-lower levels of TNF in their sera after endotoxin challenge.

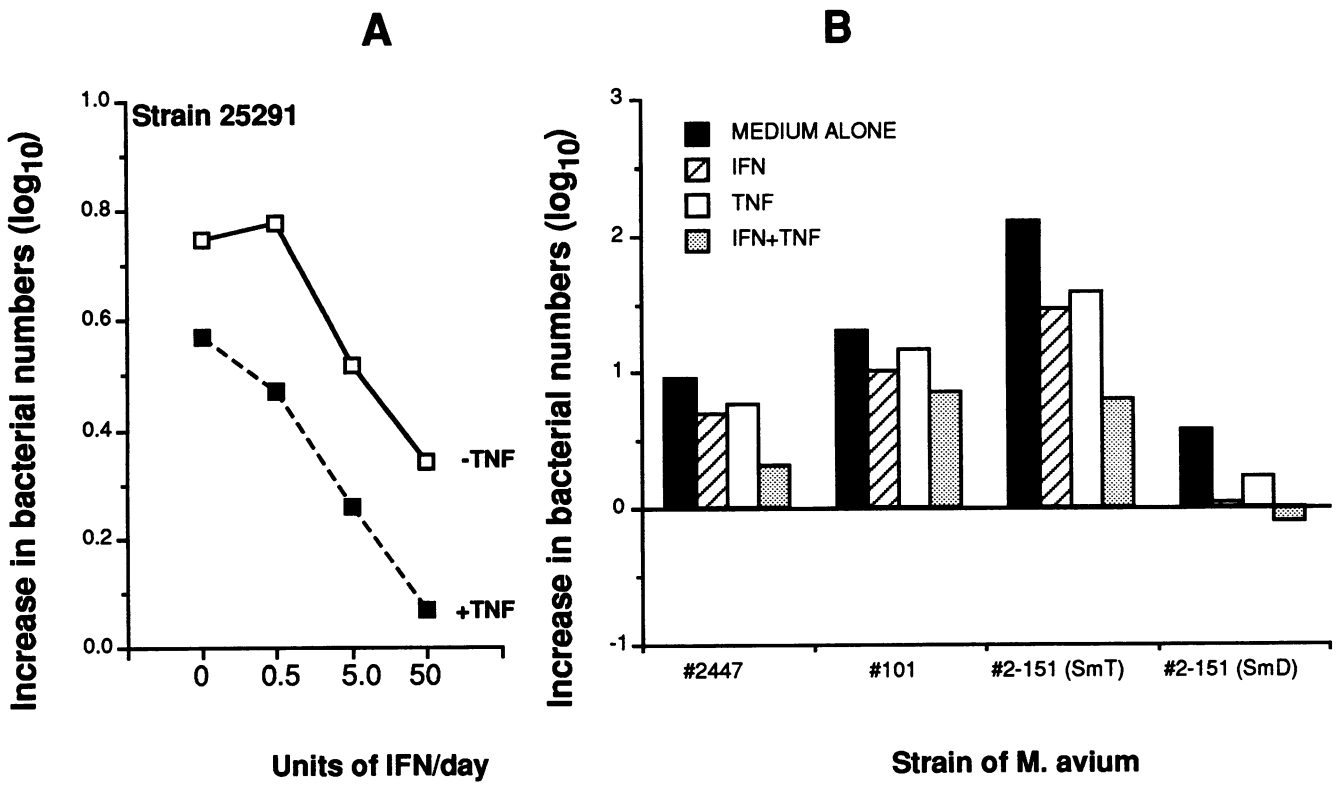

FIG. 7. Evidence of additive effects of IFN-γ and TNF-α on the induction of bacteriostasis in macrophages in vitro. (A) Increase in *M. avium* numbers (log_{10}) in bone marrow macrophages treated with increasing doses of IFN-γ in the absence or presence of a fixed dose of TNF-α (50 U/day) and infected for 7 days. (B) Growth (7 days) of different strains of *M. avium* in bone marrow macrophages treated with IFN-γ (100 U/day) with or without TNF-α (50 U/day). In panel A, the growth observed in IFN-γ-treated cultures is compared with the growth observed in cultures not treated with this cytokine (either in the absence or in the presence of TNF-α). In panel B, the growth observed in macrophages treated with cytokines is compared with that observed in control macrophages. Statistically significant differences are labeled * ($P < 0.05$) or ** ($P < 0.01$). SmT, smooth, transparent; SmD, smooth, domed.

DISCUSSION

This report shows that resistance to *M. avium* infection evolves through two stages, one of innate immunity and a second of acquired $CD4^+$ T-cell-mediated resistance. The former involves protective effects mediated by both IFN-γ and TNF-α, and the latter requires IFN-γ (at least initially) and possibly other T-cell-derived cytokines. The cytokines implicated in the early control of mycobacterial infection are most likely produced by cells which are involved with the innate immunity responses (NK cells, phagocytes, and possibly other cells) or T cells stimulated to secrete cytokines by mechanisms that do not involve specific recognition of the antigen (1). Even though immunodeficient mice may be functionally vicarious to compensate for the defect at the level of the lymphocytes and may thus exhibit abnormally high NK activity, our experiments with neutralizing antibodies in SCID mice suggest a role for NK cells in early IFN-γ-mediated protection against *M. avium* infection (4–6). In conformity with the results shown above, we have observed greater expression of IFN-γ in infected SCID mice than in uninfected SCID mice (10a), suggesting that these animals do indeed respond to the infection with an IFN-γ-secretory response. Thus, our results further illustrate the participation of cytokines in the innate-immunity phase of an immune response, such as has been extensively studied in the listeria model (4, 5, 13, 26). Furthermore, they show how the innate resistance retards the infection until the immune response takes over.

The protective role of TNF-α seems to be more modest and transient than that of IFN-γ and is apparently restricted to the innate phase of the resistance to infection. The neutralization of TNF-α by the antibody used was probably effective, since administration of the same MAb prevented the detection of biologically active TNF in infected animals treated with lipopolysaccharide, which otherwise had high levels of TNF, as shown here. Furthermore, we have observed a dramatic effect of the same antibody given under the same conditions in the case of the *M. tuberculosis* infection of mice (1a). The reverse transcription-PCR data also showed that the variation in the expression levels of this cytokine during infection was modest, suggesting that *M. avium*, as opposed to *M. tuberculosis*, is a poor trigger for the synthesis of TNF-α. In fact, *M. avium* is not toxic to the host, as evidenced by the high numbers of mycobacteria that infected organs may exhibit during some *M. avium* infections (3), suggesting that the TNF-α levels triggered in vivo are indeed low. However, mRNA levels may be difficult to interpret in the case of TNF-α, as far as extrapolating the results to net cytokine production is concerned. We observed strain-associated differences in basal levels of expression of this cytokine. BALB/c mice had lower levels, and infection resulted in a net increase of expression. C57BL/6 mice, on the other hand, had high initial message levels, and these levels were first decreased during the infection before following a kinetics of expression similar to the one observed in BALB/c mice. It has been shown that TNF production is

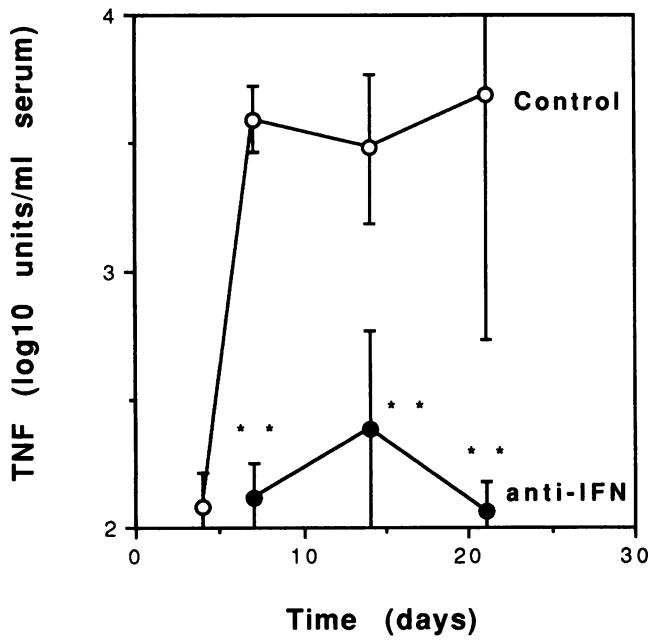

FIG. 8. Priming for TNF-α production after lipopolysaccharide challenge of *M. avium*-infected C57BL/6 mice treated with GL113 or anti-IFN every 2 weeks. The anti-IFN-γ antibody significantly blocked in vivo priming (**, $P < 0.01$).

regulated at the level of translation and that macrophages may possess cytoplasmic mRNA for this cytokine without concomitant protein synthesis (16). We suggest that mRNA for TNF-α found in uninfected mice is posttranscriptionally regulated so that it is not translated into biologically active protein (we did not detect significant TNF in endotoxin sera of uninfected mice) and that a different mRNA product that is translated into protein emerges during infection. This would account for the fact that TNF-α neutralization results in exacerbation of the infection in the absence of significant differences in mRNA levels between control and infected mice. Finally, it should be emphasized that the course of *M. avium* infection is rather indolent and that small changes in cytokine expression maintained for relatively long periods may be more important than a short peak of expression.

Between the third and fourth weeks of infection, specific T cells able to confer protection against *M. avium* began to be present, first in the spleen and later in the liver. This was paralleled by higher levels of expression of both IFN-γ and TNF-α, which had begun being synthesized earlier. The response to a secondary infection in immunized animals showed a more prompt protective effect of these two cytokines, as well as a fast induction of their expression typical of an anamnestic response. Interestingly, IFN-γ production was turned off after bacteriostasis developed. Full bacteriostasis depended on the activity of specifically induced T cells that either produced larger amounts of IFN-γ or secreted additional cytokines which would then induce full bacteriostasis. The data presented here suggest that an unidentified cytokine may be produced by protective T cells during the adaptive response to the infection and that this cytokine may be responsible for the induction of complete bacteriostasis. First, when the neutralization of IFN-γ was begun late in the infection, when T-cell-mediated protection was already detectable, there was no significant enhancement of mycobacterial proliferation, showing that IFN-γ is not necessary for the maintenance of the bacteriostatic state. Second, the levels of mRNA for IFN-γ in CD4-depleted mice were not significantly depressed compared with those in thymectomized mice, even though these two groups of animals showed different susceptibilities to *M. avium*. Third, the enhancement of bacterial proliferation by administration of anti-IFN-γ antibodies (and anti-TNF-α antibodies as well) detected at day 30 of infection and the data obtained by reverse transcription-PCR clearly evidenced the involvement of this cytokine(s) during the early phase of the infection; however, despite their activity, only partial, not complete, bacteriostasis was observed. The nature of the putative cytokine is still unknown, but a role for GM-CSF or IL-4 seems unlikely, as deduced from our results, despite in vitro evidence of a protective effect of these two cytokines against *M. avium* (2, 9, 12). Although the efficacy of the neutralizing effects of anti-IL-4 or anti-GM-CSF antibodies was not proven, these antibodies were used at concentrations shown to be effective for other antibodies and, in addition, the same antibodies have been used in other models with positive effects. It should, however, be stressed that both IFN-γ and TNF-α still need to be present at some point during the infection for induction of protection, as has been shown by the complete abrogation of any protective effects by combined anti-IFN-γ and anti-TNF-α antibody administration to immunized animals.

The cooperation between IFN-γ and TNF-α in the induction of protection against *M. avium* infection was shown to involve two distinct mechanisms. In the first place, IFN-γ was involved in priming of the macrophages for secretion of TNF-α. In addition to this mechanism, both cytokines were able to potentiate each other's effects in the induction of mycobacteriostatic activity in in vitro-cultured macrophages.

In conclusion, our data suggest the following interpretation of the response to *M. avium* 2447 infection. Early after inoculation of the microbe, cells other than T cells nonspecifically secrete IFN-γ, which then primes macrophages for TNF-α production. Both cytokines then act on infected macrophages in concert to induce partial bacteriostasis, retarding bacterial proliferation to some extent. Later, T cells are induced specifically and are responsible for more extensive IFN-γ production, as well as secretion of other cytokines able to provide infected macrophages with full bacteriostatic activities.

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REFERENCES

1. **Akuffo, H. O.** 1992. Non-parasite-specific cytokine responses may influence disease outcome following infection. Immunol. Rev. **127**:51–68.
1a. **Appelberg, R., D. Ordway, and I. M. Orme.** Unpublished data.
2. **Appelberg, R., and I. M. Orme.** 1993. Effector mechanisms involved in cytokine-mediated bacteriostasis of *Mycobacterium avium* infections in murine macrophages. Immunology **80**:352–359.
3. **Appelberg, R., and J. Pedrosa.** 1992. Induction and expression of protective T cells during *Mycobacterium avium* infections in mice. Clin. Exp. Immunol. **87**:379–385.
4. **Bancroft, G. J., R. D. Schreiber, G. C. Bosma, M. J. Bosma, and E. R. Unanue.** 1987. A T cell-independent mechanism of macrophage activation by interferon-γ. J. Immunol. **139**:1104–1107.
5. **Bancroft, G. J., K. C. F. Sheenan, R. D. Schreiber, and E. R. Unanue.** 1989. Tumor necrosis factor is involved in the T cell-

independent pathway of macrophage activation in SCID mice. J. Immunol. **143**:127–130.
6. **Bermudez, L. E. M., P. Kolonowski, and L. S. Young.** 1990. Natural killer cell activity and macrophage-dependent inhibition of growth or killing of *Mycobacterium avium* complex in a mouse model. J. Leukocyte Biol. **47**:135–141.
7. **Bermudez, L. E. M., P. Stevens, P. Kolonowski, M. Wu, and L. S. Young.** 1989. Treatment of experimental disseminated *Mycobacterium avium* complex infection in mice with recombinant IL-2 and tumor necrosis factor. J. Immunol. **143**:2996–3000.
8. **Bermudez, L. E. M., and L. S. Young.** 1988. Tumor necrosis factor, alone or in combination with IL-2, but not IFN-γ, is associated with macrophage killing of *Mycobacterium avium* complex. J. Immunol. **140**:3006–3013.
9. **Bermudez, L. E. M., and L. S. Young.** 1990. Recombinant granulocyte-macrophage colony-stimulating factor activates human macrophages to inhibit growth or kill *Mycobacterium avium* complex. J. Leukocyte Biol. **48**:67–73.
10. **Blanchard, D. K., M. B. Michelini-Norris, and J. Y. Djeu.** 1991. Interferon decreases the growth inhibition of *Mycobacterium avium-intracellulare* complex by fresh human monocytes but not by culture-derived macrophages. J. Infect. Dis. **164**:152–157.
10a. **Castro, A. G., P. Minóprio, and R. Appelberg.** Unpublished data.
11. **Denis, M.** 1991. Modulation of *Mycobacterium avium* growth in vivo by cytokines: involvement of tumour necrosis factor in resistance to atypical mycobacteria. Clin. Exp. Immunol. **83**:466–471.
12. **Denis, M., and E. O. Gregg.** 1991. Modulation of *Mycobacterium avium* growth in murine macrophages: reversal of unresponsiveness to interferon-gamma by indomethacin or interleukin-4. J. Leukocyte Biol. **49**:65–72.
13. **Dunn, P. L., and R. J. North.** 1991. Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. Infect. Immun. **59**:2892–2900.
14. **Edwards, C. K., H. B. Hedegaard, A. Zlotnik, P. R. Gangadharam, R. B. Johnston, and M. J. Pabst.** 1986. Chronic infection due to *Mycobacterium intracellulare* in mice: association with macrophage release of prostaglandin E_2 and reversal by injection of indomethacin, muramyl dipeptide, or interferon-γ. J. Immunol. **136**: 1820–1827.
15. **Flory, C. M., R. D. Hubbard, and F. M. Collins.** 1992. Effects of in vivo T lymphocyte subset depletion on mycobacterial infections in mice. J. Leukocyte Biol. **51**:225–229.
16. **Han, J., T. Brown, and B. Beutler.** 1990. Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level. J. Exp. Med. **171**:465–475.
17. **Harshan, K. V., and P. R. J. Gangadharam.** 1991. In vivo depletion of natural killer cell activity leads to enhanced multiplication of *Mycobacterium avium* complex in mice. Infect. Immun. **59**:2818–2821.
18. **Horsburgh, C. R.** 1991. *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. N. Engl. J. Med. **324**: 1332–1338.
19. **Jacobson, M. A., P. C. Hopewell, D. M. Yajko, W. K. Hadley, E. Lazarus, P. K. Mohanty, G. W. Modin, D. W. Feigal, P. S. Cusick, and M. A. Sande.** 1991. Natural history of disseminated *Mycobacterium avium* complex infection in AIDS. J. Infect. Dis. **164**:994–998.
20. **Matthews, N., and M. L. Neale.** 1987. Cytotoxicity assays for tumour necrosis factor and lymphotoxin, p. 221–225. *In* M. J. Clemens, A. G. Morris, and A. J. H. Gearing (ed.), Lymphokines and interferons. A practical approach. IRL Press, Oxford.
21. **Murphy, E., S. Hieny, A. Sher, and A. O'Garra.** 1993. Detection of in vivo expression of interleukin-10 using a semi-quantitative polymerase chain reaction method in *Schistosoma mansoni* infected mice. J. Immunol. Methods **162**:211–223.
22. **Nightingale, S. D., L. T. Byrd, P. M. Southern, J. D. Jockusch, S. X. Cal, and B. A. Wynne.** 1992. Incidence of *Mycobacterium avium-intracellulare* complex bacteremia in human immunodeficiency virus-positive patients. J. Infect. Dis. **165**:1082–1085.
23. **Orme, I. M., S. K. Furney, and A. D. Roberts.** 1992. Dissemination of enteric *Mycobacterium avium* infections in mice rendered immunodeficient by thymectomy and CD4 depletion or by prior infection with murine AIDS retrovirus. Infect. Immun. **60**:4747–4753.
24. **Squires, K. E., S. T. Brown, D. Armstrong, W. F. Murphy, and H. W. Murray.** 1992. Interferon-γ treatment for *Mycobacterium avium-intracellulare* complex bacillemia in patients with AIDS. J. Infect. Dis. **166**:686–687.
25. **Toba, H., J. T. Crawford, and J. J. Ellner.** 1989. Pathogenicity of *Mycobacterium avium* for human monocytes: absence of macrophage-activating factor activity of gamma interferon. Infect. Immun. **57**:239–244.
26. **Wherry, J. C., R. D. Schreiber, and E. R. Unanue.** 1991. Regulation of gamma interferon production by natural killer cells in SCID mice: roles of tumor necrosis factor and bacterial stimuli. Infect. Immun. **59**:1709–1715.