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Interleukin-12 Modulates the Protective Immune Response in SCID Mice Infected with *Histoplasma capsulatum*

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Infection with *Histoplasma capsulatum* results in a subclinical infection in immunocompetent hosts due to an effective cellular immune response. By contrast, immunodeficient individuals can have a severe disseminated and potentially fatal disease. In a previous study, it was demonstrated that normal mice infected intravenously with *H. capsulatum* and treated with interleukin-12 (IL-12) at the time of infection were protected from a fatal outcome. In this study, we examined the immunomodulatory effects of IL-12 on disseminated histoplasmosis in immunodeficient SCID mice. SCID mice infected with *H. capsulatum* and treated with IL-12 showed an increase in survival and a reduction in the colony counts of *H. capsulatum* in internal organs at 14 days after infection. The protective effect of IL-12 was abrogated if animals were also treated with a neutralizing antibody to gamma interferon (IFN-γ). IL-12 treatment also resulted in an increase in mRNA expression and protein production for IFN-γ, tumor necrosis factor alpha (TNF-α), and nitric oxide from spleen cells. When IL-12 was combined with amphotericin B (AmB) treatment, there was a significant increase in survival compared with either modality alone. Moreover, combined treatment resulted in an increase in both IFN-γ and TNF-α production, as well as in a substantial reduction in *H. capsulatum* burden at 35 and 90 days postinfection. This study demonstrates that IL-12 modulates the protective immune response to histoplasmosis in SCID mice and also suggests that IL-12 in combination with AmB may be useful as a treatment for *H. capsulatum* in immunodeficient hosts.

Histoplasmosis, a respiratory fungal infection, is acquired by inhalation of conidial or mycelial fragments of *Histoplasma capsulatum*. The organism then takes up residence in mononuclear phagocytic cells, and conversion to the yeast form occurs. At this stage, the cellular immune response of the host determines the degree of clinical manifestations in histoplasmosis. Infection of immunocompetent hosts either resolves spontaneously or is associated with an influenza-like illness; however, immunocompromised individuals with impaired cellular immunity develop a disseminated disease caused by hematogenous dissemination to extrapulmonary organs, which can lead to a fatal outcome. The emergence of disseminated histoplasmosis has become an alarming complication in human immunodeficiency virus (HIV)-infected individuals and in other immunocompromised hosts living in certain geographic regions of the United States (17, 22, 35).

The interaction of T cells and macrophages and the production of soluble mediators such as gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) from these cells have been shown to be important in mediating immunity to infection (11). The defect in cellular immunity that occurs in the course of HIV infection makes current treatment inadequate to eradicate the infection. Current treatments for histoplasmosis in HIV-infected individuals include antifungal agents such as amphotericin B (AmB) and itraconazole for initial treatment of severe and mild disease, respectively (22, 34, 35); however, antifungal treatment such as AmB fails to induce an initial remission in a small percentage of patients (35). Moreover, relapse occurs in most patients infected with HIV, suggesting that in the absence of intact cellular immunity, antifungal treatment alone will not eradicate the infection (35). In addition, AmB therapy is associated with significant morbidity and usually requires a course of hospitalization for its administration.

We recently demonstrated that normal mice infected with *H. capsulatum* and treated with interleukin-12 (IL-12) at the time of infection had both increased survival and a decreased tissue burden of *H. capsulatum* as assessed by quantitative culture (40). IL-12 mediated its effect by increasing the amount of IFN-γ produced from both total spleen cells and $CD4^+$ T cells. The increase in IFN-γ from total spleen cells could have been from $CD8^+$ T cells and NK cells, as well as from $CD4^+$ T cells; indeed, much of the original work on IL-12 was on its effect of increasing IFN-γ production by NK cells (37). Moreover, two of the first experiments showing IL-12 efficacy on infections in vivo used SCID mice infected with *Listeria monocytogenes* (33) or *Toxoplasma gondii* (13). In each of these models, it was shown that IL-12 was having its effect by increasing IFN-γ production from NK cells. In this study, we used an experimental model of severe combined immunodeficiency (SCID) mice to examine the IL-12 modulation of protective immune responses and cytokine production in animals infected with *H. capsulatum*. We show that either IL-12 or AmB treatment prolongs survival and decreases the tissue burden of *H. capsulatum*. Moreover, treatment with both IL-12 and AmB resulted in a substantial increase in survival, as well as in a decrease in *H. capsulatum* burden. Interestingly, the combined therapy also resulted in an increase in both IFN-γ and TNF-α production from spleen cells stimulated in vitro. These results demonstrate that IL-12 is a key immunomodulator of protective immune responses to histoplasmosis and also suggest that combination therapy with IL-12 and AmB might be useful for patients with impaired T-cell immunity.

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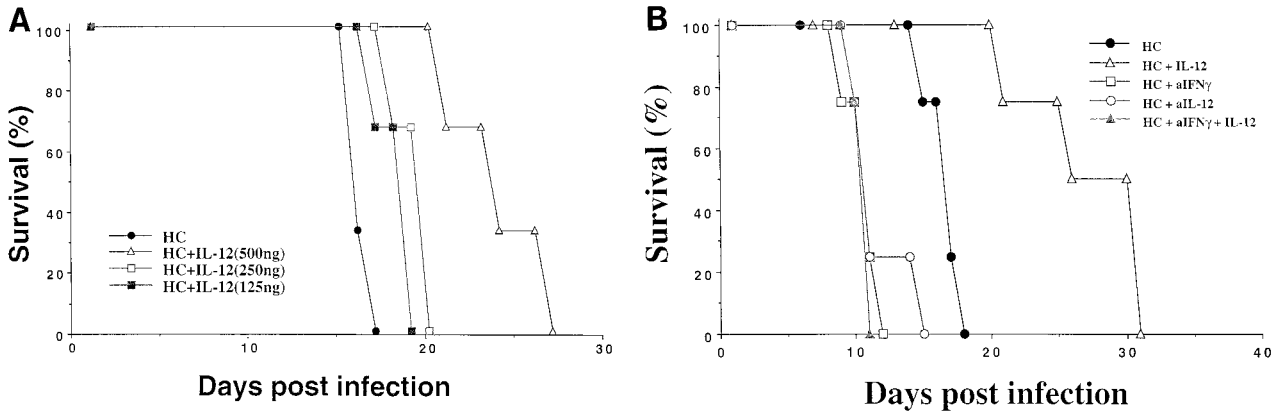

FIG. 1. IL-12 prolongs survival of SCID mice infected with *H. capsulatum* (HC). Groups of four mice were injected i.v. with 5×10^4 yeast cells in 0.5 cc of PBS. (A) Mice were administered IL-12 i.p. at a dosage of 500, 250, or 125 ng at the time of infection and once a week for 2 weeks. Similar results were seen in four separate experiments. (B) Mice were infected in a similar manner as above and treated with IL-12 at a dosage of 500 ng at the time of infection and weekly for two additional doses. Additionally, infected mice were treated at the time of infection with a single injection i.p. of 1 mg of antibody to IFN-γ (aIFNγ), alone or with IL-12, or antibody to IL-12 (aIL-12). Results are pooled from six separate experiments.

MATERIALS AND METHODS

Mice and infection. Virus-free female SCID mice 6 to 10 weeks of age were purchased from Taconic (Germantown, N.Y.) and kept in the Animal Care Facility of the National Institute of Allergy and Infectious Diseases under pathogen-free conditions. Mice were inoculated intravenously (i.v.) with 5×10^4 *H. capsulatum* yeast cells in 0.5 cc of sterile phosphate-buffered saline (PBS). It should be noted that we encountered no spontaneous deaths in uninfected control mice.

Media. Hanks balanced salt solution (Biofluids, Inc., Rockville, Md.) was used as a wash medium. Complete medium, consisting of RPMI 1640 (Biofluids, Inc.) supplemented with 10% fetal calf serum (Biofluids, Inc.), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), and 2-mercaptoethanol (0.05 mM), was used for culturing spleen cells.

Preparation and quantitation of *H. capsulatum*. The yeast phase of *H. capsulatum* (strain GS-57) was used in all experiments as previously described (40). Inocula for mice were grown on brain heart infusion agar slants (Remel, Richmond, Va.) for 48 h at 37°C, after which the culture was incubated on a gyrating shaker for another 48 h at 37°C. Aliquots of 50 ml of broth containing the yeast cells were centrifuged, and the pellets were washed three times in PBS. Cells were counted by using a hemocytometer, and appropriate dilutions were made in PBS to inoculate the mice. Quantitation of *H. capsulatum* was performed as previously described (40). Briefly, mice infected with *H. capsulatum* and treated with IL-12 and/or AmB were sacrificed at various times postinfection, and their spleens were removed. One-third of the spleen was taken from each of three animals per group, and these were combined and homogenized in a sterile mortar with PBS to prepare a 1:10 (wt/vol) suspension. Tenfold dilutions in PBS were plated in duplicate at 0.05 ml/plate on brain heart infusion medium supplemented with serum albumin growth factor and chloramphenicol (40) and incubated for 7 days at 30°C. Colonies were enumerated, and counts were recorded as CFUs.

Treatment of mice. Murine IL-12 was a generous gift from the Genetics Institute (Cambridge, Mass.). Mice were injected intraperitoneally (i.p.) with 500, 250, or 125 ng in 0.5 ml of PBS at the time of infection and once a week for the following 2 weeks. In most experiments, mice were injected with 500 ng in 0.5 ml of PBS at the time of infection and subsequently with 250 ng once a week for 2 weeks. In some experiments, mice were also treated at the time of infection with 1 mg of antibody i.p. All neutralizing antibodies were purified from ascites by ammonium sulfate precipitation. Rat anti-mouse IFN-γ (anti-IFN-γ) monoclonal antibody XMG1.2 (4) was used to neutralize IFN-γ in vivo. The preparation used had been titered in a viral neutralization assay in which 1.23 ng of the antibody was required for 50% inhibition of activity of 1 U of IFN-γ. Purified monoclonal antibodies against murine IL-12 (C17.8) (39) and TNF-α (HT-11-22) (30) were obtained from G. Trinchieri and M. Stevenson, respectively. Anti-IL-12 antibody was given i.p. at the time of infection at a dose of 1 mg. Anti-TNF-α antibody was given at a dose sufficient to neutralize 10^5 U. AmB was purchased from Pharma-Tek Inc. (Huntington, N.Y.) and dissolved in distilled water shortly before use. Mice were treated with 100 μg of AmB i.p. on day 3 postinfection and subsequently three times a week, for a total of six doses. A similar dose and schedule was found to be efficacious in reducing infection with *Candida albicans* (2).

Measurement of lymphokine production. At various times postinfection, mice were sacrificed and spleens were taken from each mouse (at least three mice for each group). Erythrocytes were lysed, and cells were washed two times and resuspended in complete medium. Spleen cells (5×10^5) were added to flat-bottomed 96-well plates in a total volume of 0.2 ml. Heat-killed *H. capsulatum* (HKHC) (3×10^5 cells) was added to cell cultures, and plates were incubated at 37°C for 48 h, at which time supernatants were collected and stored at −20°C.

Measurement of IFN-γ was carried out by specific enzyme-linked immunosorbent assay (ELISA) as described previously (8). The lower limit of the assay was 3 to 10 U/ml. One unit of IFN-γ per milliliter is equivalent to 0.03 pg. TNF-α was measured with an ELISA kit purchased from Endogen (Cambridge, Mass.); the lower limit of detection was 10 pg/ml. The amount of either IFN-γ or TNF-α was calculated by using serial dilutions of supernatants to obtain values that fell within the linear range of the standard curve. The standard error of the mean for all experiments was found to be <10%. Nitrite concentration in the supernatants was assayed by a standard Griess reaction. Briefly, 100 μl of supernatant was added to 100 μl of mixture reagent with 1% sulfanilamide and 1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (1:1) in 2.5% H_3PO_4 (Sigma Immuno-Chemicals, St. Louis, Mo.) at room temperature for 5 min. Optical density at 540 nm was measured. NO_2 concentration was determined with reference to a standard curve by using sodium nitrite in culture media.

Cytokine mRNA measurement. Cytokine mRNA levels were determined by semiquantitative reverse transcriptase PCR techniques as previously described (40). Briefly, spleens (three per group) from SCID mice infected with *H. capsulatum* were removed on day 14 postinfection, and cell suspensions were prepared after lysis of erythrocytes. Cells (10^7 per sample) were then pelleted by centrifugation and resuspended in 1 ml of RNAzol B (Tel-Test, Friendswood, Tex.) for RNA isolation. One microgram of total RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, Md.). The reaction mixture was then diluted 1:8, and 10 μl was used for specific semiquantitative amplification of cytokine mRNA with *Taq* DNA polymerase (Promega, Madison, Wis.). PCR products were subjected to Southern blot analysis and were subsequently probed with internal cytokine-specific oligonucleotides and visualized with the ECL chemiluminescence detection system (Amersham, Arlington Heights, Ill.). Nucleotide sequences for sense primers, antisense primers, and probes for hypoxanthine phosphoribosyltransferase (23 cycles), IFN-γ (28 cycles), IL-12 p40 (30 cycles), IL-4 (35 cycles), IL-10 (35 cycles), transforming growth factor β (TGF-β) (40 cycles), and TNF-α (35 cycles) were as previously described (13). Cycle numbers, indicated within parentheses, were determined experimentally.

Statistics. Statistical evaluation of differences between means of experimental groups was done by the use of analyses of variance and multiple Student's *t* tests. Comparisons were made between treated and untreated infected mice. The log-rank was used for statistical analysis of mortality.

RESULTS

IL-12 prolongs survival in SCID mice infected with *H. capsulatum*. In a previous study, we demonstrated that normal C57BL/6 mice infected with *H. capsulatum* and treated with IL-12 at the time of infection were protected from a fatal outcome (40). In this study, we determined whether administration of IL-12 could influence the outcome of SCID mice infected with *H. capsulatum*. SCID mice injected i.v. with 5×10^4 *H. capsulatum* yeast cells succumbed to infection in approximately 3 weeks (Fig. 1A and B). To examine whether

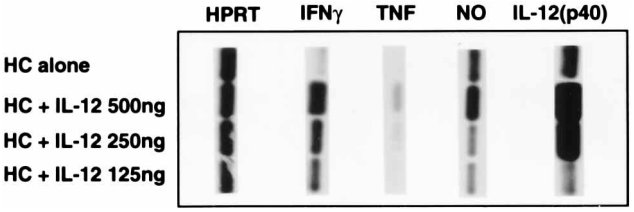

FIG. 2. IL-12 treatment increases mRNA expression for IFN-γ, TNF-α, and IL-12 (p40). SCID mice were infected with *H. capsulatum* (HC) and treated with IL-12 as described in the legend to Fig. 1. Spleen cells were prepared at day 14 postinfection. Cytokine mRNA was determined for IFN-γ, TNF-α, NO, and IL-12 (p40) by semiquantitative reverse transcriptase PCR as described in Materials and Methods. HPRT, hypoxanthine phosphoribosyltransferase.

IL-12 treatment affected mortality, mice were infected with *H. capsulatum* and treated with IL-12 at dosages of 500, 250, or 125 ng at the time of infection and once a week for the following 2 weeks (Fig. 1A). Survival of mice treated with IL-12 was increased only at the highest dosage (500 ng) (Fig. 1A). The increase in survival when the highest dosage of IL-12 was used was also seen in other experiments (Fig. 1B). The survival of mice treated with IL-12 (500 ng) at the time of infection and daily for the next 4 days (data not shown) was not different from that of mice treated weekly, for whom results are shown in Fig. 1. Our previous study using normal mice showed that infected animals treated with neutralizing antibodies to either IFN-γ or IL-12 at the time of infection had accelerated mortality, demonstrating that the infection induced both cytokines. SCID mice treated with neutralizing antibodies to either IFN-γ or IL-12 at the time of infection also had accelerated mortality (Fig. 1B). Finally, the ability of IL-12 to prolong survival was abrogated if a neutralizing antibody to IFN-γ was coadministered to the mice, demonstrating that the effect of IL-12 is likely mediated indirectly through induction of IFN-γ (Fig. 1B). The results shown in Fig. 1B are combined from six separate experiments. In these experiments, the mean numbers of days ± standard deviations until death are as follows: *H. capsulatum* alone, 17.0 ± 0.31; *H. capsulatum* plus IL-12, 27.6 ± 1.35; *H. capsulatum* plus anti-IFN-γ, 10.3 ± 0.26; *H. capsulatum* plus anti-IL-12, 11.1 ± 0.52; and *H. capsulatum* plus IL-12 plus anti-IFN-γ, 10.8 ± 0.125. All groups were significantly different ($P < 0.001$) from the *H. capsulatum*-alone group, as determined by log-rank analysis.

IL-12 increases in vivo mRNA expression and in vitro cytokine production in response to infection with *H. capsulatum*. To determine the effect of IL-12 on immune regulation in vivo, mRNA expression for various cytokines and nitric oxide (NO) was assessed from spleen cells harvested 14 days after infection. The expression of mRNA for IFN-γ, IL-12 p40, and NO showed a marked dose-dependent increase in mice treated with IL-12 (Fig. 2). Expression of TNF-α also increased following IL-12 treatment, albeit to a lesser extent than expression of the other cytokines. We also examined the time course for mRNA expression and obtained similar results at both 7 and 21 days postinfection (data not shown). The increase in IL-12 expression from mice treated with IL-12 is consistent with a positive feedback loop in which IFN-γ induced by IL-12 can act to increase endogenous IL-12 production. Taken together, these results are consistent with the hypothesis that interventions that prolong survival are mediated through an increase in IFN-γ, TNF-α, and NO, as has been previously shown for other murine models of infection (33, 37). To examine the effect of IL-12 treatment on the induction of IFN-γ and TNF-α in vitro, spleen cells were stimulated with HKHC, and IFN-γ or TNF-α production was assessed. IL-12 treatment resulted in a twofold increase in IFN-γ production in response to HKHC (Fig. 3A). Moreover, mice treated with IL-12 showed a marked increase in production of TNF-α and NO in a dose-responsive manner (Fig. 3B and C).

Effects of IL-12 on the tissue burden of *H. capsulatum*. To further characterize the biological effect of both exogenous and endogenous IL-12, the quantitative burden of *H. capsulatum* was determined from spleen cells taken at 7 and 14 days after infection from various groups of infected mice (Table 1). Mice treated with IL-12 showed a two- to threefold decrease in the CFUs of *H. capsulatum* at day 7 and a sevenfold decrease at day 14, compared with the control infected mice. In contrast, mice treated with antibodies to IL-12 or IFN-γ at the time of infection showed a two- to fivefold increase in *H. capsulatum* compared with the infected controls. Thus, the quantitative burden of *H. capsulatum* in the various groups correlates well with both the mortality and cytokine production data presented above.

Mice treated with IL-12 plus AmB have enhanced survival compared with mice treated with IL-12 or AmB alone. Although IL-12 prolonged survival in SCID mice, the animals still succumbed to the infection within 2 months. We next determined whether combination of IL-12 and AmB would have a more lasting effect on survival. Mice were treated with IL-12 and/or AmB to determine the most efficacious regimen (Fig. 4). IL-12 at a dosage of 500 ng was administered at the time of infection and weekly for 2 additional weeks. AmB was given 3 days after infection and on a schedule of three doses per week for 2 weeks. Mice treated with either IL-12 (26.0 ± 4.39 days) or AmB (37.8 ± 3.63 days) had a statistically significant ($P < 0.001$) increase in survival compared with the infected controls (16.2 ± 1.71 days). Moreover, mice treated with IL-12 plus AmB had a marked increase in survival (66.6 ± 18.04 days) compared with the infected controls or with mice given either treatment alone ($P < 0.001$). These data were pooled from three separate experiments. In two of the experiments, three of five mice remained alive more than 90 days after infection. In two additional experiments, mice treated with IL-12 and a single dose of AmB also showed prolonged survival compared with those given either treatment alone (data not shown). Thus, IL-12 and AmB are complementary in their effects on experimental histoplasmosis.

IL-12 plus AmB increases production of IFN-γ and TNF-α. Spleen cells from IL-12-treated mice stimulated in vitro with specific HKHC antigen had increased production of IFN-γ compared with spleen cells from infected controls (Fig. 5A). In contrast, spleen cells from mice treated with AmB did not show an increase in IFN-γ production. Interestingly, when both IL-12 and AmB were administered, there was a two- to threefold increase in IFN-γ compared with IL-12 alone (Fig. 5A). TNF-α production was increased in mice treated with either IL-12 or AmB (Fig. 5B), and combination treatment potentiated this increase.

To determine the effect of IL-12 and AmB treatment on in vivo expression of cytokines, mRNA expression for IL-12, IFN-γ, and TNF-α was assessed on days 14 and 35 after infection. At day 14 postinfection, IL-12 treatment caused a substantial increase in mRNA expression for IFN-γ, TNF-α, and the p40 chain of IL-12, compared with expression in the infected control animals (Fig. 6A). AmB treatment caused a level of IL-12 (p40) mRNA expression similar to that found in mice treated with IL-12. There was also a slight increase in TNF-α expression compared with that of the infected control group, but this was substantially lower than the increase in the IL-12-treated group. There was essentially no change in IFN-γ

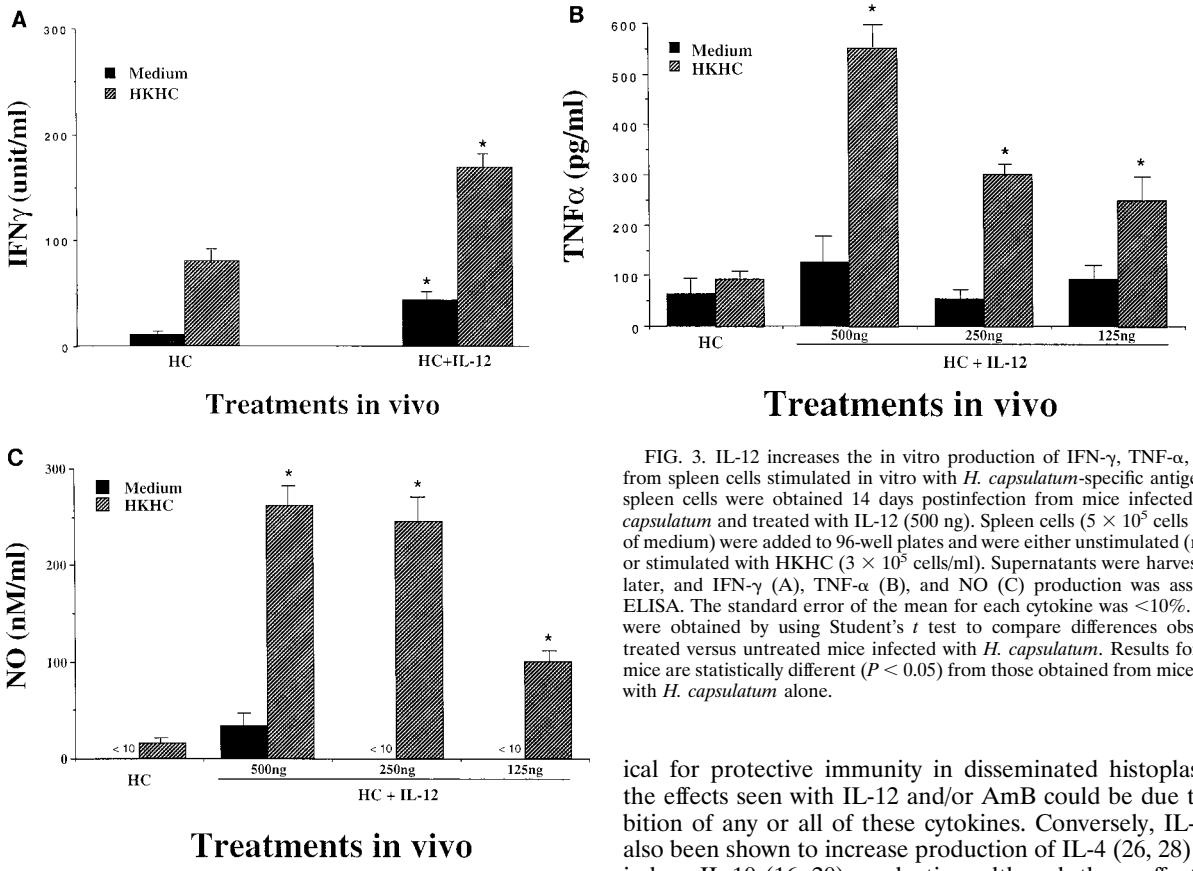

FIG. 3. IL-12 increases the in vitro production of IFN-γ, TNF-α, and NO from spleen cells stimulated in vitro with *H. capsulatum*-specific antigen. Total spleen cells were obtained 14 days postinfection from mice infected with *H. capsulatum* and treated with IL-12 (500 ng). Spleen cells (5×10^5 cells in 0.2 ml of medium) were added to 96-well plates and were either unstimulated (medium) or stimulated with HKHC (3×10^5 cells/ml). Supernatants were harvested 48 h later, and IFN-γ (A), TNF-α (B), and NO (C) production was assessed by ELISA. The standard error of the mean for each cytokine was <10%. *P* values were obtained by using Student's *t* test to compare differences observed in treated versus untreated mice infected with *H. capsulatum*. Results for treated mice are statistically different ($P < 0.05$) from those obtained from mice infected with *H. capsulatum* alone.

mRNA expression compared with that in the control group. When mRNA expression from mice treated with both IL-12 and AmB was measured, a slight increase in IFN-γ and a significant increase in TNF-α expression, compared with levels with either treatment alone, were observed. At day 35 postinfection, animals treated with both IL-12 and AmB had increased mRNA expression for both IFN-γ and TNF-α, compared with those receiving AmB alone. These results suggest that the combination of IL-12 and AmB induces an increase in IFN-γ and TNF-α above that seen with either treatment alone.

The cytokines IL-4 (25), IL-10 (23), and TGF-β (1, 9, 15, 24, 27) have been shown to act in a counterregulatory manner in suppressing IFN-γ production. Since IFN-γ production is critical for protective immunity in disseminated histoplasmosis, the effects seen with IL-12 and/or AmB could be due to inhibition of any or all of these cytokines. Conversely, IL-12 has also been shown to increase production of IL-4 (26, 28) and to induce IL-10 (16, 20) production, although these effects were on $CD4^+$ T cells (16, 26, 28). Nevertheless, it was of interest to determine what effect IL-12 treatment had on these counterregulatory cytokines (Fig. 6B). The expression of mRNA for IL-10 was slightly lower in animals treated with AmB alone and AmB plus IL-12 than in the infected controls or in animals treated with IL-12 alone. We were unable to detect significant mRNA for IL-4 in any of the groups tested. Finally, expression of TGF-β was also similar in all groups tested. Thus, IL-12 and AmB treatment did not significantly alter the expression of IL-4, IL-10, and TGF-β compared with expression in the in-

TABLE 1. Effect of IL-12 on quantitative burden of *H. capsulatum* in SCID mice[a]

Treatment	*H. capsulatum* CFU	
	Day 7	Day 14
H. capsulatum alone	1.6×10^6	1.5×10^7
H. capsulatum + IL-12	6.0×10^5	2.3×10^6
H. capsulatum + anti-IL-12	3.8×10^6	—[b]
H. capsulatum + anti-IFN-γ	7.8×10^6	—

[a] Mice were infected with *H. capsulatum* and treated with IL-12 or various anticytokines. At various times postinfection, mice were sacrificed, and portions of spleen cells from each mouse were combined and plated at various concentrations on agar plates as described in Materials and Methods. One week later, CFUs were counted.

[b] —, no data. All mice succumbed to infection at this time point.

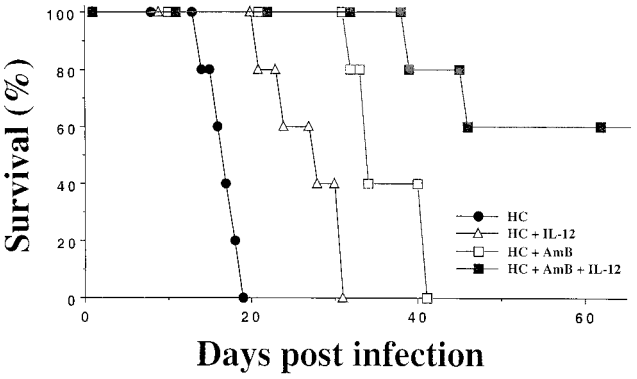

FIG. 4. IL-12 and AmB prolong survival of SCID mice infected with *H. capsulatum* (HC). Groups of five mice were injected i.v. with 5×10^4 yeast cells in 0.5 cc of PBS. Mice were left untreated or were treated with IL-12, AmB, or both. IL-12 was administered i.p. at a dosage of 500 ng at the time of infection and once a week for 3 weeks. AmB (100 μg) was administered i.p. 3 days after infection and three times per week for 2 weeks with or without IL-12. Results are pooled from three separate experiments.

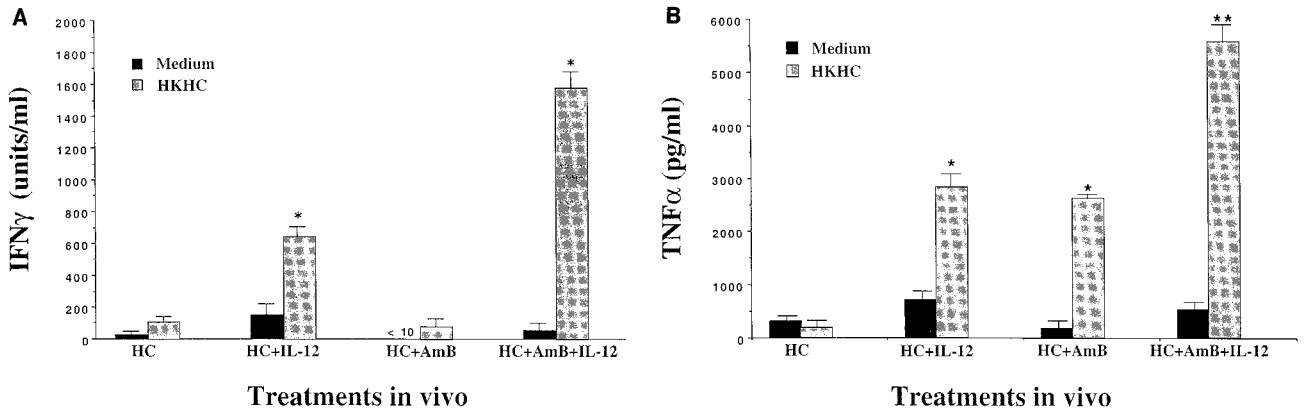

FIG. 5. IL-12 and AmB increase production of IFN-γ and TNF-α. Total spleen cells were obtained 14 days postinfection from mice infected with *H. capsulatum* (HC) and treated with IL-12 (500 ng) and/or AmB. Cells (5×10^5 in 0.2 ml of medium) were added to 96-well plates and left unstimulated (medium) or stimulated with HKHC (3×10^5 cells/ml). Supernatants were harvested 48 h later, and IFN-γ (A) or TNF-α (B) production was assessed by ELISA. Statistical analysis was performed as described in the legend to Fig. 3.

fected controls. Our results showing that mRNA for TGF-β is not affected by IL-12 treatment are also consistent with recent data reported by Wynn et al., who showed that mRNA expression of TGF-β in the livers of normal mice injected with *Schistosoma mansoni* eggs was not appreciably affected by cotreatment with IL-12 (38). It should be noted, however, that although the level of mRNA expression for TGF-β was not affected by IL-12 or AmB, the expression of active TGF-β protein is highly regulated. Thus, the possibility that IL-12 has a role in affecting production of active TGF-β should not be precluded.

Effects of IL-12 and AmB on the tissue burden of *H. capsulatum*. Figures 4 through 6 demonstrate that the combination of AmB and IL-12 prolongs survival and increases both IFN-γ and TNF-α expression. To extend these findings, the CFUs of *H. capsulatum* from spleens were assessed 14 and 35 days after infection. Table 1 shows that IL-12 treatment caused a sevenfold reduction in *H. capsulatum* after 14 days of infection compared with the level in infected controls. In results combined from three separate experiments (Table 2), IL-12 treatment caused a 10-fold reduction in the number of *H. capsulatum* CFUs ($3.56 \times 10^6 \pm 1.3 \times 10^6$) compared with the levels in controls ($3.02 \times 10^7 \pm 2.3 \times 10^7$) at 14 days after infection ($P < 0.05$). Treatment with AmB alone ($8.85 \times 10^3 \pm 14.8 \times 10^3$ CFUs) or combined with IL-12 ($1.42 \times 10^3 \pm 23.1 \times 10^3$ CFUs) caused a 3-log-unit reduction in *H. capsulatum* tissue counts from those in mice treated with IL-12 alone at 14 days postinfection ($P < 0.05$). Interestingly, there was a further reduction in *H. capsulatum* counts in the AmB-plus-IL-12 group compared with the AmB-alone group ($P < 0.05$). The reduction in the amount of *H. capsulatum* when both AmB and IL-12 were used was also striking when quantitation was done at 35 days after infection: the combined treatment resulted in a 25-fold reduction in *H. capsulatum* counts in experiment 2, and in experiment 3, no organisms were detected in the combined treatment group. Moreover, several mice treated with both modalities were still alive 90 days after infection and remained free of infection (Fig. 4 and Table 2). Thus, the prolonged survival in mice treated with both IL-12 and AmB is likely due to increased production of cytokines with concomitant reduction in *H. capsulatum* tissue burden.

DISCUSSION

Disseminated histoplasmosis has emerged as a difficult fungal infection to eradicate in individuals infected with HIV (17, 22, 35). In our previous studies using normal C57BL/6 mice, it was shown that IL-12 given at the time of infection protected animals and led to complete eradication of the infection. The effect of IL-12 was abrogated if mice were also injected with an

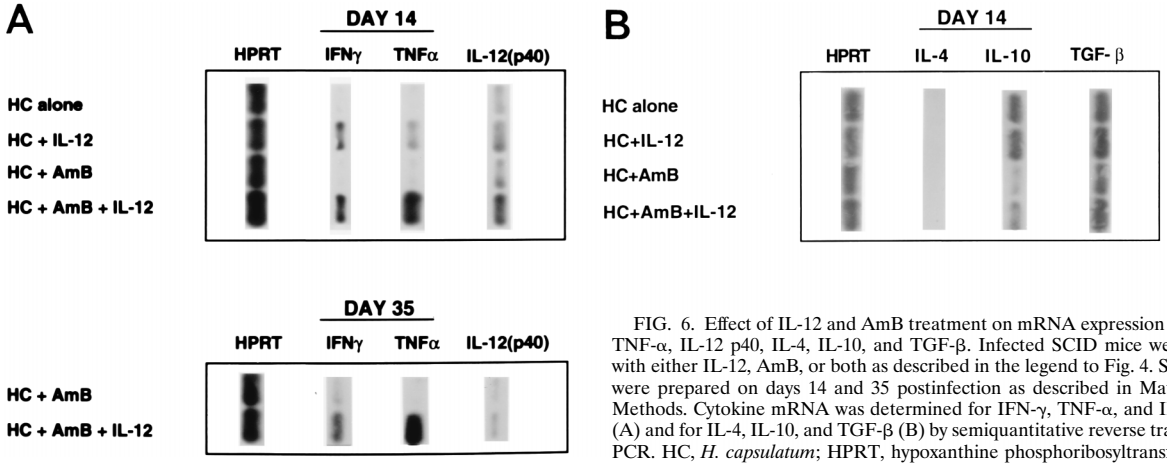

FIG. 6. Effect of IL-12 and AmB treatment on mRNA expression for IFN-γ, TNF-α, IL-12 p40, IL-4, IL-10, and TGF-β. Infected SCID mice were treated with either IL-12, AmB, or both as described in the legend to Fig. 4. Spleen cells were prepared on days 14 and 35 postinfection as described in Materials and Methods. Cytokine mRNA was determined for IFN-γ, TNF-α, and IL-12 (p40) (A) and for IL-4, IL-10, and TGF-β (B) by semiquantitative reverse transcriptase PCR. HC, *H. capsulatum*; HPRT, hypoxanthine phosphoribosyltransferase.

TABLE 2. Effect of IL-12 alone or combined with AmB on quantitative burden of *H. capsulatum* in SCID mice[a]

Treatment	*H. capsulatum* CFU					
	Expt 1	Expt 2		Expt 3		
	Day 14	Day 14	Day 35	Day 14	Day 35	Day 90
H. capsulatum alone	1.26×10^7	5.6×10^7	—[b]	2.2×10^7	—	—
H. capsulatum + IL-12	4.5×10^6	2.1×10^6	—	4.1×10^6	—	—
H. capsulatum + AmB	2.6×10^4	2.5×10^2	6.4×10^6	3.0×10^2	3.0×10^5	—
H. capsulatum + AmB + IL-12	4.1×10^3	1.5×10^2	2.4×10^5	0.3×10^2	0	0

[a] Mice were infected with *H. capsulatum* and treated with IL-12 and/or AmB. Results are reported as described in Table 1, footnote *a*.
[b] —, no data. All mice succumbed to infection at this time point.

antibody against IFN-γ, demonstrating that the effect of IL-12 was mediated through its ability to increase IFN-γ production (40). While one of the major mechanisms of IL-12 is to influence the production of IFN-γ from $CD4^+$ T cells (14, 28), it has also been shown to have an immunoregulatory role independent of T cells (13, 33). SCID mice infected with either *L. monocytogenes* or *T. gondii* and treated with IL-12 had enhanced survival through increased production of IFN-γ from NK cells (13, 33). Our initial experiments examined whether IL-12 would also influence the outcome in SCID mice infected with *H. capsulatum*. Mice infected with *H. capsulatum* and treated with IL-12 had increased production of IFN-γ, TNF-α, and NO from spleen cells as assessed by mRNA or protein. The increased production of these cytokines by IL-12 occurred in a dose-dependent manner. Treatment with IL-12 also reduced the number of CFUs of *H. capsulatum* in spleen cells of infected mice, resulting in increased survival of these mice compared with infected controls (Table 1). The effect of IL-12 was abrogated by treatment with an antibody against IFN-γ, showing that IL-12 mediated its effects indirectly through IFN-γ (Fig. 1B); however, IL-12 treatment still did not clear the infection, and mice ultimately succumbed at 30 to 40 days postinfection.

While initial treatment with AmB is often associated with clinical remission, relapse is common, demonstrating that this treatment is not curative (17, 22, 35). Moreover, treatment with AmB is often associated with significant morbidity. In the present study, we observed that *Histoplasma*-infected mice treated with AmB had increased survival compared with those receiving IL-12 alone; treatment with both IL-12 and AmB resulted in a striking increase in survival of the infected mice. This is consistent with a recent study indicating that treatment of mice with IL-12 and antifungal drugs had a beneficial effect in reducing the number of CFUs of *Cryptococcus neoformans* in the brains of animals infected with the organism (7). Interestingly, spleen cells from mice treated with both IL-12 and AmB produced more IFN-γ and TNF-α than spleen cells from mice receiving either treatment alone. Although the mechanism by which the combined treatment resulted in increased IFN-γ and TNF-α production is not clear, there is a substantial amount of literature showing that AmB has potent immunomodulatory effects (2, 19, 29). For example, it was initially shown that AmB could increase resistance to tumors and *Listeria* infection (19, 32). It was speculated at the time that the antibacterial effect of AmB may be due to a soluble mediator released from lymphocytes. In addition, there was evidence to indicate that i.p. injection of AmB induced macrophage activation (18). Furthermore, several studies have demonstrated that AmB could increase the production of TNF-α from murine macrophages (5, 6). Along the same lines, Wolf and Massof (36) showed that AmB could increase superoxide release from murine macrophages stimulated with *H. capsulatum*. Moreover, when murine macrophages were exposed to both AmB and IFN-γ, significant increases in both superoxide release and the number of actively phagocytic cells were observed, suggesting a synergistic effect of this combination. The increase in IFN-γ seen when mice were treated with IL-12 and AmB may relate to the increase in TNF-α seen with these treatments. In this regard, it is possible that the increase in TNF-α in combination with IL-12 causes increased production of IFN-γ from NK cells, as has been shown by others (13, 33).

Although these results are encouraging, there are several caveats with regard to using IL-12 as a therapeutic agent for disseminated histoplasmosis in humans. The first issue is that the protective effects of IL-12 with respect to disseminated histoplasmosis in both normal (40) and SCID mice are mediated through the induction of IFN-γ. While IFN-γ has been shown to inhibit the growth of *H. capsulatum* in murine macrophages, its role in inhibiting the growth of *H. capsulatum* in human macrophages is less clear. In two separate studies, IFN-γ was unable to activate human macrophages to inhibit *H. capsulatum* (12, 21), whereas in another study, intracellular killing of *H. capsulatum* was demonstrated when human monocytes were cultured in the presence of IFN-γ (3). A second issue is that IL-12 treatment of already established intracellular infections has been less successful than treatment given immediately before or at the time of infection (31). The studies presented in this paper assessed the effect of treating with IL-12 at the time of infection only. By contrast, treatment with AmB was initiated 3 days after infection. Mice treated with AmB at the time of infection had no detectable CFUs in their spleen cells 14 days after infection (data not shown), while mice treated at 3 days postinfection had 200 to 300 CFUs on day 14 postinfection.

The beneficial effects seen in SCID mice from the combined treatment with IL-12 and AmB may help us to understand what would occur in HIV-infected patients, since these patients at the time of clinical presentation often have a significant number of residual $CD4^+$ and NK cells. Moreover, these patients often have significant numbers of $CD8^+$ T cells at the time of clinical presentation, and there is evidence to suggest that $CD8^+$ T cells may also have a role in mediating protection against *H. capsulatum* (10). We are also encouraged that the therapeutic regimen of IL-12 administered subcutaneously once a week combined with AmB given three times per week might be better tolerated than either treatment given daily. Experiments are now in progress to evaluate whether decreasing the dose and frequency of AmB will further minimize toxicity but yield similar protective effects.

In conclusion, this study provides a reliable experimental model for examining the immunoregulatory roles of cytokines in immunodeficient host (SCID) mice infected with *H. capsulatum*. Furthermore, combined therapy with IL-12 and an antifungal drug may enable more efficient eradication of *H. cap-*

sulatum from the infected host, resulting in lower toxicity, shorter periods of hospitalization, and overall reduction in the cost to immunocompromised patients. One caveat regarding IL-12 as a therapeutic agent is that it can elicit a nonspecific proinflammatory response that could temporarily affect patients with other underlying conditions.

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