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Both Th1 and Th2 Cytokines Affect the Ability of Monoclonal Antibodies To Protect Mice against *Cryptococcus neoformans*

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Variable-region-identical mouse immunoglobulin G1 (IgG1), IgG2b, and IgG2a monoclonal antibodies to the capsular polysaccharide of *Cryptococcus neoformans* prolong the lives of mice infected with this fungus, while IgG3 is either not protective or enhances infection. $CD4^+$ T cells are required for IgG1-mediated protection, and $CD8^+$ T cells are required for IgG3-mediated enhancement. Gamma interferon is required for both effects. These findings revealed that T cells and cytokines play a role in the modulation of cryptococcal infection by antibodies and suggested that it was important to more fully define the cytokine requirements of each of the antibody isotypes. We therefore investigated the efficacy of passively administered variable-region-identical IgG1, IgG2a, IgG2b, and IgG3 monoclonal antibodies against intravenous infection with *C. neoformans* in mice genetically deficient in interleukin-12 (IL-12), IL-6, IL-4, or IL-10, as well as in the parental C57BL/6J strain. The relative inherent susceptibilities of these mouse strains to *C. neoformans* were as follows: IL-$12^{-/-}$ > IL-$6^{-/-}$ > C57BL/6J ≈ IL-$4^{-/-}$ ≫ IL-$10^{-/-}$. This is consistent with the notion that a Th1 response is necessary for natural immunity against cryptococcal infection. However, none of the IgG isotypes prolonged survival in IL-$12^{-/-}$, IL-$6^{-/-}$, or IL-$4^{-/-}$ mice, and all isotypes significantly enhanced infection in IL-$10^{-/-}$ mice. These results indicate that passive antibody-mediated protection against *C. neoformans* requires both Th1- and Th2-associated cytokines and reveal the complexity of the mechanisms through which antibodies modulate infection with this organism.

Cryptococcus neoformans is an encapsulated yeast that is a frequent cause of life-threatening meningoencephalitis in patients with impaired immunity. The prevalence of cryptococcal meningitis in patients with AIDS ranges from 8% in the United States to 30% in Africa (11, 12, 84). Current therapy is inadequate, as 10 to 20% of patients treated with antifungal drugs die from cryptococcal meningitis (10, 76). Furthermore, individuals who survive beyond the initial treatment period must be maintained on lifelong suppressive therapy to prevent relapse (62). Because of these therapeutic limitations, better treatments for *C. neoformans* infections are needed.

One new approach to improving therapy for cryptococcosis is the use of monoclonal antibodies (MAbs) to the glucuronoxylomannan (GXM) component of the *C. neoformans* capsular polysaccharide as adjuncts to antifungal drugs. Certain MAbs to GXM can protect mice against *C. neoformans* infection and enhance the efficacy of antifungal therapy (17, 18, 52–56). A murine immunoglobulin G1 (IgG1) MAb is currently undergoing phase I evaluation for the treatment of cryptococcal meningitis in patients with AIDS (7). Studies using MAbs to GXM have demonstrated that antibody-mediated protection in murine models of systemic cryptococcal infection is dependent on the antibody isotype. Comparisons of variable-region-identical antibodies of the IgG1, IgG2a, IgG2b, and IgG3 isotypes have consistently shown that all isotypes, except IgG3, prolong survival of mice infected with *C. neoformans* (61, 79, 82). This difference is not dependent on antigen clearance because all IgG isotypes accelerate clearance of GXM in infected animals in a similar manner (43). These observations indicate that functions mediated by the constant regions of these MAbs are crucial for determining their protective potential. While Fc receptors play a role in antibody-mediated protection (80), the exact mechanisms responsible for these phenomena are not understood.

It is our hope that a better understanding of the variables that mediate antibody efficacy will lead to the design of more-effective antibody-based therapeutics. Prior experiments on immunodeficient mice showed that $CD4^+$ T cells and gamma interferon (IFN-γ) are necessary for protection by IgG1 and that $CD8^+$ T cells and IFN-γ are required for enhancement of infection by IgG3 (81). These results revealed the importance of T cells and the Th1 cytokine IFN-γ in modulating the protective efficacy of the different isotypes. Before attempting to identify the detailed mechanisms responsible for the interaction of antibodies, T cells, cytokines, effector cells, and the organism, it was important to more fully define the types of cytokines that could affect this process. To do this, we investigated the capacity of passively administered IgG subclasses to protect mice deficient in either the Th1 cytokine interleukin-12 (IL-12), the proinflammatory cytokine IL-6, or the Th2 cytokines IL-4 and IL-10 against cryptococcal infection. We first studied the innate susceptibility of each of these genetically deficient mice to cryptococcal infection. The results demonstrated that *C. neoformans* infection was accelerated in IL-

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$12^{-/-}$ and IL-$6^{-/-}$ mice, while IL-$4^{-/-}$ mice were as susceptible as the background strain, C57BL/6J. In contrast, IL-$10^{-/-}$ mice were very resistant to infection. This confirmed that Th1 cytokines contributed to the natural resistance of mice to cryptococcal infection. We then examined the effect of each of the antibody isotypes and found that none of the isotypes protected IL-$12^{-/-}$, IL-$6^{-/-}$, or IL-$4^{-/-}$ mice against *C. neoformans*, while all isotypes greatly enhanced infection in IL-$10^{-/-}$ mice. These results revealed that antibody-mediated protection against *C. neoformans* is dependent on both Th1- and Th2-associated cytokines and further highlight the interdependence of cellular and antibody-mediated immunity.

MATERIALS AND METHODS

Mice. The mice with targeted disruption of specific cytokine genes used in these experiments, including IL-12p40$^{-/-}$ (46), IL-$6^{-/-}$ (38), IL-$4^{-/-}$ (42), and IL-$10^{-/-}$ (41) mice, have been reported previously. Fully backcrossed breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, Maine) and were bred and maintained in isolator cages in a pathogen-free barrier facility within the Animal Care Institute at Albert Einstein College of Medicine, where they were checked daily. All mice had been backcrossed onto C57BL/6J for at least 10 generations, and, therefore, C57BL/6J mice were used as controls. The genotype of the breeders was confirmed by PCR of tail DNA using primers described previously (38, 41, 42, 46). Mice were used at 6 to 10 weeks of age; control mice were age matched.

Antibodies. The 3E5 IgG3 hybridoma was obtained previously by fusing NSO cells to spleen cells from a mouse immunized with GXM conjugated to tetanus toxoid (8). The IgG1, IgG2b, and IgG2a switch variants of MAb 3E5 were generated by in vitro isotype switching (68, 79, 82). The variable-region sequences of these MAbs are identical, and all bind GXM (unpublished data; 82). Ascites fluid was obtained by injecting 5×10^6 hybridoma cells suspended in Hanks' buffered saline into the peritoneal cavities of pristane-primed (Sigma, St. Louis, Mo.) SCID mice. The ascites fluid was collected in a sterile fashion and centrifuged at $1{,}000 \times g$ to remove cells. Lipids and cell debris were removed with Cleanascite HC (LigoChem, Fairfield, N.J.), and the ascites fluid was sterilized by passage through a 0.2-μm-pore-size filter. Antibody concentration relative to isotype-matched standards was then determined by enzyme-linked immunosorbent assay (ELISA). Ascites fluid was stored at 4°C and checked for activity by ELISA prior to each survival experiment. Each batch of ascites fluid was tested for contaminating isotypes, which were present at <0.001%. For some experiments, antibodies were purified by protein A affinity chromatography (20).

Murine infection. *C. neoformans* serotype D strain 24067 was obtained from the American Type Culture Collection (Manassas, Va.) and stored in sucrose at −80°C. This strain was selected for study because it has been extensively analyzed (25) and was used in previous studies of antibody efficacy (23, 51, 52, 54–56, 61, 79–82). *C. neoformans* was grown at 37°C in Sabouraud's dextrose broth (Difco Laboratories, Detroit, Mich.) to log phase. Yeast cells were then washed three times with phosphate-buffered saline (PBS), and the inoculum was determined by counting in a hemocytometer. The *C. neoformans* inoculum was diluted and plated on Sabouraud's dextrose agar (Difco) to confirm CFU estimates. Organisms were suspended in PBS and injected into the lateral tail vein in a volume of 0.2 ml. For survival studies, the number of mice per group ranged from 8 to 11. Twenty-four hours prior to infection with *C. neoformans*, mice were given an intraperitoneal injection of SCID mouse ascites fluid containing 1 mg of a single 3E5 IgG isotype or, as a control, 1 ml of ascites fluid made from NSO cells, the nonproductive mouse myeloma fusion partner used to make MAb 3E5. For survival studies with cytokine-deficient mice, an additional control group of NSO cell ascites fluid-treated C57BL/6J mice was included to ensure consistency of organism inoculum and virulence between experiments. In separate experiments, we have shown that mice infected with *C. neoformans* and treated with PBS or ascites fluid from NSO cells have the same survival as untreated mice. In a few survival experiments, mice were given purified MAbs and PBS was used as the control to determine if factors in the ascites fluid other than the MAb were influencing survival. No significant differences in survival were noted between animals treated with NSO cell ascites fluid and animals treated with PBS or between animals treated with purified antibody and animals treated with the same antibody in SCID mouse ascites fluid. Serum was obtained 14 days after infection, and GXM concentration was measured by capture ELISA as previously described (9).

CFU preparation and pathological examination. Mice were killed by cervical dislocation on day 10 or 17 after infection, and their organs were removed in a sterile manner. The right upper lobe of the lung, caudal half of the spleen, accessory lobe of the liver, and right hemisphere of the brain were fixed in 10% buffered formalin and embedded in paraffin. Five mice per group were examined. Sections (5 μm thick) stained with hematoxylin and eosin (H&E) or mucicarmine were reviewed by light microscopy (one to three sections per organ). CFU were determined by homogenizing the remaining brain, lung, liver, and spleen tissue, which was then diluted in PBS and plated on Sabouraud's dextrose agar.

In vitro phagocytosis. Peritoneal macrophages were obtained by peritoneal lavage from mice 5 days after intraperitoneal stimulation with 1.5 ml of 4% thioglycolate. Thioglycolate-stimulated mice were killed, and their peritoneal cavities were washed with 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Alveolar macrophages were obtained from bronchoalveolar lavage. After the mice were killed, a 20-guage Angiocath (Becton Dickinson, Franklin Lakes, N.J.) was inserted into the trachea and the lungs were irrigated with 10 ml of PBS. Peritoneal or alveolar cells were counted, suspended in DMEM supplemented with 10% FCS, and plated on 96-well tissue culture plates (Costar, Corning, N.Y.) at a density of 3×10^4 to 4×10^4 mononuclear cells per well. Nonadherent cells were washed away after 2 h of incubation at 37°C. Adherent cells were incubated overnight without IL-10 or with 2 ng of murine recombinant IL-10 (R&D Systems, Minneapolis, Minn.)/ml. Three micrograms of purified 3E5 IgG1, IgG2a, IgG2b, or IgG3 MAb was then added to each well 20 min before adding 5×10^5 heat-killed *C. neoformans* cells per well and incubating for 4 h at 37°C. Wells were then washed three times with cold PBS to remove nonphagocytosed organisms, fixed with cold methanol for 25 min, and stained with a 1:10 solution of Giemsa (Sigma) for 20 min. The stain was then replaced with PBS. Multiple fields from three wells per condition were examined by inverted light microscopy at ×400 magnification; phagocytosis was expressed as the phagocytic index (percentage of cells with two or more internalized organisms).

Statistical methods. Data were analyzed with StatView statistical software (SAS Institute, Cary, N.C.). Serum GXM and CFU data were compared using the Mann-Whitney U test for nonparametric data. Survival data were subjected to Kaplan-Meier analysis, and statistical significance was determined by the log rank (Mantel-Cox) test. A P value of less than 0.05 was considered statistically significant.

RESULTS

IgG1, IgG2b, and IgG2a prolonged the lives of C57BL/6J mice with cryptococcal infection, while IgG3 did not. In previously reported experiments, we showed that the 3E5 IgG3 MAb to GXM does not prolong survival and that its in vitro switch variant 3E5 IgG1 protects C57BL/6J mice against intravenous cryptococcal challenge (81). To confirm these results and to further examine the efficacy of the other IgG isotypes in this mouse strain, which is the background strain for all the mice used in the experiments reported here, we gave C57BL/6J mice an intraperitoneal injection of SCID mouse ascites fluid containing 1 mg of either 3E5 IgG3 or one of its switch variants of the IgG1, IgG2b, or IgG2a isotype. SCID mouse ascites fluid made from NSO cells, the nonproductive hybridoma fusion partner used to generate MAb 3E5, was used as a control. After 24 h, animals were infected intravenously with 10^6 CFU of *C. neoformans*. The survival data in Fig. 1 demonstrate that the variable-region-identical 3E5 IgG1, IgG2b, and IgG2a MAbs significantly prolonged the lives of infected C57BL/6J mice ($P < 0.006$). IgG2a appeared to offer the most protection, but this tendency was not significant ($P = 0.08$ versus IgG1 and $P = 0.09$ versus IgG2b). IgG3, on the other hand, did not protect these mice and seemed to exacerbate infection somewhat, although this trend did not reach statistical significance ($P = 0.09$).

IL-$12^{-/-}$ and IL-$6^{-/-}$ mice were more susceptible than C57BL/6J to cryptococcal infection, while IL-$10^{-/-}$ mice were very resistant. Next, we examined the course of *C. neoformans*

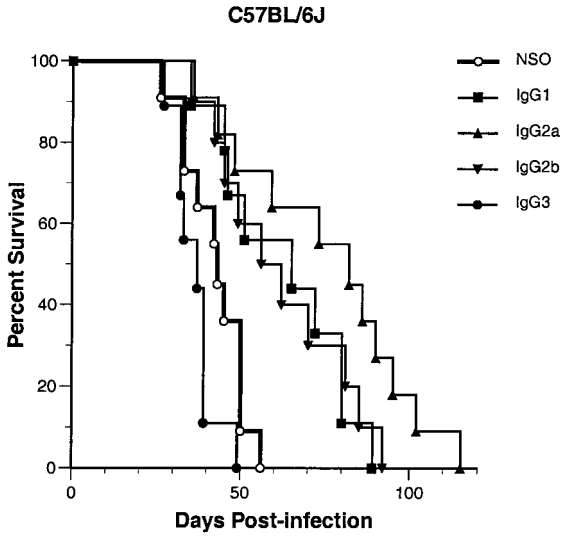

FIG. 1. Survival of MAb-treated C57BL/6J mice infected with *C. neoformans*. Mice were given an intraperitoneal injection of SCID mouse ascites fluid containing either 1 mg of the anti-GXM 3E5 IgG3, one of its in vitro switch variants of the IgG1, IgG2b, or IgG2a isotype, or 1 ml of NSO cell SCID mouse ascites fluid as a control and then 24 h later were infected intravenously with 10^6 *C. neoformans* CFU. Administration of IgG1, IgG2b, or IgG2a was significantly protective compared to the control ($P < 0.006$). Animals treated with IgG3 fared worse than control mice, but this did not reach statistical significance ($P = 0.09$). Mean survival in days by treatment group: NSO cell ascites fluid, 41; IgG1, 63; IgG2a, 75; IgG2b, 62; IgG3, 36.

infection in IL-$4^{-/-}$, IL-$6^{-/-}$, IL-$10^{-/-}$, and IL-$12^{-/-}$ mice. Decken and colleagues have recently shown that IL-$12^{-/-}$ mice are highly susceptible to cryptococcal infection, while IL-$4^{-/-}$ mice are resistant to infection (15). Blackstock et al. have reported that IL-$10^{-/-}$ mice are resistant to infection (5). The susceptibility of IL-$6^{-/-}$ mice to *C. neoformans* has not been reported previously. To compare the inherent susceptibilities of the cytokine-deficient mice with that of the parental C57BL/6J mice and to anticipate studies with MAbs, animals were treated with NSO cell ascites fluid and infected with *C. neoformans*. Figure 2A shows the survival of IL-$4^{-/-}$, IL-$6^{-/-}$, IL-$10^{-/-}$, and IL-$12^{-/-}$ mice in comparison to that of the parental C57BL/6J strain, all given 2.5×10^6 CFU of *C. neoformans*. As expected, IL-$12^{-/-}$ mice were highly susceptible to cryptococcal infection ($P < 0.0001$). IL-$6^{-/-}$ mice were also more susceptible to cryptococcal infection than the parental strain ($P = 0.02$). In contrast, IL-$4^{-/-}$ mice appeared slightly more resistant to infection than the parental strain, but the trend did not achieve statistical significance ($P = 0.4$). As reported previously (5), IL-$10^{-/-}$ mice were significantly more resistant to infection than the parental strain ($P < 0.0001$); 40% of the animals were still alive at 18 weeks, when the experiment was terminated. While CFU were not determined for these survivors, they all had significant serum GXM levels (mean = 87 μg/ml), indicating that they had not cleared the infection. Since the GXM levels all of the IL-10 $^{-/-}$ mice were low at 14 days (Fig. 2B), this suggests that there was a progressive increase in fungal burden that ultimately led to the death of the animals.

Serum GXM levels were determined on day 14 postinfection (Fig. 2B). By this time, two IL-$6^{-/-}$ mice and seven IL-$12^{-/-}$ mice had already died from the infection. In comparison to C57BL/6J mice, IL-$10^{-/-}$ mice had very low levels of circulating GXM ($P = 0.003$), while the more-susceptible IL-$6^{-/-}$ mice had a trend toward higher serum GXM levels, but this was not statistically significant ($P = 0.3$). Serum GXM levels in IL-$4^{-/-}$ mice were heterogeneous: four mice had high serum

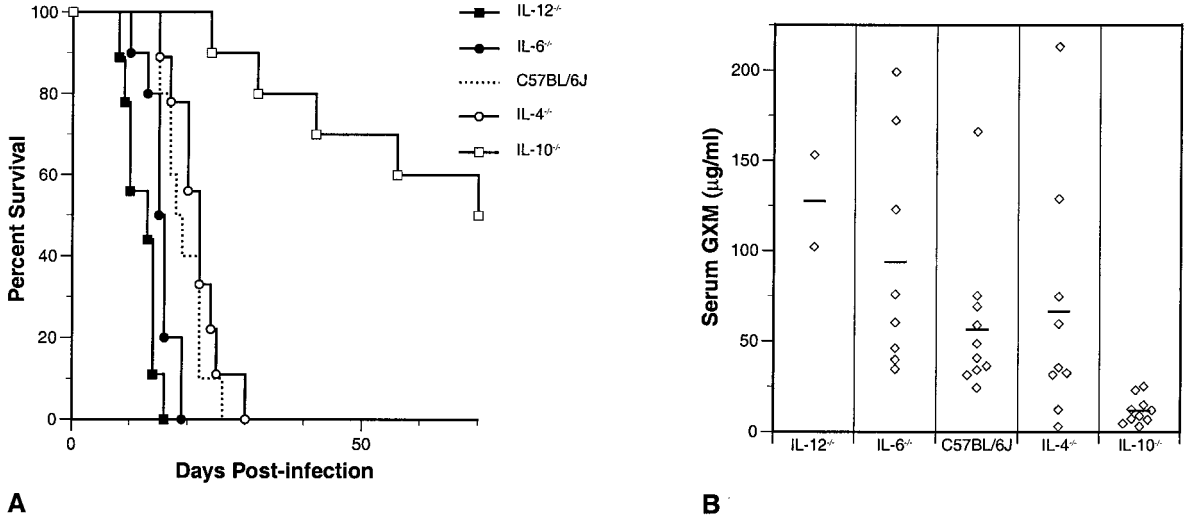

FIG. 2. (A) Survival of untreated cytokine-deficient mice and the parental C57BL/6J mice infected intravenously with 2.5×10^6 *C. neoformans* CFU. IL-$12^{-/-}$ and IL-$6^{-/-}$ mice were more susceptible to infection than control mice ($P < 0.0001$ and $P < 0.02$, respectively), while IL-$10^{-/-}$ mice were very resistant to cryptococcal challenge ($P < 0.0001$). IL-$4^{-/-}$ mice resembled the parental C57BL/6J mice ($P = 0.4$). The experiment was terminated on day 140 postinfection, when there was still 40% survival in the IL-$10^{-/-}$ mice. Mean survival in days by mouse strain: IL-$12^{-/-}$, 12; IL-$6^{-/-}$, 15; C57BL/6J, 19; IL-$4^{-/-}$, 21; IL-$10^{-/-}$, 79. (B) Serum GXM levels measured on day 14 postinfection from mice in panel A. By this time, two mice in the IL-$6^{-/-}$ group and seven in the IL-$12^{-/-}$ group had died. Horizontal lines, mean serum concentrations. In comparison to C57BL/6J, only IL-$10^{-/-}$ mice had significantly different levels of circulating GXM ($P < 0.003$).

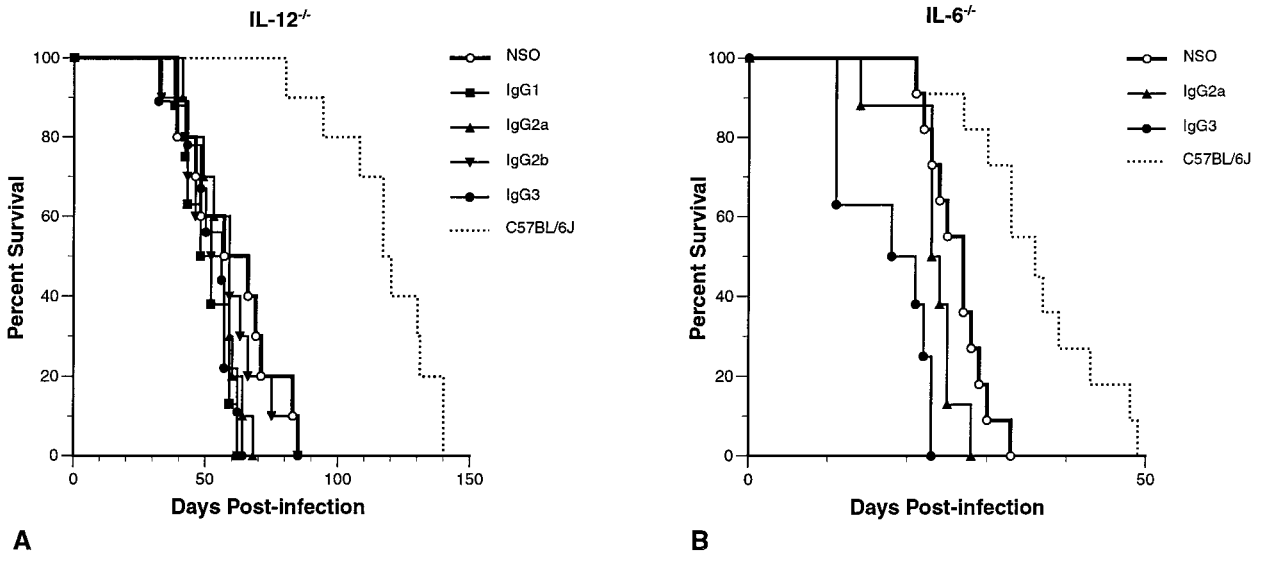

FIG. 3. Survival of MAb-treated IL-12$^{-/-}$ and IL-6$^{-/-}$ mice. (A) IL-12$^{-/-}$ mice were infected with 10^5 *C. neoformans* CFU. NSO cell ascites fluid-treated C57BL/6J given the same inoculum are also shown so that the effect of this lower inoculum on the wild-type mice can be seen. Passive-antibody administration was neither protective nor enhancing compared to the control ($P > 0.2$). Mean survival in days by treatment group: NSO cell ascites fluid, 60; IgG1, 50; IgG2a, 55; IgG2b, 56; IgG3, 52; C57BL/6J mice treated with NSO cell ascites fluid, 116. (B) Survival of MAb-treated IL-6$^{-/-}$ mice infected with 10^6 *C. neoformans* CFU. 3E5 IgG2a was not protective and 3E5 IgG3 enhanced infection in comparison to the control ($P = 0.23$ and 0.0009, respectively). Mean survival in days by treatment group: NSO cell ascites fluid, 26; IgG2a, 23; IgG3, 18; C57BL/6J mice treated with NSO cell ascites fluid, 36.

GXM (>50 μg/ml), and mice in this subgroup died earlier than the five mice with levels below 50 μg/ml ($P = 0.02$). In this experiment, serum GXM levels correlated with survival. However, in subsequent experiments using antibody treatment, serum GXM levels determined when the mice began to die did not correlate with outcome or any other measured parameter (data not shown).

IgG isotypes did not protect IL-12$^{-/-}$ mice against infection with *C. neoformans*. Given the susceptibility of IL-12$^{-/-}$ mice to *C. neoformans*, we decreased the initial inoculum 25-fold to 10^5 CFU in an attempt to detect smaller differences in antibody efficacy. This modification resulted in an increased mean survival time of control IL-12$^{-/-}$ mice from 12 to 60 days. Control NSO cell ascites fluid-treated C57BL/6J mice given the same inoculum survived substantially longer as well (mean survival = 116 days; Fig. 3A), again illustrating the increased susceptibility of IL-12$^{-/-}$ mice. None of the IgG isotypes were protective compared to control ($P > 0.2$).

In IL-6$^{-/-}$ mice, IgG2a did not protect against, while IgG3 enhanced, infection with *C. neoformans*. We studied the efficacy of our most and least protective 3E5 isotypes, IgG2a and IgG3, respectively, in IL-6$^{-/-}$ mice infected with 10^6 CFU of *C. neoformans* (Fig. 3B). Compared to control, IgG2a was not protective, while IgG3 enhanced infection ($P = 0.23$ and 0.0009, respectively). These results indicated that proinflammatory cytokine IL-6 was necessary for the protective efficacy of IgG2a but was not required for IgG3-mediated enhancement of cryptococcal infection.

IgG isotypes did not protect IL-4$^{-/-}$ mice against infection with *C. neoformans*. Mice that lack prototypic Th2 cytokine IL-4 have high circulating levels of IFN-γ (57). To our knowledge, there are no studies examining the importance of Th2 responses in passive antibody protection, but, given the seeming importance of IFN-γ in antibody protection against cryptococcal infection, we expected that these mice would be highly protected by IgG1, IgG2b, and IgG2a. To our surprise, none of the 3E5 IgG isotypes protected IL-4$^{-/-}$ mice infected with 10^6 CFU of *C. neoformans* ($P > 0.2$; Fig. 4). As we observed above (Fig. 2), IL-4$^{-/-}$ mice appeared slightly more resistant to infection than C57BL/6J mice, but at this lower inoculum the difference was statistically significant ($P = 0.03$). We repeated the experiment, this time using purified antibodies and PBS as the control with virtually identical results (data not shown), indicating that the effect was due to the MAbs and not to other factors in the ascites fluid.

All IgG isotypes significantly reduced survival of IL-10$^{-/-}$ mice infected with *C. neoformans*. Because IL-10$^{-/-}$ mice were so resistant to infection, we increased the inoculum to 5×10^6 organisms to examine antibody efficacy in these mice. C57BL/6J control mice given this inoculum were all dead by week 3 postinfection (Fig. 5A). As expected (5), the control IL-10$^{-/-}$ mice were much more resistant to infection, with a median survival of 7 weeks. Unexpectedly, we found that all isotypes enhanced infection in a highly significant fashion ($P < 0.006$). This experiment was repeated using a lower inoculum of 10^6 CFU, which resulted in longer survival times for all groups but otherwise produced very similar results (Fig. 5B). We again saw similar results when we performed this experiment with purified antibodies (data not shown). Since these findings were surprising and since we are concerned about anything that enhances infection, we carried out further experiments to try to understand the mechanism(s) underlying the enhancement of cryptococcal infection seen with antibody treatment in the IL-10$^{-/-}$ mice.

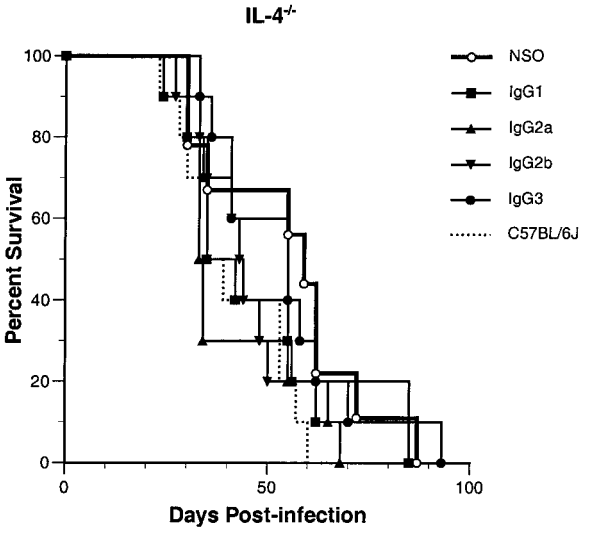

FIG. 4. Survival of MAb-treated IL-$4^{-/-}$ mice infected with 10^6 *C. neoformans* CFU. The antibody was neither protective nor enhancing compared to the control ($P > 0.2$). At this lower inoculum, control IL-$4^{-/-}$ mice were more resistant to infection than C57BL/6J mice ($P = 0.03$). Mean survival in days by treatment group: NSO cell ascites fluid, 55; IgG1, 46; IgG2a, 42; IgG2b, 45; IgG3, 54; C57BL/6J mice treated with NSO cell ascites fluid, 41.

Fungal burden in antibody-treated IL-$10^{-/-}$ mice was not different from that in control mice. Organ CFU have been correlated with antibody protection against cryptococcal infection in many of our previous studies (23, 52, 54, 55, 79, 81, 82). To determine if fungal burden or differences in cryptococcal dissemination could explain why the antibody-treated IL-$10^{-/-}$ mice were dying earlier, antibody-treated IL-$10^{-/-}$ mice were infected with 10^6 CFU of *C. neoformans* and killed when the first mouse died (day 17 postinfection) and *C. neoformans* CFU from the brain, liver, spleen, and lung were tallied (Table 1). Interestingly, there were no differences in CFU that could explain why antibody-treated animals were dying earlier, though IgG1-treated animals had significantly higher organism burdens in the liver and spleen. Despite the lack of major differences in CFU, most antibody-treated mice looked sick (as determined by lack of preening, decreased activity, and weakness), while all of the IL-$10^{-/-}$ control mice appeared robust. To determine if there were differences in fungal burden at an earlier time, we examined organ CFU on day 10 postinfection (Table 1; data from animals in the experiment are shown in Fig. 5B). The control IL-$10^{-/-}$ mice had slightly lower CFU in all organs, and, in some cases, the decrease in CFU compared to CFU in antibody-treated mice was statistically significant. We conclude that antibody-mediated enhancement of cryptococcal infection in IL-$10^{-/-}$ mice was not explained solely by differences in fungal burden.

Histopathology of antibody-treated IL-$10^{-/-}$ mice following cryptococcal infection. We examined organ histopathology from the same animals that we evaluated for fungal burden. There was little difference between C57BL/6J and IL-$10^{-/-}$ mice, regardless of treatment. In the lung and the liver, infection was characterized by the presence of diffuse foci of granulomatous inflammation, in which macrophages, epithelioid cells, and multinucleated giant cells represented the predominant cell types (Fig. 6). The cytoplasm of many of these cells stained with mucicarmine, suggesting the presence of capsular polysaccharide. These inflammatory foci also contained various proportions of neutrophils, lymphocytes, and eosinophils. In the lung, yeast cells were also seen in alveolar spaces without inflammatory cells. In animals sacrificed on day 17, both

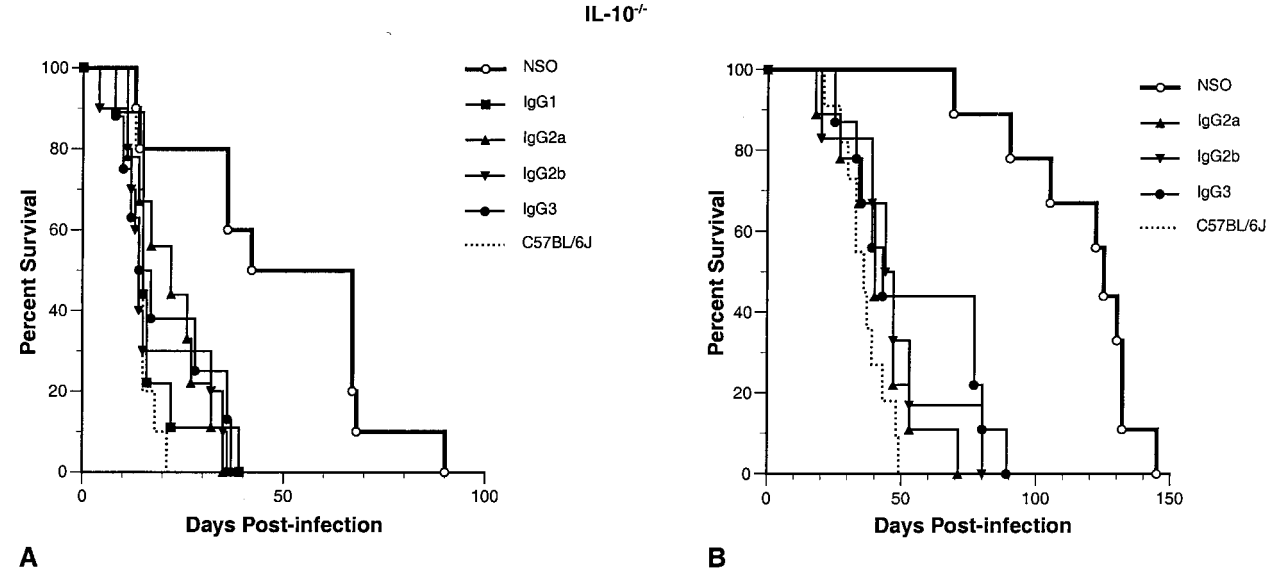

FIG. 5. (A) Survival of MAb-treated IL-$10^{-/-}$ mice infected with 5×10^6 *C. neoformans* CFU. All antibodies greatly enhanced cryptococcal infection compared to the control ($P > 0.006$). Mean survival in days by treatment group: NSO cell ascites fluid, 50; IgG1, 17; IgG2a, 22; IgG2b, 19; IgG3, 20; C57BL/6J mice treated with NSO cell ascites fluid, 15. (B) Survival of IL-$10^{-/-}$ mice treated as above but given a lower inoculum of 10^6 *C. neoformans* CFU. Again, all antibodies greatly enhanced cryptococcal infection compared to the control ($P > 0.002$). Mean survival in days by treatment group: NSO cell ascites fluid, 114; IgG2a, 42; IgG2b, 47; IgG3, 55; C57BL/6J mice treated with NSO cell ascites fluid, 37.

TABLE 1. Organ CFU from IL-10$^{-/-}$ mice infected with *C. neoformans*

Organ	Treatment[a]	Mean CFU ± SE (10^4) on day:	
		10[b]	17[c]
Brain	NSO	38 ± 16	150 ± 32
	IgG1		160 ± 28
	IgG2a	50 ± 17	150 ± 30
	IgG2b	160 ± 56	120 ± 19
	IgG3	98 ± 24	150 ± 26
	C57/NSO	58 ± 24	170 ± 23
Liver	NSO	82 ± 12	8.4 ± 3.0
	IgG1[d]		19 ± 3.0
	IgG2a	105 ± 44	7.8 ± 3.2
	IgG2b	410 ± 160	6.0 ± 1.2
	IgG3[e]	240 ± 42	7.2 ± 2.7
	C57/NSO[f]	110 ± 10	75 ± 11
Lung	NSO[g]	25 ± 6.0	110 ± 28
	IgG1		130 ± 30
	IgG2a	76 ± 25	150 ± 25
	IgG2b	110 ± 15	110 ± 36
	IgG3	100 ± 30	93 ± 15
	C57/NSO	110 ± 26	91 ± 8.6
Spleen	NSO	6.6 ± 1.0	3.4 ± 1.1
	IgG1[h]		19 ± 4.6
	IgG2a	9.2 ± 2.1	3.7 ± 1.0
	IgG2b	21 ± 11	2.4 ± 0.8
	IgG3[i]	84 ± 24	3.0 ± 0.5
	C57/NSO[j]	37 ± 15	12 ± 2.4

[a] Mice received ascites fluid containing either 1 mg of 3E5 IgG3, one of its switch variants of the IgG1, IgG2b or IgG2a isotype, or 1 ml of NSO cell ascites fluid as a control (NSO). C57BL/6J mice treated with NSO cell ascites fluid (C57/NSO) are also shown.
[b] Mice killed on day 10 postinfection. There was no IgG1 treatment group for this experiment ($n = 4$).
[c] Mice killed day 17 postinfection ($n = 5$).
[d] $P < 0.0005$ versus liver CFU for all treatment groups on day 17.
[e] $P < 0.03$ versus liver CFU for NSO and C57/NSO treatment groups on day 10.
[f] $P < 0.05$ versus liver CFU for all treatment groups on day 17.
[g] $P < 0.04$ versus lung CFU for IgG3 and C57/NSO treatment groups on day 10.
[h] $P < 0.02$ versus spleen CFU for all treatment groups except C57/NSO on day 17.
[i] $P < 0.05$ versus spleen CFU for NSO and IgG2a treatment groups on day 10.
[j] $P < 0.02$ versus spleen CFU for all treatment groups on day 17.

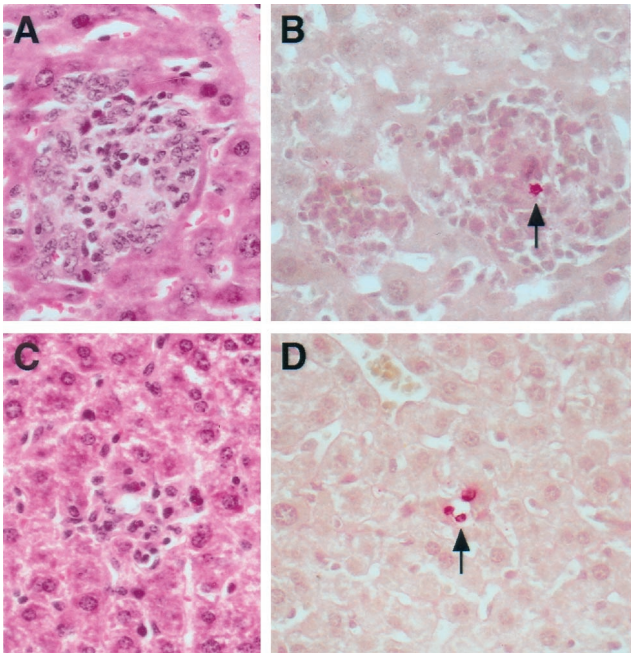

FIG. 6. Liver pathology in IL-10$^{-/-}$ mice 17 days after infection with *C. neoformans*. (A) H&E staining of the livers of IL-10$^{-/-}$ mice in the control (NSO cell ascites fluid) group showed granulomatous cryptococcal lesions that contained macrophages with a mixture of other leukocytes. (B) Mucicarmine staining showed small numbers of organisms in these lesions (arrow) and demonstrated pink cytoplasmic staining of macrophages, which is consistent with phagocytosis of cryptococcal capsular polysaccharide. (C) In contrast, H&E staining of sections from C57BL/6J mice showed smaller, less-organized inflammatory responses to cryptococcal infection. (D) Mucicarmine staining demonstrated extracellular yeast in sinusoids (arrow) without associated inflammation. Magnification (all panels), ×400.

control and MAb-treated IL-10$^{-/-}$ mice appeared to have more abundant inflammatory infiltrates near infectious foci than did the C57BL/6J mice and MAb-treated IL-10$^{-/-}$ mice had fewer free yeast cells than did control IL-10$^{-/-}$ or C57BL/6J mice. However, on day 10 postinfection, these differences in lung pathology were not observed.

In the liver, many of the granulomatous lesions were located perivascularly, and yeast cells outside of inflammatory foci were present in hepatic sinusoids and in Kupffer cells. On day 10 postinfection, the hepatic granulomas of both the MAb-treated and control IL-10$^{-/-}$ mice were more cellular and had a more epithelioid appearance than those of C57BL/6J mice, such that one of us (A.C.) was able to correctly distinguish between the IL-10$^{-/-}$ controls and C57BL/6J mice in sections from four different mice of each group in a blinded experiment. However, this difference was not noted on day 17 postinfection. In both C57BL/6J and IL-10$^{-/-}$ mice, infection in the brain was characterized by the presence of diffuse foci of infection with numerous yeast cells and minimal inflammatory response. In some lesions, the brain tissue bordering the collection of yeast cells contained intracellular yeast cells and the cytoplasm of cells with yeast stained with mucicarmine. In the spleen, yeast cells were seen in all mice in the red pulp, predominantly in the venous sinuses. In sections from mice in which yeast cells appeared to be more numerous, intra- and extracellular yeast cells were also seen in the periarteriolar lymphoid sheaths, in marginal-zone macrophages, and in lymphoid nodules. In general, the pathology did not reveal a sustained increase in inflammation in the IL-10$^{-/-}$ mice.

Levels of in vitro phagocytosis of *C. neoformans* by primary macrophages from IL-10$^{-/-}$ mice and C57BL/6J mice were similar. We compared the phagocytic properties of macrophages from IL-10$^{-/-}$ and C57BL/6J mice both with and without IL-10 pretreatment (Fig. 7). In the absence of antibodies to GXM, there was little or no phagocytosis of *C. neoformans* by alveolar or peritoneal macrophages from either C57BL/6J or IL-10$^{-/-}$ mice, even after stimulation with IL-10. In macrophages from both C57BL/6J and IL-10$^{-/-}$ mice that were not treated with IL-10, phagocytosis was greatly increased after addition of IgG1, IgG2a, and IgG2b capsule-specific antibodies and there was a small but detectable increase in phagocytosis with IgG3 (Fig. 7A). However, there were no significant differences in phagocytosis by macrophages from C57BL/6J mice

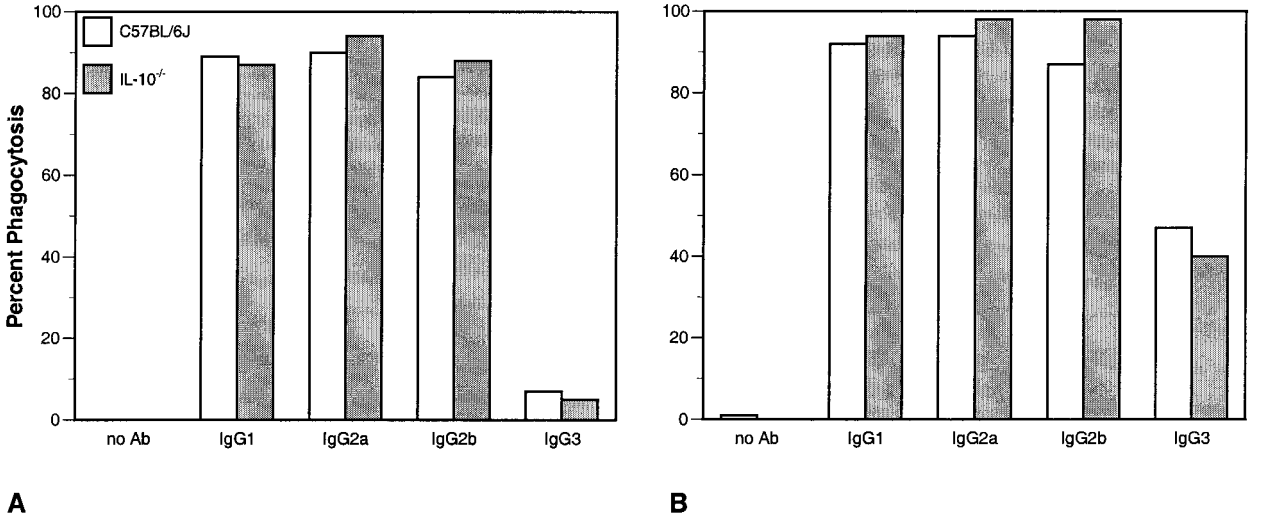

FIG. 7. Phagocytic indices of peritoneal macrophages from C57BL/6J and IL-$10^{-/-}$ mice pretreated overnight without (A) or with (B) recombinant murine IL-10 (2 ng/ml) and then incubated with either IgG1, IgG2a, IgG2b, or IgG3 3E5 MAbs to GXM and heat-killed *C. neoformans* for 4 h. This experiment was repeated three times, once with alveolar macrophages, with similar results.

and IL-$10^{-/-}$ mice in the presence of antibodies. The addition of IL-10 in vitro increased phagocytosis in both C57BL/6J and IL-$10^{-/-}$ macrophages to equivalent degrees (Fig. 7B). Stimulation with IL-10 increased the phagocytosis of IgG3-treated organisms, but there were no discernible differences in the phagocytic properties of IL-$10^{-/-}$ macrophages that explained either the resistance of IL-$10^{-/-}$ mice to cryptococcal infection or the enhancement of infection by addition of IgG1, IgG2b, or IgG2a MAbs.

DISCUSSION

There is a consensus that a Th1-driven cell-mediated response is necessary for the control of *C. neoformans* infections (reviewed in reference 10). This is consistent with the suggestion that this organism is a facultative intracellular pathogen (22). In this regard, the effective tissue response is granulomatous inflammation, which is dependent on a T-cell immune response. Numerous studies have established that T-cell deficiencies correlate with significantly enhanced susceptibility to cryptococcal infection (27, 31, 32, 81). In contrast, a role for humoral immunity in protection against infection has been more difficult to establish, and, in fact, Th2 responses are ineffective in clearing cryptococcal infection from the lung and brain (30, 34, 45). However, several studies have shown that administration of antibodies directed against the cryptococcal capsular polysaccharide can modify the course of infection to the benefit of the host by prolonging survival, clearing serum antigen, and, in some cases, reducing fungal burden (17, 18, 23, 50–54, 81, 82). Antibody efficacy has been shown to depend on such characteristics as isotype and specificity (51, 61, 79, 82) and on host factors such as T-cell immune function, the presence of IFN-γ, and Fc receptor competence (80, 81).

The different efficacies of IgG3 and the other IgG isotypes, the unexplained requirement for $CD8^+$ T cells in the IgG3-mediated enhancement of infection, and the need for IFN-γ in antibody-mediated modulation of infection suggested that cytokines might be playing a broader role than just facilitating the phagocytosis and killing of the organism by macrophages. To begin to define that broader role, we have now evaluated the contribution of cytokines IL-4, IL-6, IL-10, and IL-12 to host defense and to passive antibody efficacy with variable-region-identical MAbs representing the four murine IgG isotypes. We first examined the course of cryptococcal infection in mice deficient in IL-12, IL-6, IL-4, and IL-10 in comparison to that in C57BL/6J mice. One of the primary effects of IL-12 is to promote production of IFN-γ, which is an essential cytokine for defense against cryptococcosis (15, 29, 35, 36). IL-$12^{-/-}$ mice have impaired Th1 responses with decreased levels of IFN-γ (46) and are susceptible to diseases where IFN-γ plays an important protective role, such as infections with mycobacteria (37), *Toxoplasma gondii* (19), *Candida albicans* (48), and *Leishmania major* (47). Our observation that IL-12-deficient mice were more susceptible to infection with *C. neoformans* is consistent with these studies and confirms a recent report by Decken et al. showing increased susceptibility of both IL-$12p35^{-/-}$ and IL-$12p40^{-/-}$ mice to cryptococcal infection (15).

IL-6 is a pleiotropic cytokine with proinflammatory effects that also appears to be important in generating Th2 responses (28, 64). However, IL-$6^{-/-}$ mice are more susceptible than wild-type mice to infection by *Listeria monocytogenes* and vaccinia virus (14, 38). In both infections, Th1 responses confer resistance while Th2-associated cytokines are deleterious to the host (75). IL-6 administration reduces the severity of intracerebral *C. neoformans* infection (6), suggesting a role for this cytokine in host defense against cryptococcosis. Consistent with this view, we found IL-6-deficient mice to be more susceptible to cryptococcal infection.

IL-$4^{-/-}$ mice have deficient Th2 responses, increased serum IFN-γ, and decreased levels of IL-6 and IL-10 (40). Treatment with MAbs to IL-4 prolongs survival of mice infected with *C. neoformans* (34). Decken et al. have shown that (C57BL/6 × 129/Sv/Ev)F_2 IL-$4^{-/-}$ mice are significantly resistant to infection with *C. neoformans* (15). However, we found that IL-$4^{-/-}$

mice, backcrossed to C57BL/6J for at least 10 generations, were equally or only slightly more resistant to infection with *C. neoformans* than the parental strain. Other studies with IL-$4^{-/-}$ mice have given results that conflict with or do not seem to fit the Th1/Th2 paradigm. For example, while Kopf et al. found IL-$4^{-/-}$ mice on a BALB/c background to be more resistant to *Leishmania major* infection (39) than wild-type mice, Noben-Trauth et al. found these same mice to be as susceptible as wild type mice (60). In addition, IL-$4^{-/-}$ mice are more susceptible to *Toxoplasma gondii* infection where IFN-γ is necessary for protection (65). Our differences with Decken et al. (15) may be attributable to mouse strain, cryptococcal strain and inoculum, or both. In addition, while IL-4 is considered a prototypic Th2 cytokine, the lack of resistance by IL-$4^{-/-}$ mice to infections that require Th1 responses for protection observed by us and others is consistent with recent studies showing that endogenous IL-4 is needed to effectively sustain a protective Th1 response in candidal infection (49) and that IL-$4^{-/-}$ mice have an impaired ability to produce IFN-γ in the later stages of *Toxoplasma gondii* infection (72).

IL-10-deficient mice were significantly more resistant than the parental strain to *C. neoformans* infection (5; this paper). IL-10 is secreted by T cells, macrophages, and B1 cells and is commonly classified as a Th2-associated cytokine. While most murine studies show that IL-10 inhibits Th1, but not Th2, responses, studies with human cells indicate that IL-10 can also inhibit production of Th2-associated cytokines (16, 63, 67, 78). In addition, the observations that in mice IL-10 downregulates IL-5 production (83) and that it inhibits CD86 expression (24) suggest a broader regulatory capacity for IL-10 in humans as well as mice, and the current view is that IL-10 is a negative regulator of inflammation that acts by inhibiting release of both Th1 and Th2 proinflammatory cytokines (2, 58, 70). The intrinsic resistance of IL-$10^{-/-}$ mice to cryptococcal infection is consistent with recent reports that these mice are also protected against other infections that require a Th1 response for protection, such as those with *Candida albicans* (77), *Mycobacterium tuberculosis* (59), and *Listeria monocytogenes* (13). However, other studies with IL-$10^{-/-}$ mice illustrate the difficulty in predicting results based on the Th1/Th2 paradigm because these mice die rapidly from infection by the intracellular parasites *Toxoplasma gondii* (26) and *Trypanosoma cruzi* (33) due to systemic overproduction of inflammatory mediators such as IFN-γ and tumor necrosis factor alpha (TNF-α). We conclude that, despite the inadequacies of the Th1/Th2 model in reconciling certain findings (1, 58), our observations concerning the course of *C. neoformans* infection in cytokine-deficient mice are consistent with the belief that a Th1-driven cell-mediated response is critical for host defense against *C. neoformans*.

To our knowledge, the effects of Th1 and Th2 cytokines on antibody efficacy have not been evaluated. Passive-antibody-protection experiments have consistently shown that IgG1, IgG2a, and IgG2b are protective against cryptococcal infection in several different mouse strains including BALB/c and C57BL/6J (53, 81). Our earlier experiments with different mouse strains seem to suggest that passive antibodies are protective under both Th1 and Th2 conditions. More recently, we provided evidence that antibody-mediated protection against *C. neoformans* is dependent on cell-mediated immune responses and requires IFN-γ (81). Consistent with this finding, we observed a lack of antibody-mediated protection in IL-$12^{-/-}$ mice. However, none of the IgG isotypes were protective in mice deficient in IL-4, IL-6, and IL-10, indicating that these Th2-associated cytokines are as important for antibody-mediated protection as prototypic Th1 cytokine IFN-γ.

Probably our most dramatic finding is the observation that antibody administration greatly enhanced *C. neoformans* infection in the otherwise resistant IL-10-deficient mice. Since IL-10 upregulates macrophage expression of FcγRI and increases phagocytic capacity of macrophages (73), we considered the possibility that yeast cells were not being avidly phagocytosed in these mice. However, in vitro phagocytosis studies showed no significant difference between IL-$10^{-/-}$ and C57BL/6J mouse macrophages and organisms were seen within macrophages and Kupffer cells in the antibody-treated IL-$10^{-/-}$ mice. Given the potent anti-inflammatory role of IL-10, it is possible that the decreased survival we observed in the antibody-treated IL-$10^{-/-}$ mice resulted from increased inflammation induced by immune complexes formed when the exogenous antibody to GXM was administered. Immune complexes induce the release of many proinflammatory cytokines such as IL-1β, IL-6, and TNF-α, as well as IL-10 and vasoactive substances such as platelet-activating factor (3, 4, 74). In the absence of the inhibition of inflammation that is usually caused by IL-10, these mediators may act unopposed, thereby causing intense inflammatory damage to the host. As described in Results, all IL-$10^{-/-}$ mice showed more-abundant inflammatory infiltrates near infectious foci than did C57BL/6J mice. We did not, however, see evidence on histopathology of further increased inflammation in antibody-treated IL-$10^{-/-}$ mice compared to that in control IL-$10^{-/-}$ mice. While there were three to seven times more organisms in the spleens and livers of the IgG1-treated IL-$10^{-/-}$ mice than in controls that did not receive the antibody, there were no significant differences in the CFU in the mice treated with the other isotypes. This suggests that the enhancement of infection in the antibody-treated mice was not due to a decrease in the ability of effector cells to kill the organism in vivo in the antibody-treated IL-$10^{-/-}$ mice.

While human immunodeficiency virus (HIV) infection alters cytokine balance, the profile is more complex than polarization to Th1 or Th2 (21). Increased levels of IL-10 are associated with progressive HIV infection (69, 71). Lortholary et al. have shown that AIDS patients with disseminated cryptococcosis have elevated levels of IL-10 and TNF-α (44). While it is simplistic to look at any one factor, these studies suggest that AIDS patients with cryptococcosis are likely to have IL-10 levels that would be deleterious in cryptococcal infection but that would not appear to negatively impact passive-antibody therapy. As such, our studies provide further support for the current phase I evaluation of the murine IgG1 MAb in conjunction with antifungal therapy for the treatment of cryptococcal meningitis in AIDS patients (7). Our results also suggest that it may be important to evaluate the underlying cytokine milieu of the host before embarking on antibody therapy for *C. neoformans*. However, with further insight into the interplay of various cytokines and passive-antibody treatment, it may be possible to shift the cytokine balance to maximize the efficacy of antibodies and favorably to improve therapeutic outcome.

The Th1/Th2 paradigm has proven useful as a framework to

predict the host response to certain infections and to investigate fundamental immunologic pathways (66). However, the discrimination is an artificial one that does not apply universally (1). As our understanding of immunologic phenomena becomes increasingly sophisticated, this duality will likely become less useful as a construct for understanding the immune system. In fact, our results provide strong support for the current view that Th1-associated responses are necessary for the control of *C. neoformans* infection. However, our observations from passive-antibody studies in cytokine-deficient mice highlight a previously unsuspected dependence on the ability of the host to mount a Th2-associated response. The studies described here have alerted us to the fact that we do not understand the detailed mechanisms through which antibodies modulate cryptococcal infection in mice. It is remarkable that, although the humoral immune response has been intensely studied for over a century, we are just beginning to understand the elements involved in mediating antibody protection. Since a mouse MAb is currently being used in combination with antifungal agents to treat AIDS patients with chronic cryptococcal infection, we have used a mouse model in which the organism is administered intravenously to mimic the hematogenous spread that occurs in such patients. We have shown that the absence of both Th1 and Th2 cytokines affects the ability of antibodies of different isotypes to modify cryptococcal infection. One of the goals of treating with both antibodies and antifungals is to lower the fungal burden so that even an immunodeficient host might eradicate the organism. The administered antibodies also form antigen-antibody complexes with the organism and the shed capsular polysaccharide, which should activate both innate and adaptive immune responses and which may be protective or enhancing depending on the cytokine environment of the host. Our observations illustrate the need for additional studies to understand the variables that determine antibody efficacy. For example, antibody-treated IL-10-deficient mice should be examined for the levels of cytokines in their tissues and wild-type mice should be acutely depleted of IL-10. In addition, the cell-mediated and humoral immune responses of IL-$10^{-/-}$ mice subsequent to antibody administration should be studied. Such studies would make it less likely that unexpected defects in the development of the immune response or in the physiology of genetically defective mice are responsible for the results we have observed. In addition, detailed in vitro assays with T cells and macrophages from infected and antibody-treated mice would allow us to begin to understand the mechanisms involved. Such studies are all the more important because the results reported here may also be relevant to vaccine protocols, in particular those evaluating antibody responses using adjuvants that shift the response to Th1, as our findings suggest that the absence of IL-4 or IL-10 can neutralize antibody efficacy. Manipulation of cytokines in such systems will allow us to establish whether the protective efficacy of endogenous antibody responses is similarly affected by defects in the cytokine network.

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