# Neutralizing Antibody to Interleukin 4 Induces Systemic Protection and T Helper Type 1–associated Immunity in Murine Candidiasis

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**Summary**

An interleukin 4 (IL-4)-specific monoclonal antibody (mAb) was administered to mice infected systemically with the yeast *Candida albicans*, and the animals were monitored for mortality, development of delayed-type hypersensitivity, production of antibodies of different isotypes, release of IL-2, IL-4, IL-6, and interferon γ (IFN-γ) in vitro by splenic $CD4^+$ lymphocytes, and levels of IL-4 and IFN-γ mRNA in these cells. Neutralization of IL-4 by three weekly injections of mAb in several independent experiments resulted in an overall cure rate of 81% versus 0% of controls. Cure was associated with efficient clearance of the yeast from infected organs and histologic evidence of disease resolution, detection of strong T helper type 1 (Th1) responses, and establishment of long-lasting protective immunity. Soon after infection, and as a result of the first or second injection of mAb, there was a decrease in IL-4 mRNA in $CD4^+$ cells, which was accompanied by an increase in the levels of IFN-γ-specific transcripts. Our data thus indicate that the production of IL-4 by Th2 cells may limit Th1-associated protective immunity in murine candidiasis.

Recent evidence indicates that, early after exposure of mice, innate defense mechanisms are the primary means by which proliferation and growth of *Candida albicans* are held in check in the absence of underlying immunity, whereas later in the course of infection, clearance of the yeast is regulated, at least in part, by T cell–mediated immune responses (1). Long-term colonization of adult mice in the absence of immunosuppressive and antimicrobial treatment has been achieved by intragastric colonization of infant (2) or germ-free (3) mice, or by systemic infection with attenuated yeast cells of variant strains (4, 5). These studies have demonstrated that both specific cell-mediated immunity (6–8) and phagocytic cell activity (4, 9, 10) are instrumental in the optimal expression of acquired anti–*Candida* resistance. In particular, in a systemic *C. albicans* infection model, evidence has been obtained that preferential production of Th1 or Th2 cytokines may underlie the development of protective vs. nonprotective immunity, respectively, as elicited by concomitant chronic infection with attenuated yeast cells (11).

The role of cytokines in cutaneous lesions of murine leishmaniasis has been demonstrated by experiments in vivo, in which cytokine activities were blocked by administration of anticytokine mAbs. Anti-IFN-γ treatment of genetically resistant mice was found to result in development of progressive *Leishmania major* infection and detection of Th2 responses (12). In contrast, anti-IL-4 treatment resulted in cure of susceptible mice, and the animals demonstrated Th1 responses (13, 14). In general, these data are taken to indicate that IFN-γ and IL-4 are clearly involved in the process of subset differentiation of $CD4^+$ T cells and may exert mutually inhibitory effects on both differentiation and effector functions (14).

Following our previous observations that anti-IFN-γ treatment in vivo will block the effector functions mediated by endogenous IFN-γ in immunized mice (7) and Th2 cells may be activated and functionally interact with Th1 lymphocytes in candidal infections (11), we have investigated the effect of administration of anti-IL-4 mAb on antifungal resistance of systemically infected mice. Anti-IL-4 therapy initiated at the time of infection resulted in cure of a high percentage of mice with establishment of protective immunity. Cure was associated with the detection of Th1 responses, including strong delayed-type footpad reactions and low serum IgE. There were also early and remarkable changes in Th1 and Th2 cytokine production in vitro by $CD4^+$ cells and cytokine mRNA expression in these cells.

## Materials and Methods

*Mice.* Hybrid (BALB/cCr × DBA/2Cr)$F_1$ ($CD2F_1$) and inbred BALB/c mice were obtained from Charles River Breeding


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Laboratories (Calco, Milan, Italy). Mice of both sexes, ranging in age from 2 to 4 mo, were used.

*Yeast and Infections.* The origin, characteristics, and growth conditions of the highly pathogenic *C. albicans* (CA-6) strain used in this study have already been described in detail (6, 7). For infection, yeast cells were harvested by low-speed centrifugation (1,000 *g*), washed twice in PBS, and diluted to the desired density to be injected intravenously into mice in a volume of 500 μl. Quantification of yeast cells in organs of infected mice was performed by a plate dilution method, using Sabouraud dextrose agar (6, 7), and results (means ± SEM) were expressed as the number of CFU per organ. For histology, tissues were fixed in formalin, sectioned, and stained with periodic acid-Schiff's reagent or hematoxylin/eosin.

*Treatment of Mice with anti-IL-4 mAb.* The 11B11 hybridoma line, producing rat IgG1 anti-IL-4 mAb (15), was grown as ascites in pristane-primed irradiated BALB/c mice, and the ascites fluid was passed over a desalting column (10DG; Bio-Rad Laboratories, Richmond, CA) before antibody purification by means of affinity chromatography with protein A–Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Protein concentration was determined by measuring the OD at 280 nm. Beginning 2 d before infection, mice received 1 mg purified 11B11 mAb in 250 μl PBS intraperitoneally once per week for 3 wk. Control mice received equivalent amounts of affinity-purified rat IgG1 (Zymed Laboratories, San Francisco, CA).

*Delayed-type Hypersensitivity (DTH)[1] Assay.* A direct assay system for measuring the DTH response to cell surface antigens was employed (6) in which heat-inactivated CA-6 cells ($2 \times 10^6$/40 μl of pyrogen-free saline) were inoculated into the footpads of control or yeast-infected mice. The DTH reaction was recorded 24 h later by weighing the footpad as a measure of swelling, and the results were expressed as the increase in footpad weight (right hind) over that of the saline-injected (left hind) counterpart. Data are the means ± SEM of six to eight mice per group.

*Selection of $CD4^+$ Cells, Production of Culture Supernatants Containing Cytokine Activity, and Cytokine Assays.* $CD4^+$ lymphocytes were positively selected from pools of spleen cells by means of a panning procedure using anti-murine CD4 mAb GK1.5, which resulted in a >95% pure population on FACS® analysis (Becton Dickinson & Co., Mountain View, CA) (6, 7). Supernatants from mixed lymphocyte *Candida* cultures were obtained by culturing $CD4^+$ lymphocytes in the presence of yeast cells and accessory macrophages (6, 11). Source and characteristics of the anticytokine antibody reagents used in IFN-γ, IL-2, IL-4, and IL-6 assays were as described (6, 7, 11). Briefly, cell supernatants were tested for their concentration of IFN-γ by two-site ELISA using rat anti-murine IFN-γ mAb R4-6A2 as the primary antibody and biotinylated monoclonal AN-18.17.24 as the secondary antibody. IL-2 and IL-4 were measured using the HT2 T lymphocyte line that proliferates in response to either of these cytokines (16). mAbs S4B6 (anti-IL-2) and 11B11 (anti-IL-4), at neutralizing concentrations, were used to establish monospecificity. IL-6 activity was determined by its capacity to support the growth of the murine IL-6–dependent cell line 7TD1 (17). Proliferation was assayed using the tetrazolium-based colorimetric assay. All cytokine titers were expressed as units per milliliter, calculated by reference to standard curves constructed with known amounts of the recombinant cytokines (Genentech, Inc., South San Francisco, CA).

*Antibody Assay.* A micro-ELISA procedure was used to quantitate specific antibodies in the sera of yeast-infected mice (11). The assay involved coating the microtiter plate wells with heat-killed CA-6 cells, addition of appropriate dilutions of test antisera (from a pool of two to three mice per group), and a further reaction with alkaline phosphatase–conjugated rabbit anti-mouse IgG, IgG1, IgG2a (Zymed Laboratories), or with biotinylated rat monoclonal anti-mouse IgE (Sanbio bv Biological Products, Uden, The Netherlands) followed by an avidin-alkaline phosphatase conjugate (Vector Laboratories, Burlingame, CA). After addition of the substrate solution, the OD of triplicate samples was read with an ELISA reader, using a 405-nm filter.

*RNA Preparation and PCR.* $CD4^+$ lymphocytes, >95% pure, were obtained from spleen cells pooled from two to three animals, and $2 \times 10^7$ cells were subjected to RNA extraction by the guanidium thiocyanate-phenol-chloroform procedure (18). Amplification of synthesized cDNA from each sample was carried out as reported (19), using 5′ sense primers and 3′ antisense primers from Clontech Laboratories (Palo Alto, CA). Briefly, 5 μl of cDNA was added to a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.0 mM $MgCl_2$, 0.01% gelatin, 0.2 mM deoxynucleoside triphosphates, 1 μM of each primer, and 2.5 U AmpliTaq polymerase (Perkin-Elmer Corp., Hayward, CA). Each 100-μl sample was overlayed with 75 μl mineral oil (Sigma Chemical Co., St. Louis, MO) and incubated in a DNA thermal cycler (480; Perkin-Elmer Corp.) for a total of 40 cycles, each cycle consisting of 1 min at 94°C, 1 min at 60°C (β-actin and IFN-γ) or 67°C (IL-4), and 1 min at 72°C. The amplified DNA size, as compared with a positive control (Clontech Laboratories), was 540 bp for β-actin, 399 bp for IL-4, and 460 bp for IFN-γ. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

## Results

*Effect of In Vivo Neutralization of IL-4 on the Course of Inoculation Candidiasis.* In six independent experiments, $CD2F_1$ mice received three weekly injections of anti-IL-4 (11B11) or control antibodies starting 2 d before intravenous challenge with different numbers ($5–50 \times 10^4$) of CA-6 blastospores. While all control mice developed progressive, disseminated fatal candidiasis with median survival times of 5–18 d depending on challenge dose, the overall survival rate of anti-IL-4-treated mice (totalling 54) was 81%. The results of three representative experiments are summarized in Table 1. In experiments not reported here, we also found that the beneficial effects of IL-4 depletion were essentially lost when the size of the challenge inoculum was such (e.g., $10^6$ cells or more) as to result in animals' death within the first 24–96 h of infection. However, once cured by anti-IL-4 treatment, survivor mice would consistently resist challenge with $10^6$ CA-6 cells as performed 12 wk after primary infection (Table 1).

Quantitative cultures of kidneys and brains from control and anti-IL-4-treated mice were established 6 h and 1, 2, or 3 wk after injection of $7.5 \times 10^4$ CA-6 cells. Fig. 1 summarizes the results of one experiment representative of three, and shows that mAb treatment was associated with a dramatic reduction in fungal load in the organs of infected mice. At 3 wk, when no survivors were found in the control group (median survival time of 14.5 d), mice on anti-IL-4 therapy had almost completely cleared yeast cells from their organs. Examination of the kidneys from these mice provided histologic evidence of disease resolution (Fig. 2).

[1] *Abbreviation used in this paper:* DTH, delayed-type hypersensitivity.

**Table 1.** *Effect of Anti-IL-4 Treatment on Mouse Resistance to Inoculation Candidiasis*

| Challenge dose | Treatment | MST | D/T |
|---|---|---|---|
| | | *d* | |
| $5 \times 10^5$ | None | 5.5 | 8/8 |
| $5 \times 10^5$ | Rat IgG1 | 5.5 | 8/8 |
| $5 \times 10^5$ | mAb 11B11 | >60 | 3/9* |
| $10^5$ | None | 10 | 8/8 |
| $10^5$ | Rat IgG1 | 10 | 10/10 |
| $10^5$ | mAb 11B11 | >60 | 2/10 |
| $5 \times 10^4$ | None | 16.5 | 8/8 |
| $5 \times 10^4$ | Rat IgG1 | 15 | 8/8 |
| $5 \times 10^4$ | mAb 11B11 | >60 | 0/8* |

Mice challenged with various doses of CA-6 cells received anti-IL-4 (11B11) or control (rat IgG1) treatment. Median survival times (MST) and number of dead mice at 60 d over total animals tested (D/T) were recorded.
* Survivors resisted subsequent challenge with $10^6$ yeast cells (see text).

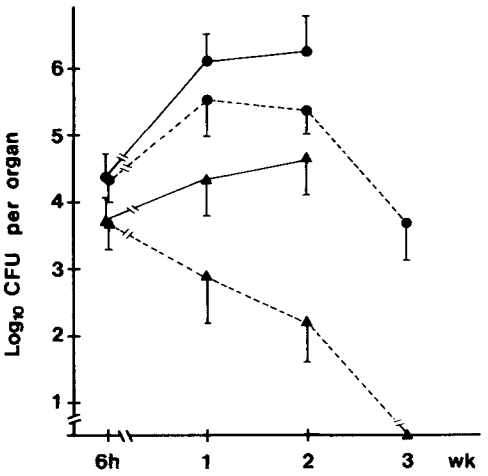


**Figure 1.** Recovery of CFU from kidneys (●) and brain (▲) of CA-6-infected mice treated with anti-IL-4 (*dashed lines*) or control (*solid lines*) antibodies. The assay was performed at 6 h and 1, 2, or 3 wk postinfection. Values are the mean of ± SEM for eight mice per group (six for control mice at 2 wk).

*Effect of Anti-IL-4 Treatment on Footpad Reaction to the Yeast.* To assess the possible effect of anti-IL-4 treatment on development of DTH, mice challenged with $7.5 \times 10^4$ CA-6 cells, and treated with control or 11B11 antibodies, were injected intra-footpad with heat-inactivated blastospores 1, 2, or 3 wk after infection. The increase in footpad weight was measured 24 h later (Fig. 3 *A*). Only mice receiving anti-IL-4 therapy and surviving CA-6 challenge developed significant footpad responses to the yeast. In addition, purified $CD4^+$ lymphocytes from cured mice could adoptively transfer DTH reactivity to naive mice challenged intra-footpad with CA-6 cells 24 h after lymphocyte transfer (Fig. 3 *B*). Upon systemic challenge with viable CA-6, enhanced clearance of the yeast from infected organs could be documented in the adoptively transferred mice (data not shown).

*Effect of Anti-IL-4 Treatment on Serum Levels of Candida-specific Antibodies.* We next measured the levels of yeast-specific antibody levels in sera of mice infected with *C. albicans* and treated with control or 11B1 antibodies. At 1, 2, and 3 wk after injection of $7.5 \times 10^4$ CA-6 cells, sera were collected and assayed for their contents of total IgG, Ig2a, IgG1, and IgE. Fig. 4 shows that, in the 2 wk preceding their death, control mice exhibited higher IgG1 levels and lower IgG2a levels in comparison with IL-4-depleted animals. Significant levels of IgE were also found. However, anti-IL-4 treatment of infected mice resulted in complete suppression of IgE production.

*Effect of Anti-IL-4 Treatment on Cytokine Production In Vitro.* To assess the effect of IL-4 depletion on cytokine release in vitro by purified $CD4^+$ lymphocytes from infected mice, mixed lymphocyte yeast cell cultures were established in the presence of accessory cells at 1, 2, and 3 wk after injection of $7.5 \times 10^4$ CA-6 cells. Appropriate controls included $CD4^+$ cells from intact donors and yeast-primed $CD4^+$ lymphocytes cultured with accessory cells but no fungal antigen. After 24 h of incubation, culture supernatants were assayed for their contents of IFN-γ, IL-2, IL-4, and IL-6. Fig. 5 shows that anti-IL-4 treatment resulted in enhanced production of Th1 cytokines throughout the 3-wk observation period. In contrast, mice treated with control antibodies predominantly produced IL-4 and IL-6 during the 2 wk preceding their death.

*Expression of IL-4 and IFN-γ Genes in IL-4-depleted Mice.* To examine the patterns of cytokine gene transcription in IL-4-depleted mice, we extracted total RNA from $CD4^+$ cells recovered at different times after infection, reverse transcribed the polyadenylated mRNA to obtain cDNA, and detected cDNA by PCR with IL-4- and IFN-γ-specific primers. PCR products were verified by probing samples transferred to a nylon membrane with a $^{32}P$-labeled internal oligonucleotide. To provide meaningful comparisons between control and IL-4-depleted mice, the cDNA samples were normalized to the β-actin PCR product, a marker for all cells. Fig. 6 shows the results obtained with RNA preparations from two independent experiments, each involving challenge of control and anti-IL-4-treated mice with $7.5 \times 10^4$ yeast cells, and harvest of $CD4^+$ lymphocytes 3 and 10 d after infection. Although the PCR products were not quantitated, the results apparently indicate that decreased IL-4 mRNA levels were present in IL-4-depleted mice starting from 3 d postinfection, the decrease representing a manifold difference on day 10 (after the second mAb injection). A reciprocal increase in IFN-γ-specific transcripts was apparently observed as a result of IL-4 depletion, with substantially higher levels of message on day 10.

## Discussion

Although superficial and systemic mycoses in humans are associated with different predisposing conditions (20), one

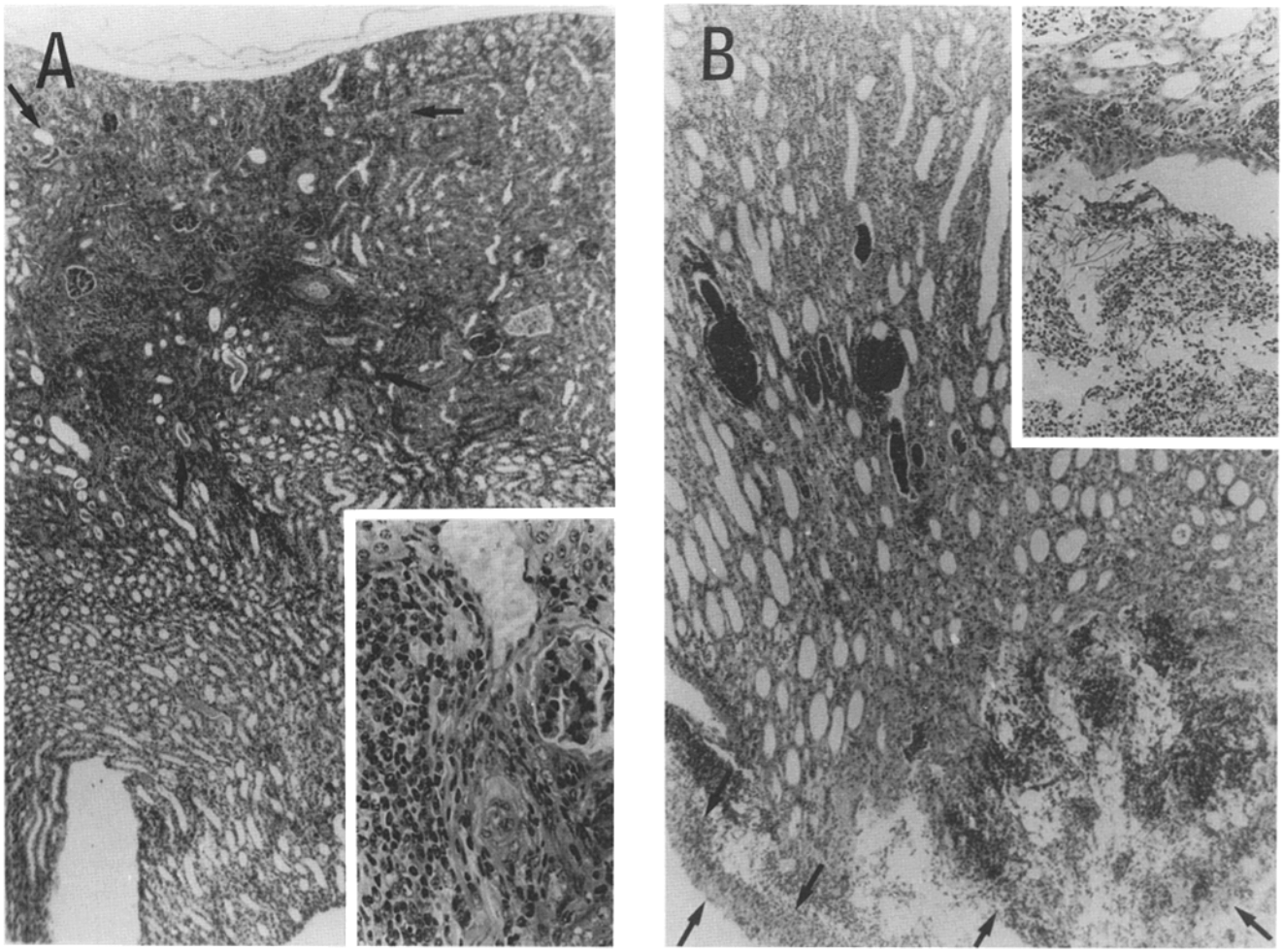


**Figure 2.** Effect of anti-IL-4 treatment on renal candidiasis. Tissue sections were prepared from the kidneys (largest transverse diameter) of 3-wk-infected mice treated with weekly injections of 11B11 mAb (*A*). For comparison, kidneys were also examined from control mice at the time of death (*B*). (*A*) An inflammatory area in the cortex (delimited by *arrows*), but no evidence of fungal growth (hematoxylin/eosin; ×75). (*Inset*) Magnification of a lesion with a predominantly lymphomononuclear infiltrate (×340). (*B*) Large aggregates of fungal yeasts and hyphae in the pelvis (*arrows*), abscesses, areas of dense leukocytic filtration, and papillary necrosis (×70). (*Insert*) Numerous inflammatory leukocytes with filamentous forms of the fungus (×140).

feature common to all forms of candidiasis is that the intrinsic candidacidal activity of granulocytes and macrophages (the major effector cell types) is rather limited (1), and that full expression of their function is contingent upon augmentation by cytokines released by antigen-specific and nonspecific cells (21, 22). In addition, both specific and nonspecific modulation of immune responses have been reported as a consequence of *C. albicans* infection in mice. Garner et al. (23) showed that mannan administered intravenously to naive mice induced a population of suppressor T lymphocytes that, when transferred to immunized mice, suppressed antigen-specific DTH responses. Ashman (24) suggested that active down-regulation of protective immunity might occur in mice that are genetically susceptible to candidiasis and are characterized by high antibody levels and poor DTH responses. Our laboratory has recently found that activation of Th2 cells occurs in mice infected with variants of the yeast that establish persistent colonization yet do not elicit protective immunity, and $CD4^+$ cells from these mice were found to suppress *Candida*-specific DTH responses (11).

In the present study we administered an anti-IL-4 mAb during the course of an otherwise lethal infection with the yeast. Provided the challenge inoculum did not result in short-term mortality, a vast majority (~80%) of the anti-IL-4-treated mice began to actively eliminate yeast cells from their organs, resolved their infections, and demonstrated Th1 responses. In contrast, mice treated with control antibody experienced progressive, disseminated fatal candidiasis, and were characterized by weak or absent footpad responses, and higher IgG and IgE levels. The protective effect of anti-IL-4 treatment required the antibody to be given within approximately the first 2–3 d after infection, under which conditions a single antibody injection was almost as effective as the weekly administration regimen employed in this study (Romani et al., manuscript in preparation). Splenic $CD4^+$ lymphocytes from infected and IL-4-depleted mice harvested at 1, 2, and

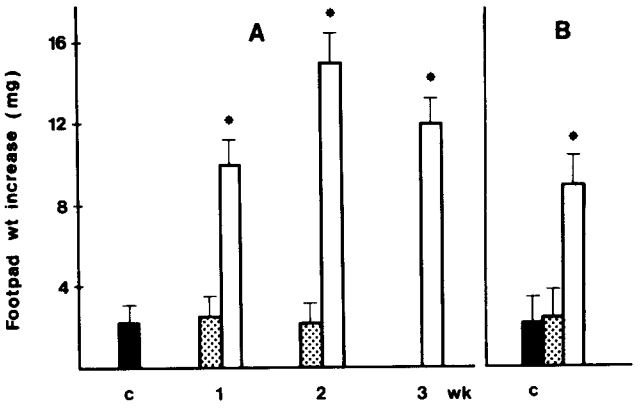


**Figure 3.** DTH in yeast-infected mice treated with anti-IL-4 mAb (*A*) or in mice adoptively transferred with CD4+ lymphocytes from these animals (*B*). (*A*) At different times after CA-6 challenge, mice receiving 11B11 (□) or control (▩) antibodies were injected intra-footpad with inactivated yeast cells 24 h before measurement of their footpad response. As a negative control, uninfected mice (*c*) were assayed. (*B*) Prospective recipients of an intra-footpad yeast challenge received an intravenous injection of $10^7$ CD4+ lymphocytes from 2-wk-infected mice treated with 11B11 (□) or control (▩) antibodies. CD4+ cells from intact mice (*c*) were also transferred. *Significant difference ($p < 0.01$) from value obtained in control mice (*c*), as determined by Student's *t* test. Essentially the same results were obtained in two additional experiments.

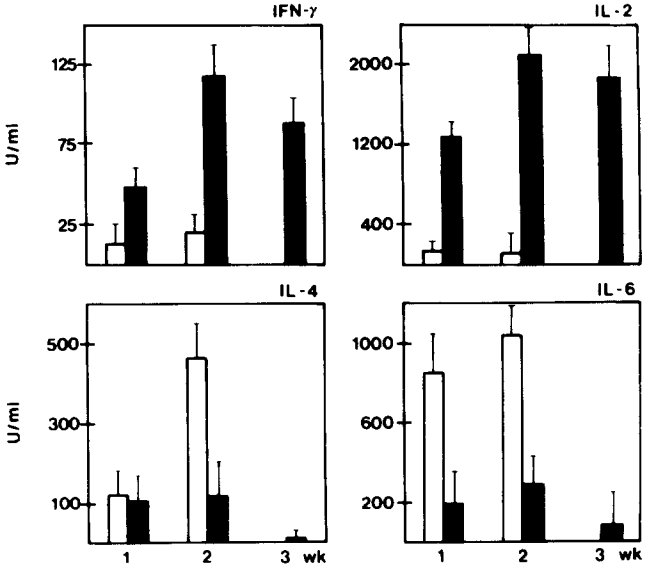


**Figure 5.** Th1 and Th2 cytokine production in yeast-infected mice treated with 11B11 (■) or control (□) antibodies. Mixed CD4+ lymphocyte yeast cell cultures were established at 1, 2, and 3 wk postinfection, and supernatants were assayed for IFN-γ, IL-2, IL-4, and IL-6 contents. Each value represents the mean ± SEM of four to six separate experiments, each involving replicate measurements. Cytokine production in control cultures (see text) was consistently negligible.

3 wk postinfection produced high IFN-γ and IL-2 levels, and substantially reduced amounts of Th2 cytokines, IL-4, and IL-6. At 3 and 10 d postinfection, CD4+ cells from mice treated with anti-IL-4 mAb exhibited reciprocal changes in IL-4 and IFN-γ mRNA expression that represented mani-

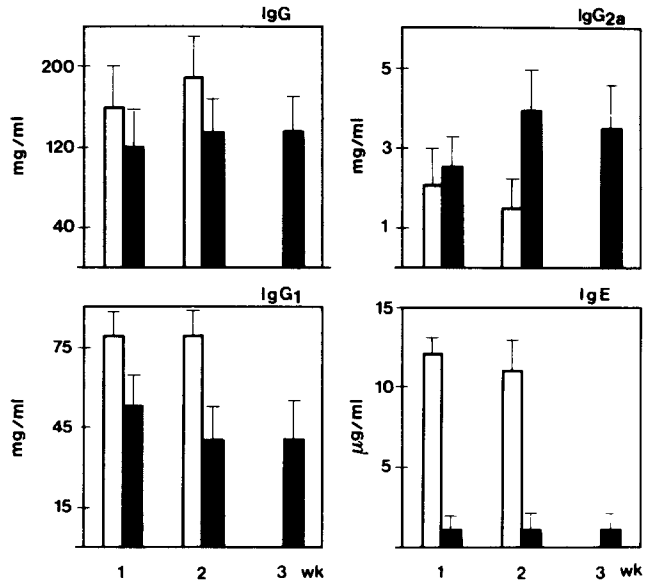


**Figure 4.** Serum Ig levels in yeast-infected mice treated with 11B11 (■) or control (□) antibodies. At 1, 2, and 3 wk after injection of 7.5 × $10^4$ CA-6 blastospores, sera were assayed for total IgG or IgG2a, IgG1, and IgE titers. Values represent the means ± SEM of three experiments. Specific antibody levels in uninfected mice were negligible or undetectable.

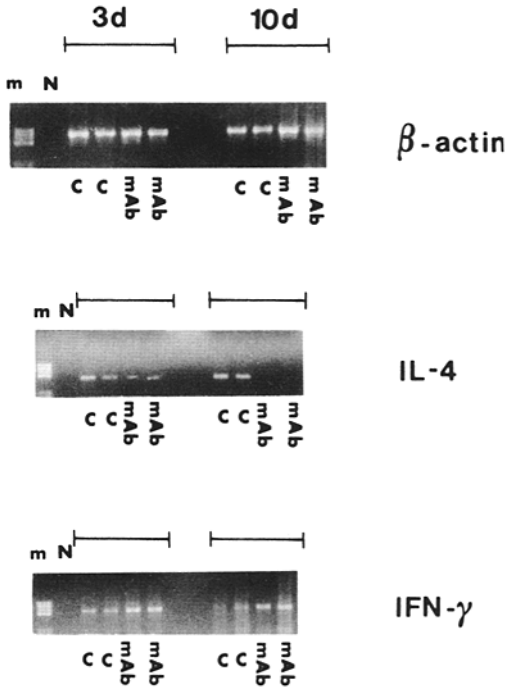


**Figure 6.** IL-4 and IFN-γ mRNA expression in CD4+ cells revealed by PCR normalized to β-actin. RNA was isolated from lymphocytes of control (*C*) or 11B11 mAb–treated mice at 3 and 10 d after infection, and the resulting cDNA was used in the PCR with cytokine-specific primers. After amplification, 10 μl of the reaction mix was removed, analyzed by 1.5% agarose gel electrophoresis, and visualized by ethidium bromide staining. The assay was performed with two different RNA preparations, and a second assay with the same RNA preparations gave analogous results. m, marker track (kilobase ladder consisting of pBR322 DNA cut with HaeIII); N, no DNA added to the amplification mix during PCR.

fold differences with respect to control lymphocytes on day 10 postinfection.

In murine cutaneous leishmaniasis, the early and permanent conversion of nonhealer into healer mice induced by anti-IL-4 treatment (13, 25), and the reversion of the effect by anti-IFN-γ (14), indicates that the alteration of the disease outcome is primarily the consequence of changes in the basic nature of the T cell response, with a shift from a predominant Th2 to a Th1 response. A similar mechanism of anti-IL-4 activity might apply to mouse infection with *C. albicans*, although in the present study we have not examined the role of T cell–derived IFN-γ in the efficacy of anti-IL-4 therapy. The striking difference in IL-4 mRNA observed at 10 d could indicate that IL-4 depletion leads to blockade of Th2 cell expansion. Because IL-4 is required for maturation of $CD4^+$ precursors to the Th2 phenotype, neutralization of this factor would be expected to block both Th2 expansion and the subsequent formation of Th2-derived factors, such as IL-4 and IL-10, that limit protective Th1 responses (14, 26). In addition, the increase in IFN-γ mRNA might indicate, and favor, the development of Th1 reactivity, as IFN-γ is crucially involved in the initial phases of Th responses in vivo (27). Finally, neutralization of IL-4 could further benefit the infected host by blocking the adverse effects of IL-4 on macrophage activation (28) and killing of phagocytosed yeast cells.

Elevated IgE, IgA, and IgG responses to the yeast show an inverse relationship with delayed skin responses in atopics (29), in women suffering from recurrent *Candida* vaginitis (20, 30), and in patients with chronic mucocutaneous candidiasis (20). This suggests that humoral and cell-mediated immune responses to *Candida* in humans may be reciprocally regulated. Our present data that neutralization of endogenous IL-4 has therapeutic value in a murine model of systemic candidiasis could provide a means for improving our understanding of the host/parasite relationship, and also could offer the basis for attempting new strategies in the treatment of human candidiasis.

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