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IL-12 Promotes Drug-Induced Clearance of *Mycobacterium avium* Infection in Mice

T. Mark Doherty¹ and Alan Sher

The intracellular pathogen *Mycobacterium avium* is a major cause of opportunistic infection in AIDS patients and is difficult to manage using conventional chemotherapeutic approaches. In the current study, we describe a strategy for the treatment of *M. avium* in T cell-deficient hosts based on the simultaneous administration of antibiotics and the immunomodulatory cytokine IL-12. In contrast to SCID mice, which were partially resistant, animals lacking a functional IL-12 p40 gene were found to be highly susceptible to *M. avium* infection, suggesting that the cytokine can control bacterial growth even in immunodeficient mice. Indeed, rIL-12 that was injected into infected SCID mice in high doses caused small but significant reductions in splenic pathogen loads. Moreover, a lower dose of IL-12, when combined with the antimycobacterial drugs clarithromycin or rifabutin, induced a decrease in bacterial numbers that was significantly greater than that resulting from the administration of the cytokine or drug alone. A similar synergistic effect of IL-12 and antibiotics was seen when immunocompetent mice were treated with the same regimen. The activity of IL-12 in these experiments was shown to be dependent upon the induction of endogenous IFN-γ. Nevertheless, IFN-γ itself, even when given at a higher dose than IL-12, failed to significantly enhance antibiotic clearance of bacteria. Together these findings suggest that IL-12 may be a particularly potent adjunct for chemotherapy of *M. avium* infection in immunocompromised individuals and may result in more effective control of the pathogen without the need for increased drug dosage. *The Journal of Immunology*, 1998, 160: 5428–5435.

*Mycobacterium avium* is a facultative intracellular bacterium that normally poses a health problem only to severely immunocompromised individuals (1–5). However, with the growth of the AIDS epidemic, *M. avium* is now recognized as an important opportunistic infection (4, 6). The clinical management of *M. avium* infections in AIDS patients has proved difficult, because a complete cure is rarely achieved with the antibiotic regimens available (7), a scenario which also favors the development of drug resistance (8, 9). Indeed, the current treatment of the pathogen in HIV-positive individuals normally involves the simultaneous administration of multiple antimycobacterial drugs that often produce significant side effects (10). Macrolide antibiotics have consistently proved to be the most efficacious against *M. avium* infection, but they are generally supplemented with other drugs to augment their effectiveness and to inhibit the development of resistance by the bacteria. (11–14). Rifabutin in particular has been commonly used to supplement macrolide antibiotic treatment (15).

As with most intracellular infections, the control of *M. avium* in immunocompetent hosts appears to depend upon the induction of cell-mediated mechanisms as opposed to humoral immune mechanisms (16–18). Thus, in both human disease (19, 20) and murine experimental models, *M. avium* infection stimulates the production of macrophage-activating lymphokines (IFN-γ, TNF-α) as well as microbiocidal products such as nitric oxide and/or reactive oxygen intermediates (21–26). That such responses are important in the control of infection is evidenced by the increased bacterial growth seen in mice that are deficient in T cells or the production of IFN-γ (24, 27–29) or in patients with defects in the generation or effector functions of Th1 responses (30). Studies in mice treated with neutralizing mAb to IL-12 also show decreased resistance to the pathogen (22, 31). The latter cytokine is thought to play a pivotal role in the induction of most IFN-γ-dependent, cell-mediated responses (32). It is presumed that the loss of control of *M. avium* infection in late-stage AIDS patients reflects an impairment in those CD4-dependent effector mechanisms that are similar to those studied in vitro and in experimental laboratory hosts (5).

In a previous study, we systematically compared the growth of *M. avium* in mice with genetically engineered defects in lymphokine synthesis and macrophage effector function (24). Interestingly, T cell-deficient SCID mice, while more susceptible to infection than wild-type (wt)² animals, were nevertheless more resistant than comparably infected IFN-γ-deficient mice. This finding suggested that in the absence of T cells, *M. avium* infection can be partially controlled by IFN-γ that is derived from NK cells, which are known to serve as an alternative source of this cytokine. Since IL-12 is an important stimulator of NK-produced IFN-γ (33, 34), we have considered possibly using the former cytokine to enhance resistance to the bacterium in T cell-deficient hosts.

In the present report, we demonstrate that rIL-12 does indeed augment resistance to *M. avium* infection in SCID mice and, more importantly, acts synergistically to promote bacterial clearance when combined with conventional antibiotics. Our findings suggest that combined IL-12/drug treatment may offer a highly effective strategy for the management of atypical mycobacterioses in immunocompromised individuals.

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² Abbreviations used in this paper: wt, wild-type; GKO, IFN-γ knockout; BL/SCID mice, C57BL/6-SCID/Sld mice; MAG, mycobacterial Ag; KO, knockout; HPRT, hypoxanthine phosphoribosyltransferase.
Materials and Methods

Animals

C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). C57BL/6-SCID/Sj1 (BL/SCID) mice were initially purchased from The Jackson Laboratory (Bar Harbor, ME). Mice that were deficient for expression of the IFN-γ gene and backcrossed onto the C57BL/6 genome by the seventh generation were derived from stocks originally provided by D. Dalton and S. Stewart (Genentech, San Bruno, CA). These IFN-γ knockout (GKO) mice were genotyped by the PCR of blood as previously described (35). Mice with targeted disruptions of the IL-12 p40 gene (IL-12 knockout [KO]) were generously provided by J. Magram (Hoffman-La Roche, Nutley, NJ). All animals were maintained in specific-pathogen-free quarters and housed in the same animal facility at the National Institute of Allergy and Infectious Diseases (NIAID); all mice were age- and sex-matched for experiments and used at 6 to 10 wk of age.

Bacterial strain and Ag

The M. avium strain 2–151 SmT was kindly provided by Dr. A. Cooper (Colorado State University, Colorado). Stocks were prepared according to the following protocol for use in all subsequent experiments. BL/SCID mice were infected i.v. with 10^7 CFU/mL, spleens were taken after 6 wk, and single-cell suspensions were prepared and plated on Middlebrook 7H11 agar (National Institutes of Health media unit, Bethesda, MD). Bacterial colonies were harvested from agar plates after 2 wk, dispersed by 2 × 5 s uses of a probe sonicator (Heat Systems, Farmingdale, NY), frozen in saline at 10^9 to 10^10 CFU/mL (determined after sonication), and stored frozen at −70°C.

Mycobacterial Ag (MAg) was made by freeze-thaw lysis of M. avium at 10^6 CFU/mL. Bacteria were quick-frozen in 0.5 mL aliquots in PBS (Life Technologies, Gaithersburg, MD) using an ethanol/dry ice mixture and subsequently thawed at 37°C. After three cycles, the lysate was disrupted by 3 × 10 s uses of a probe sonicator. The protein concentration was determined using the bicinchoninic acid assay (36) and adjusted to 7.5 µg/mL. MAg was used to stimulate spleen cells at a final concentration of 50 µg/mL.

Drugs and cytokine treatment

Mice were injected i.v. with 10^7 CFU of the M. avium strain 2–151 SmT. After 2 wk, the animals had developed a disseminated infection, and bacteria were readily demonstrable in a variety of organs. Treatment was subsequently initiated with either clarithromycin, rifabutin, IL-12, IFN-γ, or combinations of drug and cytokine that were administered i.p. in 200 µL of saline at the doses indicated. Since neither rifabutin nor clarithromycin is readily soluble in an aqueous solution, the former was dissolved in DMSO, while the latter was with 95% ethanol. Both were subsequently diluted to 1% in saline as suspensions in accordance with the manufacturer’s instructions. Injections were repeated on alternate days for three sequential doses. After a rest period of 2 days, another three doses were administered on the same schedule. Control animals were given saline alone at the same time intervals. Preliminary experiments, which tested drugs in the range of 5 to 200 mg/kg by several different routes (s.c., peritoneal, or gavage) on daily or alternate day administration, indicated that this regimen was highly efficacious while producing a minimum of apparent side effects (data not shown). Mice were sacrificed for the analysis of infection and immune responses at 2 days after the final injection.

In some experiments, mice were simultaneously treated with the neutralizing anti-IFN-γ mAb (XMG.6) or with a control mAb (GL113) (cell lines provided by R. Coffman, DNAX, Palo Alto, CA); the mAbs were given i.p. at 1 mg/mouse at the same time as the cytokine and drug injections.

Measurement of bacterial infection in vivo

The progress of bacterial growth was monitored at various time points by assaying bacterial CFUs in the spleen and lungs of infected mice. Organs were weighed, and then a small portion of each tissue was removed, embedded in paraffin for sectioning, and stained with hematoxylin and eosin or the Fite acid-fast method (American Histolabs, Gaithersburg, MD). The remainder of each organ was homogenized by maceration through a fine mesh stainless steel sieve into complete RPMI 1640 (Life Technologies, Grand Island, NY) (including 2 mM glutamine, 30 µg/mL of ampicillin, 10% FCS, and 50 µM 2-ME); the cells were counted, and an aliquot was diluted to 10^9/mL in buffered saline for the enumeration of viable bacterial counts. Triplicate, serial 10-fold dilutions of the cells were then performed on Middlebrook 7H11 agar plates which were incubated at 37°C for 2 to 3 wk. Colonies were then counted, and the number of CFUs per organ were calculated. The detection limit of this protocol is ~100 CFU/sample, although the precise limit is defined by the number of cells contained in the target organ, as this number is typically better in early infection (as low as 10 CFU/sample).

Measurement of IFN-γ expression

The relative level of IFN-γ mRNA in spleen cells was assayed by RT-PCR as previously described (37). Briefly, single-cell suspensions that had been prepared as described above were thoroughly washed, and total RNA was prepared by lysis of 10^6 cells in RNA-STAT-60 (Tel-Test ‘B’, Friendswood, TX) followed by precipitation from the aqueous phase as recommended by the manufacturer. Recovered RNA was resuspended in diethyl pyrocarbonate-treated, distilled, deionized water and cDNA synthesized. PCRs were performed on serial dilutions of cDNA (from 10 µl) in a final volume of 50 µl, and a sample (10 µl) of each PCR reaction was electrophoresed through a 1.0% agarose gel and visualized with ethidium bromide. The number of cycles of PCR amplification used was first determined by amplifying cDNA through 24 to 36 cycles and comparing the product obtained to a standard curve from LPS-stimulated spleen cells. The number of cycles of amplification was chosen to give a PCR product that was easily detected in a gel while remaining on the linear part of the amplification curve. Ethidium bromide-stained gels were photographed with an Eagle Eye II Still Video System (Stratagene, La Jolla, CA), and the intensity of fluorescence was determined using the associated EagleSoft software. To ensure that equivalent amounts of cDNA were used in each reaction, PCR was performed for hypoxanthine phosphoribosyltransferase (HPRT) from each sample, and the cDNA was adjusted to equivalent levels. Each pair of primers spanned at least one intron, which allowed mRNA to be distinguished from any contaminating genomic DNA, and all primers were synthesized at NIAID. The cycle number and sequences used were: HPRT (30 cycles): HPRT sense GTT GGA TAC AGG CCA GAC TTT GGT GT; HPRT antisense GAG GGT AGG CTC GCC TAT AGG CT; IFN-γ (29–30 cycles): IFN-γ sense TGG AGG AAC TCG CAA AAG GAT GAT; IFN-γ antisense TGG GGA CAC TCT CCT CCC CAC.

IFN-γ protein levels were also assayed after the restimulation of spleen cells in vitro. Briefly, splenocyte suspensions (2 × 10^6/mL) in complete RPMI 1640 medium were distributed in 96-well plates (Costar, Cambridge, MA) and exposed to MAg at 50 µg/mL in a total culture volume of 200 µL. Supernatants were collected at 72 h, and IFN-γ levels were assayed by a two-site sandwich ELISA as previously described (38).

Results

Decreased resistance of IL-12-deficient mice to M. avium infection

In a previous study of genetically immunodeficient animals, we found that both GKO and BL/SCID mice are defective in their control of chronic M. avium infection (24). To comparatively assess the contribution of IL-12 to host resistance, we studied the growth of the organism in mice lacking a functional IL-12 p40 gene (39, 40). These animals were each i.v. inoculated with 10^7 CFU of the virulent 2–151 SmT strain. As positive or negative controls, we simultaneously infected BL/SCID, GKO, or wt mice that also share the same C57BL/6 genetic background. Animals were sacrificed at regular intervals after inoculation, and the growth of the bacteria in spleens was determined by the titration of single-cell suspensions onto 7H11 agar and expressed as bacterial load per whole organ. Qualitatively similar results were obtained when the data were expressed as bacterial load either per gram of tissue or per million cells (data not shown). In addition, RT-PCR was performed on RNA that had been extracted from spleen cells to measure the expression of the IFN-γ gene.

As expected on the basis of previous studies, all of the mice strains tested showed an initial increase in bacterial numbers after infection (Fig. 1, top panel). In C57BL/6 mice, growth slowed as the infection was gradually brought under control. As described previously, this correlated with both the induction of a strong IFN-γ response and the development of significant pathology, which were characterized by a monocytic infiltration of infected organs and an enlargement of the liver and spleen (24, 41). Bacterial numbers in the spleens of IL-12 KO, GKO, and BL/SCID...
mice, in contrast to the C57BL/6 control mice, continued to increase up to 8 wk postinfection (Fig. 1, top panel). Infected IL-12 KO and GKO mice displayed nearly identical growth kinetics, confirming the critical function of both cytokines in the development of resistance. Consistent with the known role of IL-12 in the induction of IFN-γ (42), spleen cells from the infected IL-12 KO mice produced IFN-γ mRNA in much lower quantities than did the equivalent cell populations from the infected control animals (Fig. 1, bottom panel). Interestingly, infected BL/SCID mice showed bacterial growth kinetics that were distinct from both IL-12 KO and GKO animals, displaying an intermediate pattern of susceptibility (Fig. 1, top panel). A similar difference in infection was seen in the lungs of these strains, where again, BL/SCID mice characteristically developed bacterial loads that were intermediate between the wt C57BL/6 and the gene KO mice (data not shown). Furthermore, spleen cells from infected BL/SCID mice produced IFN-γ mRNA at levels that were intermediate between that synthesized by splenocytes from infected wt and IL-12 KO animals.

FACS analysis and in vitro restimulation with Ag failed to show any evidence for the development of a specific T cell response in BL/SCID mice (data not shown), indicating that the partial resistance of these animals cannot be accounted for by “leakiness” of the SCID mutation. Instead, the above observations suggested that the residual control of bacterial growth in BL/SCID mice is due to the production of IFN-γ from a non-T cell (i.e., NK cell) source, a response which is characteristically dependent upon IL-12 (43). Synergistic effect of combined IL-12/antibiotic treatment on bacterial loads in BL/SCID mice

To directly assess whether IL-12 can mediate resistance in T-deficient hosts, BL/SCID mice were infected i.v. with 10^7 virulent M. avium and 2 wk later treated for an additional 2 wk with 500 ng/mouse of exogenous murine rIL-12 that was delivered i.p. in 200 μl of buffered saline on a daily basis. This protocol resulted in a significant (p < 0.05) reduction in splenic bacterial colony counts vs those measured in control infected animals that had been injected with saline alone (mean CFU ± SE from two experiments: IL-12 group = 1.3 ± 0.27 × 10^4; control group = 6.8 ± 0.64 × 10^4). Although they survived the treatment, the mice receiving IL-12 at this dose displayed noticeable wasting and splenomegaly when compared with control animals (data not shown).

Although the effect of the high-dose IL-12 treatment on M. avium infection in BL/SCID mice was not dramatic, we hypothesized that the cytokine might induce a more marked increase in bacterial clearance when combined with known antimycobacterial drugs. In performing this analysis, the frequency of IL-12 administration and consequently the total dose was reduced by half (i.e., to 500 ng/mouse on alternate days) to minimize side effects and simplify the treatment regimen. When administered at this dose, IL-12 caused no mortality, weight loss, or change in outward appearance of the animals in the study, while still resulting in a noticeable increase in spleen weight (data not shown). Clarithromycin, a macrolide antibiotic that has been shown to be effective against M. avium infections in both human and mouse (44, 45) was used in the experiment and administered i.p. simultaneously with the IL-12. The dose of the drug chosen (200 mg/kg) was considered to be optimal according to preliminary experiments.

As shown in Figure 2, top panel, treatment with IL-12 at the lower dose caused only a minor reduction in splenic bacterial counts vs the recoveries from control mice. In contrast, when combined with clarithromycin, even the lowered IL-12 treatment resulted in a dramatic decrease in splenic bacterial colonies, >100-fold compared with the numbers determined in control animals. This reduction was highly significant (p < 0.001) with regard to the clearance of bacteria induced by the drug alone at this dose. The same synergistic effect of combined IL-12/clarithromycin treatment was reproducibly observed in four separate experiments.

Since the standard procedure for the clinical treatment of M. avium infection involves the administration of multiple antibiotics to increase efficacy and protect against the growth of drug-resistant variants, we tested the same IL-12 regimen with a second, unrelated antibiotic, rifabutin. As shown in Figure 3, top panel, combined IL-12/rifabutin administration again resulted in a >100-fold decrease in colony counts which was significant (p < 0.01) vs that induced by the drug alone.

The synergistic effect of IL-12 and clarithromycin was also discernible at lower antibiotic concentrations, where combined treatment with 100 mg/kg promoted bacterial clearance more effectively than 200 mg/kg of drug given alone (Fig. 4, top panel). In contrast, synergy with IL-12 was evident only at the highest dose of rifabutin tested because of the lower efficiency of this drug (Fig. 4, bottom panel). Together, these findings indicated that IL-12 can
enhance the efficacy of *M. avium* chemotherapy in immunodeficient hosts, and that this effect is not a peculiarity of the specific drug or regimen employed.

As shown in the middle panel to Figure 2 and the bottom panel to Figure 3, combined IL-12/drug treatment was also highly effective in promoting bacterial clearance in immunocompetent C57BL/6 mice; in the case of animals receiving clarithromycin, this treatment reduced bacterial counts to below the level of detection (≤1 CFU per 10^7 spleen cells). Thus, the protocol can also be efficacious in hosts that are already mounting an immune response to the infection.

**Dependence of synergistic effect of IL-12 treatment on IFN-γ**

To assess the role of IFN-γ induction in the combined effect of IL-12 and drugs on *M. avium* infection in immunodeficient hosts, we simultaneously administered neutralizing anti-IFN-γ mAbs to IL-12/clarithromycin treated, infected BL/SCID mice. As shown in Figure 2, *top panel*, this procedure completely ablated the contribution of IL-12 to the reduction in splenic colony counts that were induced by combined cytokine/drug therapy, while animals receiving control Abs were unaffected. Moreover, combined IL-12/clarithromycin treatment failed to augment bacterial clearance beyond the level induced by drug alone in IFN-γ-deficient (GKO) mice (Fig. 2, *bottom panel*). Interestingly, treating GKO mice with IL-12 in the absence of drug caused a significant enhancement of bacterial recovery. This effect is most likely due to the noticeable influx of macrophages into the spleens of IL-12-treated animals, which, in the case of GKO mice, would allow expanded growth of the pathogen in the absence of immune control.

To confirm that IFN-γ was indeed induced in mice treated with IL-12, mRNA that was specific for the cytokine was quantitated in the spleen cells of infected BL/SCID mice by semiquantitative RT-PCR. In addition, secretion of IFN-γ by the same spleen cells was measured after restimulation with MAg in vitro. As shown in Figure 5, levels of IFN-γ message and protein were significantly augmented in animals treated with IL-12 alone or with IL-12 plus clarithromycin. In both cases, this increase was partially blocked by the in vivo injection of anti-IFN-γ mAbs, suggesting a role for the cytokine in maintaining levels of IL-12 and, consequently,
IFN-γ itself in infected hosts. When administered in combination with IL-12, clarithromycin caused a moderate decrease in IFN-γ message and protein levels compared with that seen with IL-12 treatment alone, perhaps due to reduced induction of the lymphokine because of the drop in bacterial numbers.

Substitution of IFN-γ for IL-12 results in a loss of therapeutic efficacy

Since the effect of IL-12 on drug-induced bacterial clearance is dependent upon IFN-γ, we also evaluated the therapeutic activity of the latter cytokine in the same antibiotic treatment protocol. In these experiments, a dose of IFN-γ (1.0 μg/mouse/injection) that was twice that of IL-12 was employed. As shown in Figure 6, this higher dose of IFN-γ failed to promote bacterial clearance in GKO mice, suggesting a requirement for greater levels of the cytokine to reconstitute the animals. Interestingly, IFN-γ also failed to show any beneficial effects in C57BL/6 mice, perhaps since endogenous IFN-γ levels were already very high in this strain (Fig. 1, bottom panel). In contrast, IFN-γ alone did have a small but significant therapeutic benefit in BL/SCID mice, possibly by augmenting the intermediate levels of the cytokine expressed by that strain (Fig. 6 and bottom panel to Fig. 1). Nevertheless, in all three mouse strains tested, IFN-γ administration clearly failed to enhance the clearance of M. avium that was induced by the antibiotic (clarithromycin) (Fig. 6). Thus, even when employed at a higher dose than IL-12, IFN-γ cannot effectively substitute for that cytokine in promoting drug therapy of M. avium infection.

Discussion

IL-12 is known to be a crucial cytokine in the induction of host resistance to intracellular pathogens (46); for this reason, it has
become an important candidate as an immunotherapeutic agent for a number of major infectious diseases (47). In addition to its function in promoting the cell-mediated immune control of pathogen growth, IL-12 has recently been considered as a potential adjunct to antimicrobial chemotherapy. In a pioneering study, Nabors et al. showed that IL-12 promotes the immunologic clearance of established *Leishmania major* infection in susceptible BALB/c mice as mediated by treatment with the antimonial, pentostam (48). These authors suggested that IL-12 functions in this model by converting an exacerbative Th2 response into a curative Th1 response, an effect that is dependent upon concomitant drug treatment to reduce Ag load. In a different although related study, Clemons et al. demonstrated that IL-12 treatment enhanced the efficacy of fluconazole treatment on central nervous system infection with *Cryptococcus neoformans* in BALB/c mice, although the mechanism of action of the cytokine was not explored (49).

In the present report, we have extended the concept of combined IL-12/drug therapy to the treatment of an important opportunistic pathogen, *M. avium*, in a T cell-deficient setting which is similar to that expected in late-stage AIDS patients who are the primary victims of atypical mycobacterioses. In contrast to the previous studies cited above, the effects of IL-12 and drug observed in the SCID mouse model we employed cannot be due to immunoregulatory changes in T lymphocyte function but instead must involve the induction of alternative pathways of resistance revealed in an immunodeficient milieu. Previous work employing neutralizing mAb had suggested a role for endogenous IL-12 in host resistance to *M. avium* (22, 31) based on elevated bacterial counts. In the current study, this function of IL-12 was confirmed in our model of *M. avium* infection using mice with a defective IL-12 p40 gene. These animals showed a >1000-fold increase in splenic bacterial loads and importantly were indistinguishable from IFN-γ-deficient mice in their high susceptibility to infection (Fig. 1, top panel). Moreover, both IL-12- and IFN-γ-deficient animals became more heavily infected than SCID mice, suggesting that the latter animals retain a partially effective IL-12/IFN-γ-dependent resistance mechanism. Indeed, we have previously shown that infected SCID mice are only partially impaired in their mRNA responses for both cytokines (24), and their reduced production of IFN-γ message relative to wt mice was confirmed here (Fig. 1, bottom panel). Interestingly, IL-12 KO mice also retain a partial IFN-γ response but at a level that is significantly lower than that observed in the SCID animals (Fig. 1, bottom panel). Thus, it seemed logical to assume that the defective resistance of SCID mice reflects a deficiency in IL-12/IFN-γ production that might be corrected by the exogenous administration of one of the cytokines.

When administered to previously infected SCID mice, rIL-12 in high doses (500 ng/mouse, daily) did indeed cause a fivefold reduction in splenic bacterial loads as measured 2 wk later. The use of higher doses and longer treatment periods was impractical given the limited supply of cytokine, although a larger reduction in CFUs was observed in a single experiment in which cytokine was given at the same dose for 4 wk (data not shown). These results support earlier observations (23) showing that exogenous IL-12 treatment augments the resistance of BALB/c and DBA/2 mice to *M. avium* infection and extend these findings by demonstrating that comparable effects on bacterial growth can be induced in the absence of functional T cells. Nevertheless, it is doubtful that long-term treatment with IL-12 would be economically practical or sufficiently efficacious to justify the use of such a regimen in AIDS patients with atypical mycobacterioses. Moreover, a clinical trial of such a procedure would require the removal of patients from antibiotic therapy, an unethical practice. The latter considerations further support the rationale for combined IL-12/drug therapy.

The major finding of the current study is that the treatment of *M. avium*-infected, T cell-deficient mice with rIL-12 and clarithromycin results in a striking reduction in splenic bacterial loads. This effect was observed using a suboptimal, lowered total dose of cyto- tkine (500 ng/mouse, three times per week) and was clearly greater than that induced by the drug or cytokine alone (Fig. 2). Moreover, the same drug/ cytokine synergy was observed in immunocompetent C57BL/6 mice as well as with a different antimycobacterial agent, rifabutin (Figs. 3 and 4), suggesting that IL-12 coadministration may have broad application as an adjunct for chemotherapy of *M. avium* infections.

Although our experiments in GKO mice and with IFN-γ neutralization indicate that IL-12 mediates this synergistic effect through the induction of the former lymphokine, the mechanism by which IFN-γ enhances drug activity is not yet clear. An obvious explanation is that drug treatment lowers the resistance of the organism to microbicidal killing by IFN-γ-activated macrophages or, conversely, that IFN-γ-induced macrophage activation prevents the recovery of bacteria that have been partially crippled by drug treatment. Alternatively, as suggested by the results of previous studies using IFN-γ in combination with other antimicrobials (50), the cytokine may enhance the uptake of antibiotics into infected cells. Although the participation of one or several of the above mechanisms would seem likely, other data suggest that synergy in the activity of IL-12 and antibiotics in the therapy of *M. avium* may have a quite different explanation. Thus, as reported previously (24), we have found that the virulent bacterial strain used in our in vivo mouse infections appears to be refractory to in vitro killing by IFN-γ-activated macrophages. Moreover, our preliminary attempts to demonstrate an enhancement of drug (clarithromycin)-mediated intracellular killing of *M. avium* in vitro by the addition of IFN-γ to the cultures have failed to reveal significant effects (data not shown). Therefore, it is possible that IL-12 augments bacterial clearance by an IFN-γ-dependent mechanism that is unrelated to the direct microbicidal function of the macrophage.

The data demonstrating that IL-12 mediates its effects on *M. avium* through the induction of IFN-γ raise the question of whether IFN-γ itself would be equal or superior to IL-12 in synergizing with antibiotics for bacterial clearance. To address this issue, infected mice were treated with clarithromycin and IFN-γ at a level that was twice that of the IL-12 employed in identically performed drug/cytokine experiments. Interestingly, no significant effects of IFN-γ on bacterial clearance were observed in the presence of drug with that dose and regimen (Fig. 6), suggesting that IL-12 is a more effective immunotherapeutic agent. Since it is known that IL-12 has a longer half-life than IFN-γ (51) and, as a upstream inducer, should trigger larger amounts of the latter cytokine than the dose injected, one possibility is that IL-12 administration results in a higher, sustained level of IFN-γ than that achieved by direct IFN-γ treatment. Alternatively, IL-12 is known to have several physiologic effects (e.g., on hemopoiesis (41, 52) and nitric oxide induction via TNF-α (53)) that are not shared by IFN-γ, and it is conceivable that one of these activities may synergize with the IFN-γ-mediated functions of the cytokine in promoting bacterial clearance. Detailed dose-response studies comparing the effects of IFN-γ and IL-12 administration on the host response to *M. avium* in drug-treated animals will be required to address these questions.

Although the mechanisms underlying the efficacy of combined IL-12/antibiotic treatment remain to be elucidated, our current findings suggest that the strategy is worthy of further investigation as an approach for improving the management of this important opportunistic pathogen in AIDS patients. While considerable toxicity was encountered in the initial clinical trials with IL-12, this
References


