# Induction of Protective Th1 Responses to *Candida albicans* by Antifungal Therapy Alone or in Combination with an Interleukin-4 Antagonist

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**Resistance or susceptibility to disseminated and mucosal *Candida albicans* infections in mice correlates with the development of protective or nonprotective T helper (Th) cell responses. To determine whether immunomodulatory activity on Th cell functions is an effect beyond that provided by antifungal therapy, mice with disseminated or gastrointestinal infection were treated with amphotericin B or fluconazole and assessed for mortality, fungus burden in the organs, and parameters of Th cell–dependent immunity. Both antimycotics produced protective CD4$^+$ Th1 cell responses, as revealed by increased production of interleukin (IL)-12 and interferon-$\gamma$, decreased production of IL-4, delayed-type hypersensitivity to fungal antigen, and the disappearance of antigen-specific IgE. Concomitant neutralization of endogenous IL-4 greatly increased the antifungal efficacy and the Th1-promoting activity of both agents. These results indicate that successful antifungal therapy alone or in combination with cytokine antagonists may rely on the induction of an appropriate Th antifungal cell response.**

Deep-seated candidal infections are important causes of infectious morbidity and mortality in immunocompromised patients [1, 2]. Several classes of antifungal compounds, including triazoles, echinocandins, and amphotericin B liposomal formulations, are being developed for management of invasive candidiasis [3–5]. Nevertheless, the mortality associated with systemic *Candida albicans* infection is >50% [6]. Several factors restrict the use and efficacy of the available antifungal agents [7], including their decreased efficacy in eradicating systemic infections in severely immunocompromised hosts [8–10]. Hence, alternative strategies would be useful for the treatment of these infections.

Recent studies have increased our understanding of the immunopathology of fungal infections [11], thus offering opportunities for immunobased therapeutic strategies. It has been demonstrated that T helper (Th) cells play a central role in regulating immune responses to *C. albicans* by secreting cytokines that modulate the development and activity of immune effectors. The outcome of systemic and mucosal *C. albicans* infections in mice was found to be largely dependent upon which of the two major CD4$^+$ Th cell subsets predominates: Th1 cell development leads to resistance and onset of protective immunity, whereas Th2 cell responses are associated with susceptibility to candidal diseases [12, 13]. Many factors control Th cell subset development and regulation in murine candidiasis [14]. Among these are cytokines, which act as modulators of antifungal effector functions and as key regulators in the development of the different Th cell subsets from precursor Th cells. Thus, neutralization of interferon-$\gamma$ (IFN-$\gamma$) [15], transforming growth factor-$\beta$ [16], or interleukin (IL)-12 [17] and administration of IL-4 or IL-10 [18] early in infection led to the onset of Th2 responses; administration of transforming growth factor-$\beta$ [16] and endogenous IL-4 [19, 20] or IL-10 [21] neutralization resulted in the onset of Th1 responses.

The effects of cytokines on Th cell responses in vivo were found to be so dependent on the timing of cytokine appearance that factors affecting the type and timing of cytokine appearance profoundly influenced the induction and expression of Th functions in mice with candidiasis [22]. One such factor is represented by the dose of *C. albicans* administered. Low levels of infection caused mice, which normally experience severe disease and mount a dominant Th2 response, to mount a Th1 response [14, 22], a finding similar to that obtained in murine leishmaniasis [23]. Therefore, regulation of the initial fungus burden may affect Th subset differentiation, as clearly evidenced in neutrophil-depleted, normally resistant mice mounting a Th2 rather than a Th1 cell response to a given dose of *C. albicans* [14].

Because amphotericin B and fluconazole possess potent anticandidal activity in vivo and in vitro [24] and because amphotericin B increases host resistance in *C. albicans*–infected mice [25], we assessed the immunomodulatory functions of these agents on Th cell responses in mice with candidiasis. To this purpose, mice with disseminated or gastrointestinal infections were given preventive or early treatment with either agent alone or in combination with an IL-4 antagonist, soluble IL-4 receptor (sIL-4R). Mice were monitored for mortality,

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fungus burden in the organs, and type of $CD4^+$ Th cell development.

## Materials and Methods

*Mice.* Specific-pathogen free BALB/c mice were obtained from Charles River Breeding Laboratories (Calco, Milan, Italy). Female mice, 2–4 weeks old, were used. The mutant mouse strain 129/Sv/Ev, which is deficient in expression of the $\alpha$ chain of the IFN-$\gamma$ receptor (IFN-$\gamma$ $R^{-}/^{-}$) [26], and wild-type strain 129/Sv/Ev, which is not deficient in expression of the $\alpha$ chain of the IFN-$\gamma$ receptor (IFN-$\gamma$ $R^{+}/^{+}$), were a gift of Manfred Kopf (Basel Institute for Immunology, Basel, Switzerland).

*Antifungal agents.* Amphotericin B (Fungizone) was purchased from Bristol-Myers Squibb (Sermoneta, Italy). A standard solution of the drug was prepared by dissolving 50 mg of amphotericin B with 41 mg of sodium deoxycholate in 10 mL of distilled water. Further dilutions were made with a 5% glucose–water solution. Fluconazole was supplied by Bioindustria Farmaceutici (Novi Ligure, Italy); it was provided in a 50-mL nonpyrogenic water solution containing 100 mg of fluconazole and 450 mg of sodium chloride. Further dilutions were made in PBS.

*Yeast, infections and enumeration of colony-forming units (cfu).* The *C. albicans* strain used in this study was isolated from a clinical specimen and identified as described previously [25]. The yeast was grown at 28°C under slight agitation in low-glucose Winge medium (BBL Microbiology Systems, Cockeysville, MD; 0.2% [wt/vol] glucose and 0.3% [wt/vol] yeast extract) until a stationary phase of growth was reached (~24 h). Under these conditions, the cultures yielded $\sim 2.8 \times 10^8$ cells/mL, and the organism grew as an essentially pure yeast-phase population. After the 24-h culture, cells were harvested by low-speed centrifugation (1000 *g*), washed twice in saline, and diluted to the desired density for intravenous (iv) injection (0.5-mL volume/mouse) via the lateral tail vein. The viability of the cells was >95% by trypan blue exclusion and quantitative cultures.

Resistance to reinfection was assessed by injecting mice that survived the primary infection with $10^6$ *C. albicans* cells 14 days after the primary challenge. Alimentary tracts of mice were colonized by oral intragastric (ig) inoculation with $10^8$ viable yeast cells delivered via an 18-gauge, 4-cm-long plastic catheter, as described [20, 27, 28]. Yeast cells in organs of infected mice (6–8 per group) were quantitated by a plate-dilution method, using Sabouraud dextrose agar, and results (mean ± SE) were expressed as cfu per organ.

*Disseminated candidiasis model.* Mice were infected iv with $2 \times 10^5$ *C. albicans* blastoconidia, an inoculum size that results in a median survival time of 12–17 days for infected mice, as indicated by pilot experiments. Treatments with antifungal agents were as follows.

*Preventive treatment.* Various doses (10, 1, or 0.1 mg/kg) of amphotericin B were given intraperitoneally (ip) to mice on day 7 before the infectious challenge. For fluconazole, 20 mg/kg was given ig daily for 7 days before infection.

*Early treatment.* Eight normal mice per experiment were left untreated; the rest were randomly separated into treatment groups (8/group) and given either one ip dose (10, 1, or 0.1 mg/kg) of amphotericin B 24 h after inoculation or daily ig doses (20 or 2 mg/kg) of fluconazole on days 1–14 after inoculation. MICs of amphotericin B and fluconazole for the *C. albicans* isolate were determined by the broth dilution antifungal susceptibility test as previously described [29]. The MIC at 24 h was $\leqslant 0.15$ $\mu$g/mL for amphotericin B and $\leqslant 0.24$ $\mu$g/mL for fluconazole.

*Gastrointestinal candidiasis model.* Mice with gastrointestinal infection were treated with either a 10 mg/kg ip dose of amphotericin B 7 days before or 24 h after inoculation or with a 2 mg/kg ig dose of fluconazole on days 1–14 after infection. Doses, routes of administration, and treatment schedules were selected on the basis of our previous experience [25] for amphotericin B and by reference to published work for fluconazole [30]. Control mice received vehicle alone.

*Combination therapy with sIL-4R.* Treatment with sIL-4R (Behringwerke, Marburg/Lahn, Germany) was done as described [20, 22]. In the disseminated infection group, mice were treated ip with 0.1 mg/kg amphotericin B 7 days before iv challenge and then injected ip with sIL-4R (10 $\mu$g/injection) on the day of and 1, 2, and 3 days after infection. In the gastrointestinal infection group, mice were treated daily from day 1 to 14 after challenge with 2 mg/kg fluconazole ig and with sIL-4R ip (10 $\mu$g/injection) on the day of and 1, 2, 3, and 5 days after infection.

*Purification and culture of $CD4^+$ T cells.* $CD4^+$ lymphocytes were positively selected from pools of spleen cells by means of a panning procedure using purified anti-murine CD4 monoclonal antibody (MAb) GK1.5, which resulted in a >95% pure population as determined by flow cytometry. $CD4^+$ cells ($5 \times 10^6$) were cultured in the presence of $5 \times 10^5$ irradiated (2000 rad) splenocytes and heat-inactivated yeast cells. Cytokines were measured in supernatants collected after 48 h, as described [16, 19–21].

*Cytokine assays.* IFN-$\gamma$ and IL-4 levels were determined in supernatants by means of a cytokine-specific ELISA, using pairs of anti-cytokine MAbs, as previously described [16, 19–21]. For serum IL-12p70 measurement, a modified antibody-capture bioassay was used as described [16]. Cytokine titers were calculated by reference to standard curves constructed with known amounts of recombinant cytokines (IFN-$\gamma$ and IL-4 [PharMingen, San Diego] and IL-12 [Genetics Institute, Cambridge, MA]).

*IgE assay.* A micro-ELISA procedure was used to quantitate yeast-specific IgE in the sera of mice [19–21]. The assay involved coating the microtiter plate wells with *Candida* antigen, adding appropriate dilutions of test antisera, and reacting further with biotinylated rat anti-mouse IgE MAb (Sanbio Biological Products, Uden, Netherlands) and an avidin–alkaline phosphatase conjugate. After addition of the substrate solution, the optical density of triplicate samples was read with an ELISA reader, using a 405-nm filter.

*Delayed-type hypersensitivity (DTH) assay.* The direct assay system for measuring the DTH response to cell surface antigens was used [15, 19–21]. Whole heat-inactivated *C. albicans* cells ($2 \times 10^6$) were inoculated into the footpads of *C. albicans*–infected mice in a volume of 40 $\mu$L. The DTH reaction was recorded 24 h later by weighing the footpads as a measure of swelling, and the results were expressed as the increase in footpad weight over that of the saline-injected counterpart. Data were expressed as the means ± SEs of 6 mice per group.

*RNA preparation and reverse transcriptase (RT)–polymerase chain reaction (PCR).* Macrophages (obtained by 2-h plastic adherence, >95% pure on esterase staining) from pooled spleens of 3 to 4 animals were subjected to RNA extraction by the guanidium thiocyanate–phenol–chloroform procedure as previously described [31]. In brief, 3 $\mu$g of total RNA was incubated with 0.5 $\mu$g of oligo(dT) (Pharmacia, Uppsala, Sweden) for 3 min at 65°C and chilled on ice for 5 min. Each sample was then incubated for 2 h at 42°C after the addition of 20 U RNase inhibitors (Boehringer Mannheim, Milan, Italy), 1.5 m*M* each dNTP, 25 U of avian myeloblastosis virus RT (Boehringer Mannheim), and RT buffer (50 m*M* TRIS-HCl, pH 8.3, 8 m*M* $MgCl_2$, 30 m*M* KCl, and 10 m*M* dithiothreitol, final concentrations) in a final volume of 20 $\mu$L. cDNA was diluted to a total volume of 500 $\mu$L with TE buffer (10 m*M* TRIS-HCl, 1 m*M* EDTA, pH 8.0) and frozen at −20°C until used.

Synthesized cDNA from each sample was amplified as described previously [32]. In brief, 5 $\mu$L of cDNA was added to a reaction mixture containing 50 m*M* KCl, 10 m*M* TRIS-HCl (pH 8.3), 3.0 m*M* $MgCl_2$, 0.01% gelatin, 0.2 m*M* each dNTP, 1 $\mu$*M* each primer, and 2.5 U AmpliTaq polymerase (Perkin-Elmer, Hayward, CA). Each 100-$\mu$L sample was overlaid with 75 $\mu$L of mineral oil (Sigma, St. Louis) and incubated in a DNA Thermal Cycler 480 (Perkin-Elmer) for a total of 30 cycles for each cytokine. For $\beta$-actin and IL-12, the cycles and temperatures were as described [33]. The $\beta$-actin primers were used as a control for RT and for the PCR reaction itself and also for comparing the amount of products from samples obtained with the same primer. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR-assisted mRNA amplification was repeated at least twice for at least 2 separately prepared cDNA samples for each experiment. Data are representative of at least three different experiments.

*Statistical analysis.* Survival and organ clearance data from each group of treated mice were compared with those from uninfected mice by use of the Mann-Whitney *U* test; significance was defined as $P < .05$ or $P < .01$. Student's *t* test was used to determine significance between cytokine production by controls and treatment groups. In vivo groups consisted of 6–8 animals. Unless otherwise specified, the data reported were pooled from 3–5 experiments.

## Results

*Effects of amphotericin B and fluconazole on the course and outcome of systemic candidiasis.* Mice with systemic infection were given preventive or early treatment with amphotericin B (10, 1, or 0.1 mg/kg) or fluconazole (20 or 2 mg/kg). The effect of treatments was evaluated in terms of survival from primary and secondary infections (table 1) and in terms of fungus burden in the kidneys (figure 1). Preventive treatment with 0.1 mg/kg of amphotericin B did not modify the survival of mice to yeast challenge. In contrast, treatments with the higher doses (1 or 10 mg/kg) resulted in cure of mice, as more than 85% survived primary infection, a result in line with previous findings [25].

When survivors were assessed for resistance to reinfection, they were found to be partially (10 mg/kg dose) or completely (1 mg/kg) resistant. Early treatment (24 h after infection) with any of the doses of amphotericin B resulted in cure of mice from the primary infection. However, only mice treated with the higher doses showed increased resistance to reinfection. Preventive treatment with fluconazole did not affect survival from infection. In contrast, early treatment with 20 mg/kg of fluconazole resulted in cure of mice from primary infection and resistance to reinfection, and treatments with 0.2 or 2 mg/kg significantly increased survival time.

To correlate these findings with fungal growth in the organs, fungus cells were quantitated in the kidneys of infected mice at 1 and 2 weeks after infection (figure 1). No fungal growth was evident in mice treated with 10 mg/kg of amphotericin B either before or after infection. A persistent and significant reduction of fungal growth was observed after preventive or early treatment with 1 mg/kg of amphotericin B or after early, but not preventive, treatment with 0.1 mg/kg. For fluconazole, early treatment with 20 mg/kg significantly inhibited fungal growth in the organs at both 1 and 2 weeks after infection. At 2 mg/kg, significant inhibition was observed only at 1 week; no significant effects on fungal growth were observed when fluconazole (20 mg/kg) was given before infection (data not shown).

*Effects of amphotericin B and fluconazole on the course of gastrointestinal candidiasis.* To evaluate the effects of amphotericin B and fluconazole on the course of mucosal candidiasis, we used an experimental model of infection in which adult immunocompetent mice were inoculated ig with the fungus [20, 27, 28]. This results in a transient colonization of the intestinal organs, particularly the stomach. Amphotericin B (10 mg/kg) was administered either 7 days before or 1 day after challenge, and fluconazole (2 mg/kg) was given daily from day 1 to 14 after infection. At 1, 2, and 4 weeks after infection, the number of fungal cells was determined in the stomach of infected animals. The results (figure 2) indicate that preventive, but not early, treatment with amphotericin B significantly inhibited fungal growth at 2 and 4 weeks after infection. In contrast, early treatment with fluconazole was highly effective in inhibiting fungal growth. Together, these results indicate the high efficacy of amphotericin B and fluconazole against murine disseminated and gastrointestinal *C. albicans* infections. Moreover, they show that treatment with either agent may result in the development of resistance to subsequent reinfection in mice with disseminated candidiasis.

*Effects of amphotericin B and fluconazole on anticandidal Th-dependent cell functions.* Because the occurrence of protective and nonprotective immunity to *C. albicans* is associated in mice with preferential expansion of Th1 and Th2 cells, respectively [12–14], parameters of specific $CD4^+$ Th cell immunity were examined in iv infected mice given antifungal treatment. To this purpose, IFN-$\gamma$ (Th1) and IL-4 (Th2) cyto-

**Table 1.** Efficacy of amphotericin B or fluconazole against disseminated candidiasis.

| Antifungal agent, day(s) of treatment, dose | Resistance to infection: Primary MST | Primary D/T | Secondary MST | Secondary D/T |
|---|---|---|---|---|
| Controls | 16 | 24/24 | — | — |
| Amphotericin B | | | | |
| Day 7 before challenge | | | | |
| 10 mg/kg | >60 | 3/24 | 26* | 8/8 |
| 1 mg/kg | >60 | 3/24 | >60 | 1/8 |
| 0.1 mg/kg | 17 | 24/24 | — | — |
| Day 1 after challenge | | | | |
| 10 mg/kg | >60 | 0/24 | 27* | 8/8 |
| 1 mg/kg | >60 | 0/24 | 25* | 8/8 |
| 0.1 mg/kg | >60 | 4/24 | 11 | 8/8 |
| Fluconazole | | | | |
| Day 7 before to day 0 of challenge | | | | |
| 20 mg/kg | 15 | 24/24 | — | — |
| Days 1-14 after challenge | | | | |
| 20 mg/kg | >60 | 0/24 | >60 | 2/8 |
| 2 mg/kg | 26† | 21/24 | — | — |
| 0.2 mg/kg | 26† | 22/24 | — | — |
| Controls | | | 9 | 8/8 |

NOTE. Amphotericin B was given intraperitoneally; fluconazole was given intragastrically. For primary infection, BALB/c mice were infected intravenously (iv) with $2 \times 10^5$ *C. albicans* cells. For secondary infection, parallel group of mice was reinfected with $10^6$ cells 14 days after primary infection. MST = median survival time (days); D/T = no. of dead mice/no. of mice infected.

* $P < .01$ (reinfected vs. infected mice).

† $P < .05$ (treated vs. untreated infected mice).

kine production by CD4$^+$ cells, serum levels of *Candida*-specific IgE, and development of DTH reactivity were assessed in mice given curative treatment (i.e., 1 mg/kg amphotericin B 7 days before infection or 20 mg/kg fluconazole daily on days 1–14 after infection) or noncurative treatment (i.e., 0.1 mg/kg amphotericin B or 2 mg/kg fluconazole given as above). CD4$^+$ T cells were purified from spleens of infected and treated mice at 1 and 2 weeks after infection. Cells were antigen-stimulated in vitro before assessment of IFN-$\gamma$ and IL-4 production in the culture supernatants.

As shown in figure 3, IL-4 production increased and IFN-$\gamma$ production decreased in infected, nontreated mice over the

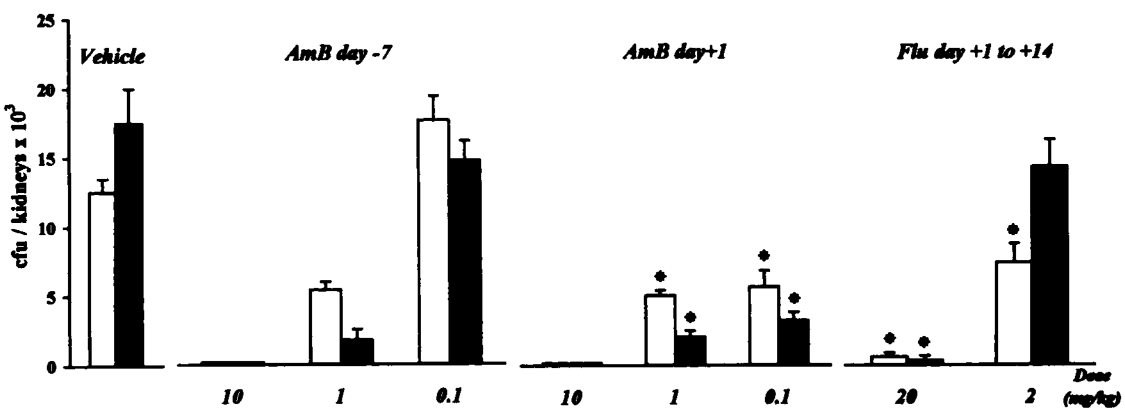


**Figure 1.** No. of *C. albicans* cfu recovered from kidneys of BALB/c mice treated or not treated with different doses of amphotericin B (AmB) intraperitoneally or fluconazole (Flu) intragastrically at various days before (−) or after (+) intravenous infection with $2 \times 10^5$ yeast cells. cfu were counted at 1 (open bars) and 2 (solid bars) weeks after infection. Values are means ± SEs. * $P < .01$ (AmB or Flu vs. vehicle).

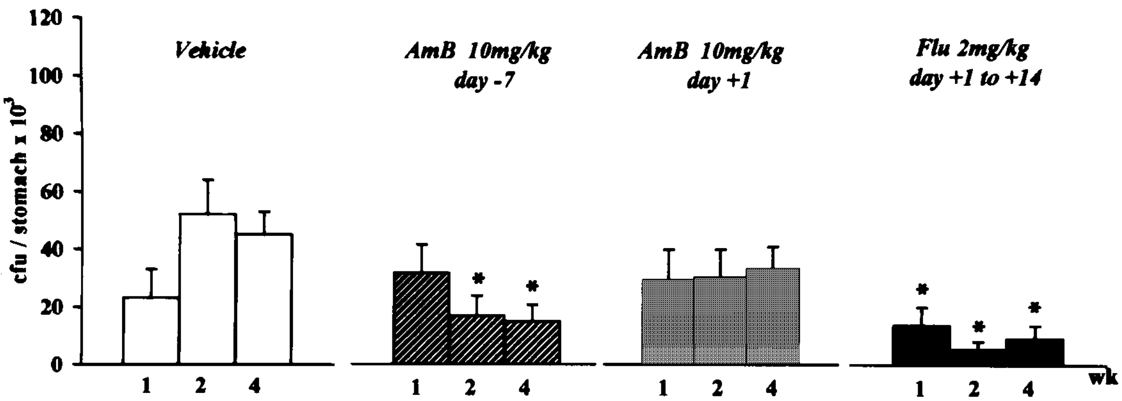


**Figure 2.** No. of *C. albicans* cfu recovered from stomachs of BALB/c mice treated or not treated with amphotericin B (AmB) intraperitoneally or fluconazole (Flu) intragastrically at various days before (−) or after (+) intragastric infection with $10^8$ yeast cells. cfu were counted at 1, 2, or 4 weeks after infection. Values are means ± SEs. * $P < .01$ (AmB or Flu vs. vehicle).

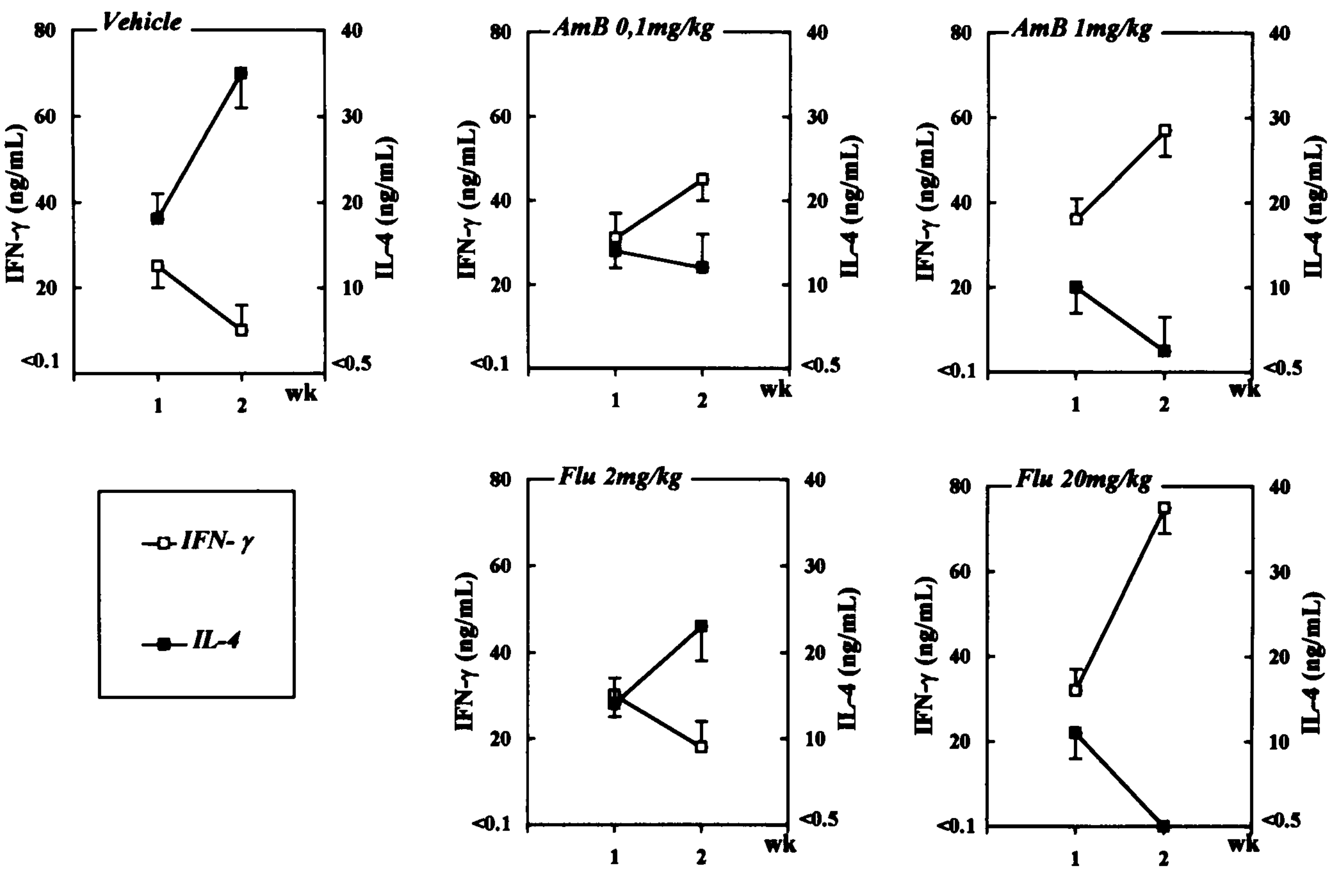


**Figure 3.** Cytokine production in BALB/c mice infected intravenously with *C. albicans* ($2 \times 10^5$) and treated or not treated with amphotericin B (AmB) or fluconazole (Flu). Treated mice received curative (1 mg/kg) or noncurative (0.1 mg/kg) doses of AmB 7 days before infection or daily curative (20 mg/kg) or noncurative (2 mg/kg) doses of Flu on days 1–14 after infection. Mixed CD4+ lymphocyte–yeast cell cultures were established at 1 and 2 weeks after infection, and supernatants were assayed for IFN-γ and IL-4 content. Each value is mean ± SE of 4 separate experiments. Cytokine levels of supernatants from mixed cultures of CD4+ cells from uninfected mice were below detection limit of assay (indicated by < on *y* axis).

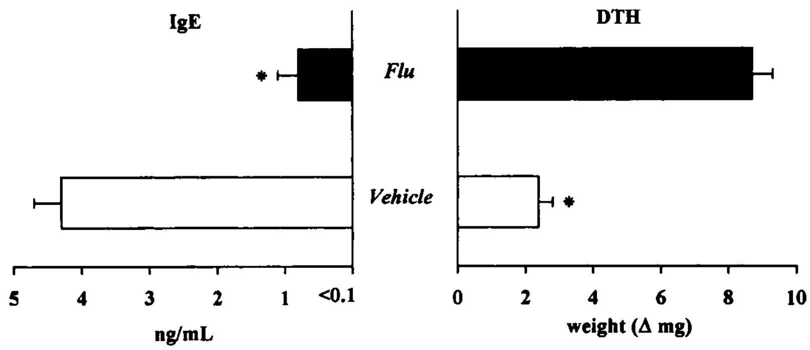


**Figure 4.** Serum antigen–specific IgE levels and delayed-type hypersensitivity (DTH) reactivity to *Candida* antigens in BALB/c mice infected intravenously with $2 \times 10^5$ *C. albicans* and treated or not with fluconazole (Flu). Flu (20 mg/kg, intragastrically) was given daily on days 1–14 after infection. Assays were done 15 days after infection. * $P < .01$ (Flu vs. vehicle).

course of the infection. The same pattern of cytokine production was observed in mice exposed to noncurative treatment with fluconazole (2 mg/kg), although the actual cytokine production was lower. For amphotericin B–treated mice, IFN-$\gamma$ production increased over the course of infection; however, the increase was not paralleled by a decrease in IL-4 production. In contrast, curative treatment with fluconazole (20 mg/kg) or amphotericin B (1 mg/kg) resulted in dramatically impaired IL-4 production and improved IFN-$\gamma$ production, particularly at 2 weeks after infection. These results were paralleled by an increased frequency of cells producing IFN-$\gamma$ and a decrease of those producing IL-4 in the spleens of mice at 2 weeks after infection (data not shown). Therefore, treatment with amphotericin B or fluconazole appears to alter the ratio between Th1 and Th2 cytokine–producing cells in *C. albicans*–infected mice.

To further document the effect of antifungal agents on Th cell subset development, serum levels of antigen-specific IgE and development of DTH reactivity were assessed 2 weeks after infection in mice given curative doses of fluconazole. Figure 4 shows that IgE serum levels were greatly reduced in mice as a consequence of treatment; conversely, a strong DTH reactivity appeared in treated mice, and a weak reactivity appeared in vehicle-injected mice (controls). These results indicate that the curative effect of the antifungal agents is associated with the occurrence of predominant anticandidal $CD4^+$ Th1 cell responses.

*Effect of amphotericin B on disseminated candidiasis in IFN-$\gamma$ $R^-/^-$ mice.* To assess the contribution of IFN-$\gamma$ production in the curative effect of antifungal chemotherapy in disseminated candidiasis, mice functionally deficient in IFN-$\gamma$, such as those deficient in the expression of the $\alpha$ chain of IFN-$\gamma$ receptor [26], were used. Seven days before iv challenge with $4 \times 10^5$ *C. albicans* cells, IFN-$\gamma$ $R^-/^-$ and IFN-$\gamma$ $R^+/^+$ mice were given curative treatment with amphotericin B (i.e., 1 mg/kg). After challenge, the animals were assessed for resistance to infection in terms of their rate of survival. Treatment with amphotericin B increased survival of susceptible wild-type mice (table 2) and decreased fungal growth in the kidneys (data not shown). None of these effects were observed in IFN-$\gamma$ $R^-/^-$ mice. Therefore, these data suggest that in the absence of a direct activity of the drug on fungal cells, the curative effect of amphotericin B greatly relies on its ability to induce Th1 protective responses through an IFN-$\gamma$–dependent mechanism.

*Effect of concomitant treatment with sIL-4R on the efficacy of amphotericin B and fluconazole.* We have already shown that neutralization of endogenous IL-4 results in cure of mice from either disseminated [19] or gastrointestinal [20] infection. To determine whether concomitant neutralization of endogenously produced IL-4 could increase the efficacy of amphotericin B and fluconazole, mice with disseminated or gastrointestinal infection were treated with a suboptimal dose of sIL-4R and noncurative doses of amphotericin B (one 0.1 mg/kg dose 7 days before infection) or fluconazole (daily 2 mg/kg injections on days 1–14 after challenge). Mice were monitored for fungal growth in the organs. The results (figure 5) show that each treatment alone was effective, although transiently, in reducing fungal growth in vivo. However, combined treatment (antifungal plus sIL-4R) greatly increased the efficacy of both drugs, as revealed by a higher and persistent inhibition of fungal growth in the kidneys and stomach of infected mice. Thus,

**Table 2.** Effect of amphotericin B (AmB) on disseminated candidiasis in interferon-$\gamma$ receptor–deficient (IFN-$\gamma R^-/^-$) and wild-type (IFN-$\gamma R^+/^+$) mice.

| | | Resistance to infection | |
|---|---|---|---|
| Mice | AmB | MST | D/T |
| IFN-$\gamma$ $R^+/^+$ | − | 8 | 4/4 |
| IFN-$\gamma$ $R^+/^+$ | + | >60 | 1/4 |
| IFN-$\gamma$ $R^-/^-$ | − | 9 | 4/4 |
| IFN-$\gamma$ $R^-/^-$ | + | 10.5 | 4/4 |

NOTE. Mice were intravenously infected with $4 \times 10^5$ *C. albicans* cells. AmB (1 mg/kg) was given intraperitoneally 7 days before infection. MST = median survival time (days); D/T = no. of dead mice/no. of mice infected.

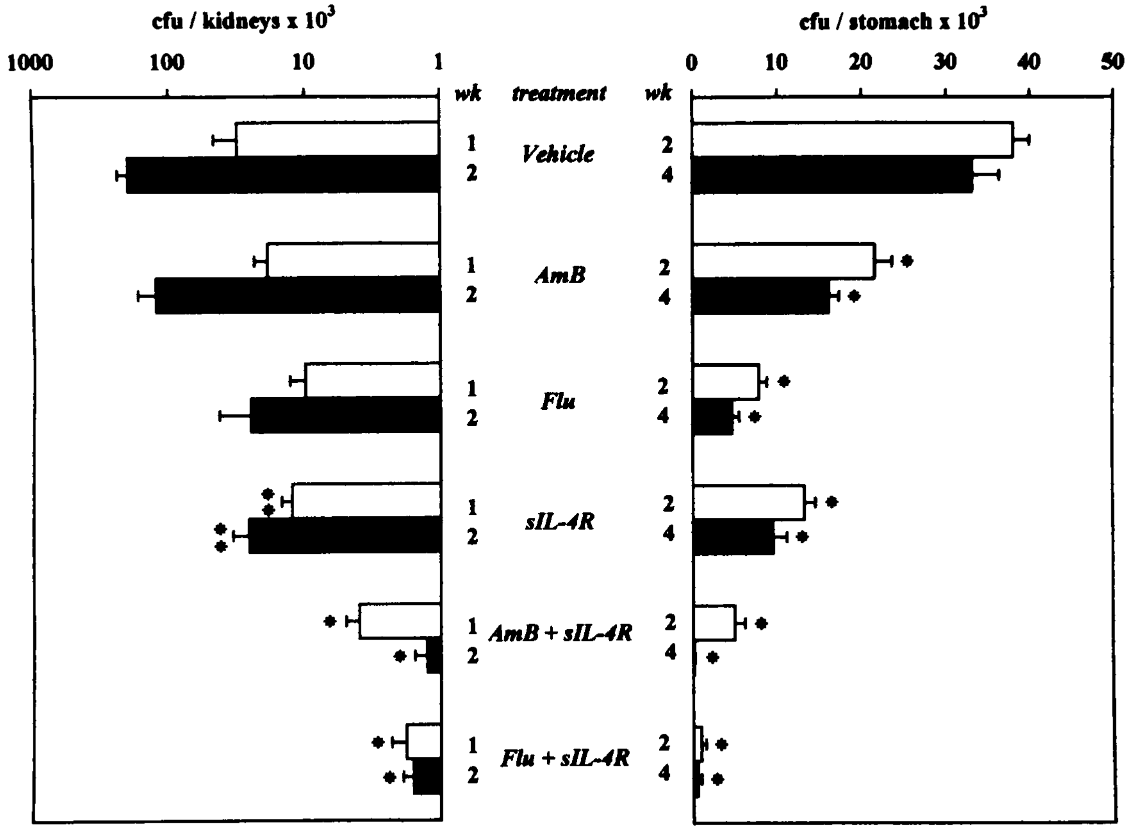


**Figure 5.** No. of *C. albicans* cfu recovered from kidneys or stomachs of BALB/c mice infected intravenously with $2 \times 10^5$ or intragastrically with $1 \times 10^8$ yeast cells and treated with antimycotic drug alone or in combination with soluble IL-4 receptor (sIL4R). Treatment doses and schedules were as follows: amphotericin B (AmB), 0.1 mg/kg 7 days before infection; fluconazole (Flu), 2 mg/kg daily on days 1–14 after infection; sIL4R, 10 $\mu$g/dose on day of and 1, 2, and 3 days after challenge. Assays were done at different weeks after infection (indicated on $x$ axis). Values are means ± SEs. * $P < .01$, ** $P < .05$ (treatments vs. vehicle).

combined treatment with sIL-4R and either agent resulted in a synergistic anticandidal effect.

To assess whether combined treatment also resulted in a modified pattern of cytokine production, serum levels of bioactive IL-12 and IL-12p40 gene expression in splenic macrophages were evaluated. Bioactive IL-12 levels were greatly increased in the serum of mice given amphotericin B or fluconazole combined with sIL-4R (figure 6). Similarly, combined treatments greatly increased the expression of IL-12p40 gene expression in macrophages. Because IL-12 is both prognostic and required for Th1 development in mice with candidiasis [17], these results further indicate that the ability of amphotericin B and fluconazole to promote anticandidal Th1 cell development is greatly potentiated by the concomitant inhibition of endogenous IL-4.

## Discussion

The appreciation of the importance of T cell function in the overall coordination of the immune response to pathogens has offered opportunities to manipulate these processes and to alter the outcome of antimicrobial host responses [34, 35]. The failure to control or resolve infections is often the result of inappropriate rather than insufficient immune responses. In fungal infections, activation of the different types of Th cell subsets has been well documented [12].

In the present study, we provide evidence that curative treatments with amphotericin B or fluconazole were associated with the occurrence of IFN-$\gamma$–dependent Th1 cell responses in mice with disseminated candidiasis. Mice cured from the primary infection acquired resistance to reinfection (table 1), and this correlated with the detection of increased production of IFN-$\gamma$, but not IL-4, by $CD4^+$ T cells from spleens (figure 3). IgE serum levels, which are often increased in human systemic fungal infections [36], decreased in cured mice. In contrast, strong DTH reactivity appeared (figure 4), thus confirming the important role of cell-mediated immunity in candidiasis [37–40]. Increased levels of circulating IL-12p70 were observed in mice with disseminated (figure 6) or gastrointestinal (data not shown) infections, thus indicating the occurrence of

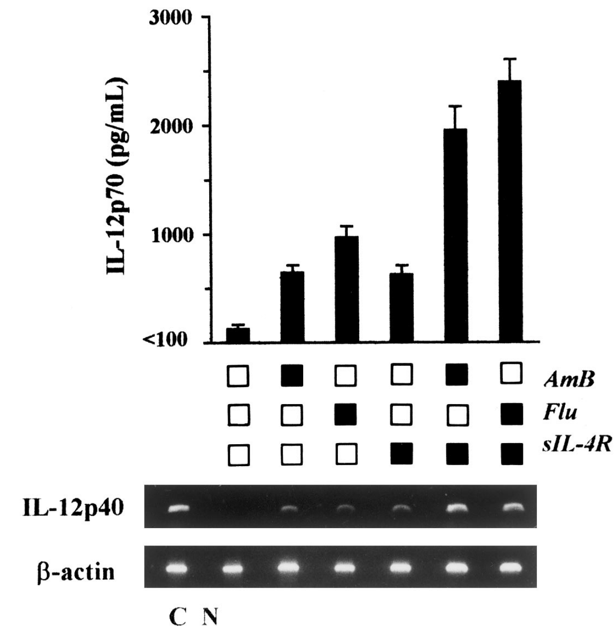


**Figure 6.** Serum levels of bioactive IL-12p70 and IL-12p40 gene expression in yeast cell–infected BALB/c mice treated with antimycotic drugs alone or in combination with soluble IL-4 receptor (sIL4R); treatment is indicated by solid squares in middle of figure. Treatment doses and schedules were as follows: amphotericin B (AmB), 0.1 mg/kg 7 days before infection; fluconazole (Flu), 2 mg/kg daily on days 1–14 after infection; sIL4R, 10 $\mu$g/dose on day of and 1, 2, and 3 days after challenge. At 2 weeks after infection, IL-12p70 was determined by means of antibody-capture assay, and IL-12p40 gene expression was determined in splenic macrophages by means of reverse transcriptase–polymerase chain reaction (PCR). IL-12p70 serum levels from uninfected naive mice were below detection limit of assay (indicated by < in $y$ axis). C = $\beta$-actin or cytokine-specific controls; N = no DNA added to amplification mix during PCR.

protective Th1 responses in both types of infections. For amphotericin B, induction of protection was not observed in mice functionally deficient in IFN-$\gamma$ (table 2), thus indicating the importance of this cytokine in the immunomodulatory activity of the drug.

Recent studies demonstrated that the initial fungus burden may affect Th subset differentiation in mice with candidiasis [14, 22]. Here we show that induction of Th1 responses correlates with a low fungus burden in the organs (figures 2 and 3), thus providing further evidence for the importance of the antigenic load in Th cell selection [14, 22].

Anaissie et al. [24] showed a positive correlation between the anticandidal activity in vitro of amphotericin B and fluconazole and their ability to prolong survival and reduce tissue counts in infected mice. Thus, the direct interaction of either drug with *C. albicans* could be a major mechanism leading to Th1 selection in mice with candidiasis. However, the results of our study do not exclude the possibility of an interaction of amphotericin B and fluconazole with host defense mechanisms, ultimately leading to an effective control of fungal growth through an action on antifungal effector functions.

Amphotericin B may considerably enhance host immune responses [41, 42], including phagocyte activation [43, 44] and cytokine production [45, 46]. In an experimental model of *C. albicans* infection, the therapeutic activity of amphotericin B greatly relied on its ability to activate antifungal effector functions of macrophages [25]. In humans, amphotericin B improved the clinical outcome of patients with documented systemic candidiasis even though serum levels were well below the MIC for the infecting pathogen [47]. In accord, the results of the present study show the high efficacy of a single administration of amphotericin B against yeast challenge 7 days after treatment, at a time when the drug concentration in plasma is barely detectable [25].

Unlike amphotericin B, fluconazole was not found to have immunostimulatory properties [41, 48, 49]. However, the notion that fluconazole is not effective against intracellular *C. albicans* [50] poses the question as to whether the efficacy of fluconazole also relies on an active immune system. Preliminary results from our laboratory indicate that treatment of intact mice with fluconazole results in an increased production of IFN-$\gamma$ and nitric oxide by nonadherent and adherent spleen cells, respectively (data not shown).

An interesting observation of the present study is that mice exposed to early treatment with the highest doses of amphotericin B, although able to survive the primary infection, failed to mount long-lasting resistance to reinfection. We have already shown that for durable anticandidal protection to occur, a persistent, albeit low, antigenic fungus load appears to be required [22]. Therefore, it is likely that the potent antifungal activity exerted by amphotericin B in these conditions may result in a rapid clearance of fungus cells, thus preventing the retention of durable anticandidal immunologic memory.

These results indicate that either amphotericin B or fluconazole may exhibit immunomodulatory functions on antifungal Th cell responses, an activity that appears to depend on both the time and dose of drug administration.

Whatever the mechanism by which amphotericin B and fluconazole induce anticandidal protective Th1 responses, this activity was potentiated by concomitant treatment with the IL-4 antagonist, sIL-4R. We showed that a dose of amphotericin B or fluconazole, ineffective in altering the course of disease, could significantly reduce the fungus load in the organs when administered with sIL-4R (figure 5). As shown in figure 3, either drug alone, at the doses used, induced only quantitative but not qualitative changes in the pattern of cytokine production by CD4$^{+}$ splenocytes, thus evidencing the failure to induce Th1 cell development. However, combination treatment with either drug and sIL4R resulted in the increased production of IL-12 (figure 6), an indicator of the occurrence of Th1 responses [17]. These results indicate that concomitant IL-4 neutralization may increase the efficacy and Th1-promoting activity of the antifungal drugs such that the levels of endogenously produced IL-12 could be predictive of the outcome of therapy. Similar results were obtained in a murine model of established *Leishmania major* infection, in which the efficacy and Th1-promoting activity of antimony therapy was increased by depletion of IL-4 [51] or concomitant treatment with IL-12 [52].

Although it is clear from the present study that sIL-4R and antifungal agents can act in synergism to inhibit a progressive infection and promote Th1 responses, the mechanism underlying this effect remains undefined. Given the detrimental role of IL-4 in murine candidiasis [18, 53], inhibition of IL-4 function during infection could be beneficial. However, a synergistic but not an additive effect was observed upon treatment with sIL-4R and antifungal agents. It is possible that the reduction of the fungus burden that occurs as a result of antifungal therapy may further decrease the production of IL-4 in infected mice, as already demonstrated [14, 22], thus amplifying the effect of sIL-4R therapy. The relative absence of inhibitory cytokines, such as IL-4, may allow for the expansion of $CD4^+$ Th1 cells in the presence of IL-12 [17, 54].

Our study reveals a new immunomodulatory function for amphotericin B and fluconazole: the ability to induce development of protective Th1 responses, an effect potentiated by concomitant IL-4 depletion. Whatever the mechanism by which this effect is obtained, these findings further indicate that interfering with the balance of Th cell subsets and their cytokines may alter the outcome of systemic and mucosal candidiasis. Results from a preliminary experiment indicate that the immunomodulatory function of amphotericin B and fluconazole as well as their synergistic relationship with sIL-4R were retained in mice infected with *C. albicans* and immunosuppressed by treatment with cyclophosphamide (unpublished data). If amphotericin B and fluconazole retain their immunomodulatory properties in immunocompromised persons, new therapeutic strategies, including combined treatment with cytokine or cytokine antagonists (or both), may be found for the treatment of fungal infections in such hosts.

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