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Antibody-Mediated Protection against *Cryptococcus neoformans* Pulmonary Infection Is Dependent on B Cells

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The pathogenesis of pulmonary *Cryptococcus neoformans* infection and the efficacy of passive immunoglobulin G1 (IgG1) administration were investigated in B-cell-deficient and C57BL/6J mice. C57BL/6J mice lived longer than B-cell-deficient mice after both intratracheal and intravenous infections. Administration of IgG1 prior to infection prolonged the survival of C57BL/6J mice but had no effect on the survival or numbers of CFU in the lungs of B-cell-deficient mice. *C. neoformans* infection in B-cell-deficient mice resulted in significantly higher levels of gamma interferon (IFN-γ), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α) than in C57BL/6J mice. IgG1 administration reduced IFN-γ and MCP-1 levels in C57BL/6J mice but not in B-cell-deficient mice. In addition, compared to its effect in C57BL/6J mice, *C. neoformans* infection in FcRγIII-deficient, athymic, and SCID mice significantly increased IFN-γ and MCP-1 levels. IgG1 administration was associated with reduced IFN-γ levels in C57BL/6J mice but not in FcRγIII-deficient, athymic, and SCID mice. These observations suggest that IgG1-mediated protection in this system is a consequence of alterations in the inflammatory response that translate into less damage to the host without directly reducing the fungal burden. For hosts with impaired immunities, the ineffectiveness of passive antibody (Ab) may reflect an inability to down-regulate inflammation and avoid self-damage. The results indicate an important role for B cells in host defense against *C. neoformans* infection and demonstrate a surprising dependence of Ab-mediated protection on B cells in this system.

The role of humoral immunity to *Cryptococcus neoformans*, an encapsulated fungal pathogen that is a relatively frequent cause of meningoencephalitis in immunocompromised patients, has historically been uncertain because of the difficulty in consistently protecting against experimental infection by passive administration of immune sera (11). Additionally, it has been difficult to unequivocally establish a role for B cells in the defense against fungal pathogens, since most studies have shown no differences in the outcomes of fungal infections in B-cell-deficient and normal mice (2, 41, 57). A breakthrough in this field occurred with the use of monoclonal antibodies (MAbs) to investigate questions related to Ab protection (17). Unlike the experiments with immune sera, MAbs have produced more-consistent results because they are constant reagents of a defined specificity and isotype. Several groups have now shown that passive administration of Ab specific for the capsular polysaccharide can modify the course of *C. neoformans* infection in mice (17, 21, 26, 37, 42–45, 50). This discovery has led to a new paradigm, one which accepts that some Abs can protect against fungi but which leaves the role of natural humoral immunity uncertain, since whether Ab responses during infection are protective is unknown. Some serological studies of human and mouse Abs provide strong circumstantial evidence that natural Ab responses contribute to protection (18, 25), while other studies suggest that Ab responses to infection are dominated by nonprotective Abs (62). The strategy of evaluating the potential of humoral immunity by testing individual MAbs has now been applied to other pathogens, but the role of natural Ab responses to these pathogens is also uncertain. MAb-mediated protection against a variety of pathogens, including *Mycobacterium tuberculosis* (53), *Listeria monocytogenes* (19), *Candida albicans* (29), *Histoplasma capsulatum* (46), and *Ehrlichia chaffeensis* (58), has now been demonstrated. However, generating MAbs does not always guarantee success, as illustrated with *Blastomyces dermatitidis*, for which the administration of MAb did not prolong survival and may even have been disease enhancing (60).

Although a consensus has emerged that certain Abs are protective against intracellular pathogens when administered before infection (12), the role of B cells in host defense remains obscure. B cells play a complex role in both the protection and the pathogenesis of infection against intracellular pathogens (12). *M. tuberculosis* infection in B-cell-deficient mice resulted in higher organ burdens of mycobacteria (56). However, Bosio et al. demonstrated that there is no difference between fungal burdens in cases of delayed inflammation and delayed extrapulmonary dissemination in B-cell-deficient mice (8). Furthermore, Allendorfer et al. demonstrated that there is no difference between B-cell-sufficient and B-cell-deficient mice during *H. capsulatum* infection (2). Additionally, B-cell-deficient mice infected with *C. albicans* are resistant to mucosal infection but susceptible to systemic infection (57). Hence, there is collective evidence that B cells play a complex role during infection with any of a variety of organisms.

The role of T cells in protection against *C. neoformans* is well established (31, 32). However, establishing the role of B cells in infection against *C. neoformans* has been more difficult. An early study using Ab-mediated depletion of B cells showed no difference between the susceptibility of mice to *C. neoformans*

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infection and that of controls whose B cells were not depleted (41). Aguirre and Johnson were able to document an effect that suggested a role for B cells against *C. neoformans* for conditions under which both T- and B-cell functions were impaired and B cells were reconstituted from normal mice (1). Our study reexamined the susceptibility of B-cell-deficient mice to pulmonary *C. neoformans* infection and found enhanced susceptibility relative to that of wild-type mice. Furthermore, we evaluated the efficacy of passive Ab administration against *C. neoformans* in B-cell-deficient mice and found that Ab administration was ineffective in prolonging survival. Our results suggest that B cells contribute to host defense against *C. neoformans* and reveal the dependence of Ab efficacy on B cells for this infection.

(The data in this paper are from a thesis submitted by J. Rivera in partial fulfillment of the requirements for the degree of doctor of philosophy in the Sue Golding Graduate Division of Medical Science, Albert Einstein College of Medicine, Yeshiva University, Bronx, N.Y.)

MATERIALS AND METHODS

***C. neoformans*.** Strain ATCC 24067 (serotype D) (American Type Culture Collection, Manassas, Va.) was grown from frozen stock in Sabouraud's dextrose broth (Difco, Detroit, Mich.) at 30°C for 48 h. This strain was selected because it has been used extensively in Ab studies and is well characterized (27). Inoculum was confirmed by counting CFU on Sabouraud's dextrose agar (Difco).

Abs. MAb 2H1 and 18B7 are immunoglobulins G1 (IgG1), and both bind the glucuronoxylomannan (GXM) component of *C. neoformans* capsular polysaccharide (44) and are protective against *C. neoformans* infection (21, 43–45; A. Casadevall, 1998, no. 230). Murine IgG1 ascites fluid was prepared by injecting hybridoma cells into the peritonea of pristine-primed BALB/c mice. NSO is the nonproducing mouse myeloma fusion partner of the IgG1 hybridomas, and ascites fluid produced using this cell line was used in some experiments as a negative control for survival experiments. For some experiments, MAbs were purified from either ascites fluid or hybridoma supernatants by use of protein G affinity chromatography (Pierce, Rockford, Ill.) as instructed by the manufacturer, and phosphate-buffered saline (PBS) was used as the control. Purified MAb 18B7 was used only for cytokine experiments with FcRγIII mice.

Mice. Six- to eight-week-old female C57BL/6-Igh-6^{tm1Cgn} (B-cell-deficient, μMT$^{-/-}$), C57BL/6J, and FcRγIII-deficient mice (C57BL/6J genetic background) were obtained from the Jackson Laboratory (Bar Harbor, Maine). B-cell-deficient mice were generated by targeted disruption of the membrane exon of the immunoglobulin μ chain gene, and as a result, they possess no mature B cells and are unable to produce Abs (34). Additional 6- to 8-week-old female BALB/c, athymic nude (BALB/c genetic background), and SCID/NCr (BALB/c genetic background) mice were obtained from the National Cancer Institute (Bethesda, Md.). All mice were housed in sterile microisolator cages in a barrier environment. These mice were kept in a specific-pathogen-free barrier facility in microisolator cages, fed irradiated rodent food, provided with autoclaved bedding, and routinely monitored for serologic evidence of exposure to common murine pathogens. The results of all routine serological testing for various murine pathogens were negative.

Survival analysis. Six- to eight-week-old B-cell-deficient and C57BL/6J mice were infected intravenously (i.v.) with 10^5 *C. neoformans* cells and intratracheally (i.t.) with 10^6 *C. neoformans* cells as previously described (21, 45). One milligram of MAb was administered intraperitoneally 24 h prior to infection. Mice were monitored daily for mortality and morbidity. Surviving mice were killed, their lungs were removed, and their upper right lobes were fixed in 10% buffered formalin (Fisher, Pittsburgh, Pa.) for histological examination.

CFU experiments. B-cell-deficient and C57BL/6J mice were given MAb and infected as described above. Mice infected with 10^5 or 10^6 yeast cells were sacrificed at day 28 or 21 postinfection, respectively. BALB/c, athymic, and SCID mice were infected as described above with 10^6 yeast cells and sacrificed on day 14. Numbers of CFU in organs were determined by homogenizing the tissue and plating it on Sabouraud's agar, as described previously (21). Paraffin-embedded lung tissue sections were stained with hematoxylin and eosin or mucicarmine for histological examination.

GXM levels and clearance. GXM clearance experiments were performed as described previously (36). Briefly, B-cell-deficient and C57BL/6J mice were treated with 1 mg of IgG1 intraperitoneally 24 h prior to i.v. administration of GXM (50 μg). Blood was obtained from the orbital sinus immediately before MAb administration and 2, 6, 16, 22, 46, 70, and 94 h after i.v. GXM administration. Serum was isolated by centrifugation of blood. Mice were sacrificed by cervical dislocation, and their organs were removed immediately. Organs were homogenized (Ultra Turrax T25 homogenizer; Janke and Kunkel, Staufen, Germany), and organ homogenate and serum were treated with proteinase K (1 mg/ml, 1 h, 37°C). A GXM-capture enzyme-linked immunosorbent assay (ELISA) was used to detect GXM in the sera, lungs, livers, spleens, and kidneys of mice as previously described except that MAb 2D10 was used for capture and MAb 2H1 was used for detection (13).

Histological examination. Formalin-fixed, paraffin-embedded tissues at 21 and 28 days after infection were examined with hematoxylin-eosin and mucicarmine for evaluation of histopathology and fungal distribution.

Cytokine and chemokine studies. B-cell-deficient, C57BL/6J, BALB/c, athymic, SCID, and FcRγIII-deficient mice were infected as described above. Mice were given IgG1 or PBS 24 h prior to infection with yeast. Sham-infected groups were given IgG1 or PBS 24 h prior to i.t. administration of PBS. Mice were sacrificed at day 14 postinfection (day 7 for FcRγIII-deficient mice), and their right lungs were homogenized in 2 ml of PBS in the presence of protease inhibitors (Complete Mini; Boehringer Mannheim, Indianapolis, Ind.). The homogenates were centrifuged at 6,000 × *g* for 10 min to remove cell debris, and the supernatant was frozen at −80°C until it was tested. The supernatants were assayed for concentrations of interleukin 2 (IL-2), IL-4, IL-6, IL-10, IL-12 p70, monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein 1α (MIP-1α) by use of ELISA kits (Pharmingen, San Diego, Calif., and R&D Systems, Inc., Minneapolis, Minn.). Samples were diluted with 1% fetal bovine serum in PBS. The detection limits of cytokine assays were 3.1 pg/ml for IL-2, 7.8 pg/ml for IL-4, 15.6 pg/ml for IL-6 and tumor necrosis factor alpha (TNF-α), 31.3 pg/ml for IL-10 and alpha interferon (IFN-α), and 62.5 pg/ml for IL-12 p40, as stated by the manufacturer. The detection limits of the chemokine assays were 4.7 pg/ml for MIP-1α and 15.6 pg/ml for MCP-1, as determined by the manufacturer. The serum obtained from CFU and GXM experiments was assayed for IgE concentrations by ELISA, as described by the manufacturer (Pharmingen).

Endotoxin precautions. For the cytokine and cellular-response experiments, great care was taken to avoid contamination with endotoxins. One person did all the work involving the purification of MAb reagents and handling of reagents in a laminar-flow hood. Solutions were made with endotoxin-free water or PBS. Extensive use was made of disposable pyrogen-free plastic ware, pipettes, pipette tips, and microcentrifuge tubes, etc. The endotoxin concentration in MAb solutions measured by the *Limulus* amoebocyte assay (BioWhittaker, Walkersville, Md.) was below the limit of detection of the assay.

Preparation of lung leukocytes. B-cell-deficient and C57BL/6J mice were given MAb and infected as described above. At day 14 postinfection, lungs were excised, minced, homogenized by use of a sterile 70-μm-pore-size nylon mesh (Becton Dickinson, Paramus, N.J.), and digested for 60 min with a 10-ml/lung concentration of digestion buffer containing RPMI medium, 10% fetal calf serum, 1 mg of collagenase (Boehringer Mannheim, Chicago, Ill.)/ml, and 30 μg of DNase I (Sigma, St. Louis, Mo.)/ml. The total cell suspension was collected by centrifugation, and erythrocytes were lysed. Briefly, erythrocytes were lysed by resuspension in ice-cold 0.17 M NH_4Cl on ice for 10 min. A 10-fold excess of RPMI solution was then added to make the solution isotonic, the cells were collected by centrifugation and suspended in staining solution (PBS, 1% fetal bovine serum), and live cells (trypan blue exclusion) were counted in a hemocytometer chamber.

Cell staining and FACS analysis. Neutrophils, eosinophils, lymphocytes, and monocytes/macrophages were visually counted from Giemsa-stained samples of lung cell suspensions centrifuged onto glass slides (cytospin; Shandon, Pittsburgh, Pa.). Approximately 200 to 400 cells were counted from randomly chosen high-power microscope fields for each sample. For fluorescence-activated cell sorter (FACS) analysis, lung leukocytes (10^6) were stained for 30 min on ice with 100 μl of one of the following Abs diluted in staining buffer: R-phycoerythrin-labeled anti-CD45 (2 μg/ml), fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 (5 μg/ml), Cy-Chrome-labeled anti-mouse CD8 (2 μg/ml), FITC-labeled anti-mouse CD19 (5 μg /ml), or FITC-labeled anti-mouse MAC-3 (5 μg/ml) (all MAbs were from Pharmingen). The samples were washed twice in staining buffer and fixed in 1% paraformaldehyde. Stained samples were stored in the dark at 4°C until they were analyzed on a Calibur FACscan flow cytometer (Becton Dickinson, Mountainview, Calif.) with CELLQuest (Becton Dickinson)

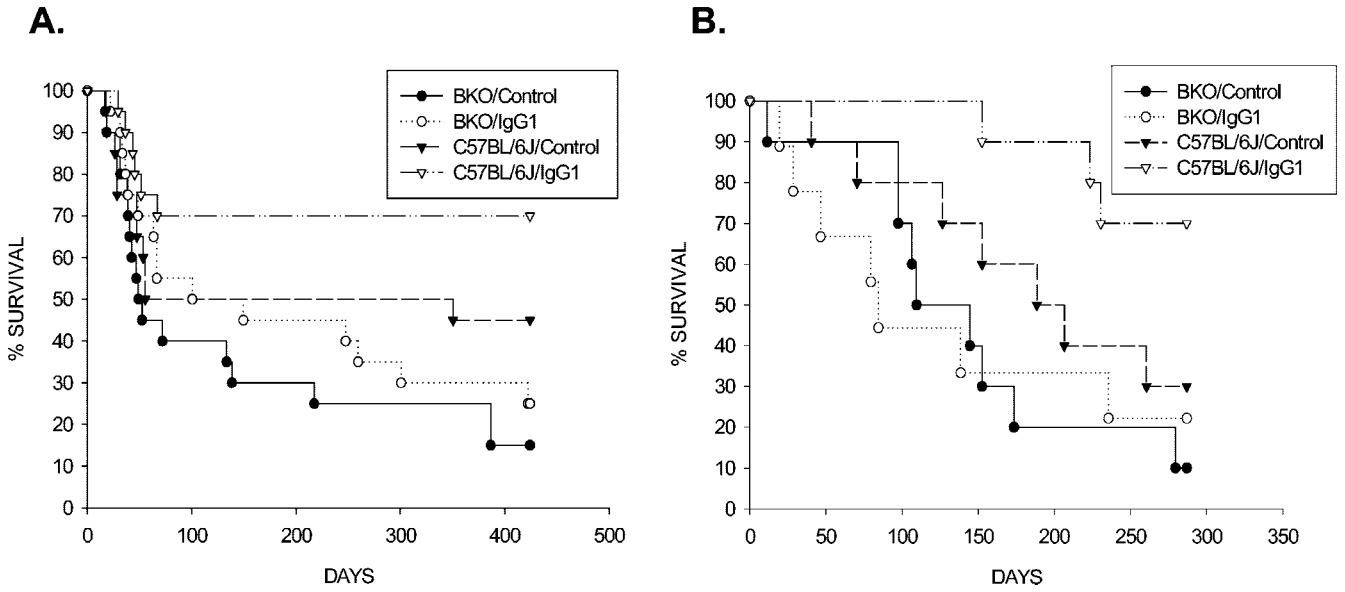

FIG. 1. Passive IgG prolongs the survival of C57BL/6J but not B-cell-deficient mice. (A) Survival following i.t. infection with 10^6 *C. neoformans* cells. This experiment was done twice, and results are representative of pooled data from two experiments (20 mice in each group). (B) Survival of C57BL/6J mice (10 mice) and B-cell-deficient mice (10 mice) following i.v. infection with 10^5 *C. neoformans* cells. BKO, B-cell-deficient mice.

software. $CD45^+$ cells within the appropriate forward and side scatter gates were analyzed. Controls consisted of isotype-matched irrelevant Abs.

Statistics. All data were analyzed by the Student *t* test, the Kruskal-Wallis test, a one-way analysis of variance (ANOVA) (primer; McGraw-Hill, New York, N.Y.), and a log rank analysis (Sigmastat, Chicago, Ill.).

RESULTS

Survival. C57BL/6J mice infected i.t. with *C. neoformans* survived longer than B-cell-deficient mice (median survival times of 56 and 49 days, respectively; $P = 0.055$) (Fig. 1A). Two experiments were done to assess the impact of passive IgG1 on the course of i.t. infection in B-cell-deficient and C57BL/6J mice. Both experiments independently showed a trend towards the prolonged survival of C57BL/6J mice treated with IgG1 and no effect on B-cell-deficient mice (median survival time of 100 days; $P = 0.30$). Combining the data made more clear the existence of a trend towards the prolonged survival of C57BL/6J mice treated with IgG1 (median survival time of >424 days) relative to that of C57BL/6J control mice, but this difference was not significant at the 0.05 level ($P = 0.11$). Since differences in mortality between groups take over a year to become evident when C57BL/6J mice are infected i.t., we opted to also evaluate survival with an i.v. model of infection. C57BL/6J mice infected i.v. with *C. neoformans* survived longer than B-cell-deficient mice (median survival times of 189 and 110 days, respectively; $P = 0.057$) (Fig. 1B). Administration of IgG1 significantly prolonged the survival of *C. neoformans*-infected C57BL/6J mice (median survival time of >287 days) relative to that of C57BL/6J control mice ($P = 0.05$), whereas IgG1 administration did not prolong the survival of B-cell-deficient mice (median survival time of 107 days; $P = 0.722$) (Fig. 1B). The experiment was terminated on day 287, at which time all surviving mice (three C57BL/6J control mice, seven C57BL/6J IgG1-treated mice, one B-cell-deficient control mouse, and two IgG1-treated B-cell-deficient mice) had detectable lung CFU, indicating chronic infection.

Fungal burden. To further understand the differences between survival rates and the failure to protect with IgG1 in B-cell-deficient mice, we examined lung and brain fungal burdens. Two independent CFU experiments are summarized in Table 1. At day 28, there was no difference between the lung

TABLE 1. Numbers of CFU in the organs of C57BL/6J and B-cell-deficient mice infected with *C. neoformans*[a]

C. neoformans infection (no. of organisms)	Group (no. of mice)	Mean no. of CFU ($\log_{10}$) in specified organs of mice ± SD			
		$\mu MT^{-/-}$		C57BL/6J	
		Brain	Lung	Brain	Lung
10^5	Control (10)	2.84 ± 0.66	6.41 ± 1.35	2.92 ± 0.83	7.10 ± 0.12
	IgG1 (10)	2.25 ± 0.40	6.90 ± 0.68	2.90 ± 1.56	7.18 ± 0.26
10^6	Control (9)	4.30 ± 0.97	7.51 ± 0.19[b]	4.24 ± 1.26	7.04 ± 0.36[b]
	IgG1 (9)	3.72 ± 1.47	7.28 ± 1.13	3.34 ± 1.59	7.10 ± 0.77

[a] $\mu MT^{-/-}$ and C57BL/6J mice received 1 mg of IgG1 or control MAb 24 h prior to infection with 10^5 or 10^6 *C. neoformans* cells. Mice were killed 28 and 21 days later, respectively. Statistical analysis was performed using the Kruskal-Wallis test and the Student *t* test.

[b] Compared with the value for C57BL/6J control mice, *P* was 0.003.

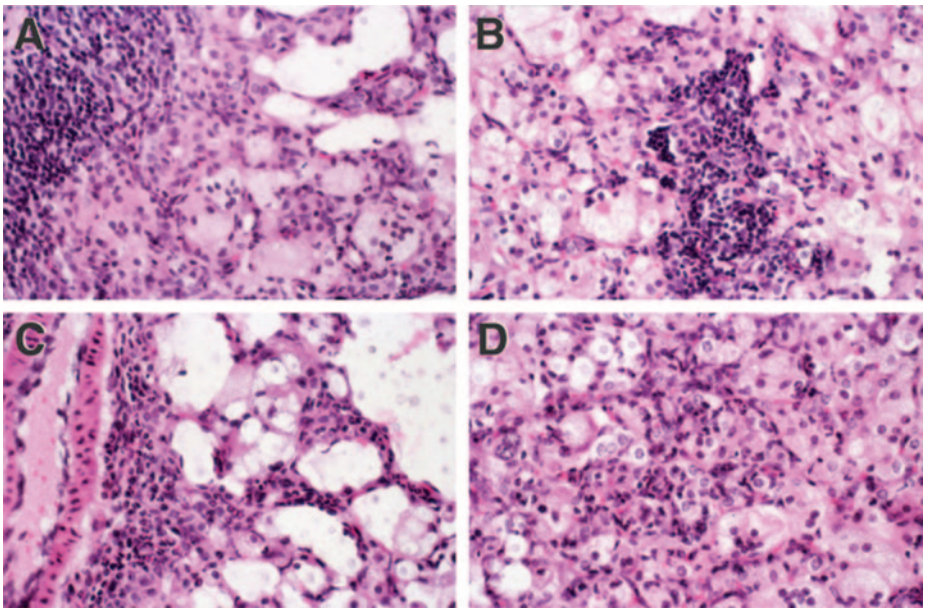

FIG. 2. Histology of lungs following i.t. infection with *C. neoformans* after staining with hematoxylin and eosin. (A) Control treatment C57BL/6J mice on day 21. Large collections of extracellular organisms are seen in airspaces, with little inflammation. (B) IgG1-treated C57BL/6J mice on day 21. Granulomatous inflammation is seen with fewer cryptococci in airspaces. The inflammatory cells include polymorphonuclear leukocytes, lymphocytes, epithelioid cells, and macrophages. (C) Control treatment B-cell-deficient mice on day 21. There are collections of yeast cells in air spaces and little inflammation. (D) IgG1-treated B-cell-deficient mice on day 21. Extensive granulomatous inflammation is associated with small collections of yeast cells. Original magnification, ×50.

fungal burdens of C57BL/6J and B-cell-deficient mice infected with 10^5 yeast cells. The administration of IgG1 did not reduce the numbers of CFU in the lungs or brains of B-cell-deficient or C57BL/6J mice. IgG1 administration had no effect on the numbers of CFU in the brains of B-cell-deficient or C57BL/6J mice. Infection with 10^6 *C. neoformans* cells resulted in higher numbers of lung CFU in B-cell-deficient control mice than in C57BL/6J control mice (Table 1). In addition, we examined lung and brain fungal burdens in athymic and SCID mice (BALB/c genetic backgrounds). IgG1 administration to athymic mice before infection had a modest effect in reducing the numbers of lung CFU ($P < 0.05$) but no effect on the numbers of brain CFU ($P = 0.224$). IgG1 administration to SCID and BALB/c mice had no effect on the numbers of brain and lung CFU.

Serum GXM levels, clearance, and distribution. There was a trend toward lower serum GXM levels in C57BL/6J mice than in B-cell-deficient mice, but this difference was not significant ($P > 0.05$). Serum GXM was rapidly cleared in both B-cell-deficient and C57BL/6J mice given IgG1 (data not shown). The overall patterns of GXM clearance were similar in B-cell-deficient and C57BL/6J mice (data not shown) and comparable to that reported previously (36).

Histological analysis. At day 28, the lungs of C57BL/6J control mice exhibited minimal inflammation, with large extracellular collections of *C. neoformans* cells in the alveolar spaces (Fig. 2A). In contrast, the lungs of C57BL/6J mice treated with IgG1 exhibited more-organized inflammation composed of macrophages and epithelioid cells and fewer extracellular collections of fungal cells (Fig. 2B). Perivascular cuffs composed of lymphocytes and polymorphonuclear leukocytes were also present. The lungs of B-cell-deficient mice exhibited minimal inflammatory responses compared to those of MAb-treated B-cell-deficient mice (Fig. 2C and D). A mixed inflammatory infiltrate composed of polymorphonuclear leukocytes, lymphocytes, macrophages, and epithelioid cells was present in the perivascular spaces and parenchyma. The lungs of infected B-cell-deficient and C57BL/6J mice at days 14 and 21 postinfection revealed similar histological findings (data not shown).

Cytokine and chemokine studies. To better understand immune responses in B-cell-deficient and C57BL/6J mice, levels of cytokine and chemokine expression were examined. At day 14 postinfection, B-cell-deficient mice had significantly higher pulmonary concentrations of IFN-γ than C57BL/6J mice irrespective of IgG1 treatment (Fig. 3, upper panel). B-cell-deficient mice also had significantly higher concentrations of MCP-1 and MIP-1α than C57BL/6J mice. MCP-1 and MIP-1α are induced in the lungs of B-cell-deficient and C57BL/6J mice infected with *C. neoformans* (Fig. 3, middle and lower panels). Additional cytokines were measured (IL-2, IL-6, IL-10, and TNF-α), but no differences were noted between infected B-cell-deficient and C57BL/6J mice (Table 2). Notably, MAb decreased IFN-γ and MCP-1 levels in the lungs of B-cell-deficient mice. IL-4, IL-10, and IL-12 concentrations were lower in the lungs of infected B-cell-deficient mice than in those of sham-infected B-cell-deficient mice. Similarly, IL-4 and IL-6 concentrations were lower in the lungs of infected C57BL/6J mice than in those of sham-infected mice. IgG1 administration had little or no effect on the levels of these cytokines during infection except for IL-10 and IL-12, which were increased in C57BL/6J mice (Table 2). To better understand the role of B cells in the regulation of Th1 responses, cytokines and chemokines were studied in mice with a C57BL/6J genetic background, as well as in athymic and SCID

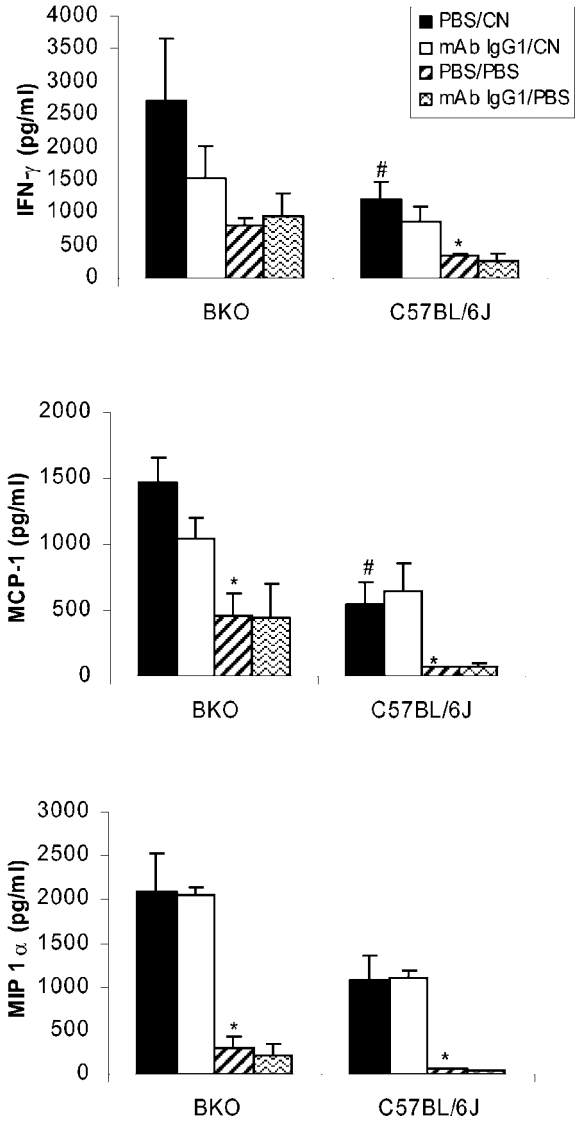

FIG. 3. Differences in levels of cytokine and chemokine expression in lungs of infected B-cell-deficient and C57BL/6J mice. Cytokine and chemokine levels were measured by ELISA at day 14 postinfection. Bars denote mean protein concentrations. In the group treated with PBS and *C. neoformans* (PBS/CN) and the group treated with IgG1 and *C. neoformans* (IgG1/CN), there were six mice each; in the group treated with PBS only (PBS/PBS) and the group treated with IgG1 and PBS (IgG1/PBS), there were three mice each. Error bars denote standard deviations. *, value is significantly different from that obtained under conditions with no IgG1; #, value is significantly different from that measured for B-cell-deficient mice. Statistics were confirmed by using one-way ANOVA. BKO, B-cell-deficient mice.

mice with a BALB/c genetic background. A prior study had shown that passive IgG1 was ineffective at mediating protection in SCID mice (61), but neither FcRγIII-deficient nor athymic mice have been studied. FcRγIII-deficient mice had significantly higher concentrations of IFN-γ in their lungs than did the C57BL/6J mice, irrespective of IgG1 treatment ($P < 0.05$). Notably, MAb treatment significantly decreased the levels of IFN-γ in FcRγIII-deficient mice ($P < 0.05$) (Fig. 4). The level of MCP-1 was significantly increased in FcRγIII-deficient mice compared to that in C57BL/6J mice ($P < 0.05$), but MAb administration had no effect. *C. neoformans* infection decreased the level of TNF-α in C57BL/6J mice but had no effect on FcRγIII-deficient mice. No differences were noted for IL-4, IL-6, and IL-10 concentrations in FcRγIII-deficient mice. Also, Ab administration had little effect on the levels of these cytokines, except for IL-6, which was decreased in C57BL/6J mice ($P < 0.001$) (data not shown). For BALB/c, athymic, and SCID mice, IgG1 administrations were associated with changes in the cytokine expression levels, but these were less pronounced than in the C57BL/6J background mice (Fig. 5). At day 14, IFN-γ and MCP-1 concentrations were increased in BALB/c, athymic, and SCID mice. Levels of TNF-α were increased in BALB/c and SCID mice ($P < 0.05$). Interestingly, IgG1 administration decreased IFN-γ and MCP-1 concentrations in BALB/c ($P = 0.04$), SCID ($P = 0.09$), and athymic ($P = 0.07$) mice. In addition, IgG1 administration decreased the level of MCP-1 in athymic mice ($P < 0.001$) and the level of TNF-α in SCID mice ($P = 0.058$). Ab administration had no effect on TNF-α levels in BALB/c and athymic mice.

Serum IgE levels. We measured serum IgE levels in infected B-cell-deficient and C57BL/6J mice in the presence and absence of IgG1. C57BL/6J mice infected with *C. neoformans* had significantly higher concentrations of serum IgE than B-cell-deficient mice (data not shown). In addition, we found a trend towards decreased serum IgE levels in IgG1-treated C57BL/6J mice, which did not reach significance. IgE levels in PBS- and IgG1-treated B-cell-deficient mice were below the level of detection by ELISA (data not shown), a result which is consistent with the inability of these mice to make immunoglobulin.

Cellular composition of lung infiltrate. Lung weights, which reflect the magnitude of the inflammatory response, GXM depositions, and levels of organ edema were similar in B-cell-deficient and C57BL/6J mice (Fig. 6A). Accordingly, we found no differences in the total numbers of cells recruited to the lungs of B-cell-deficient and C57BL/6J mice after infection (Fig. 6B). On day 14, FACS analysis of lung cells showed similar percentages of lymphocytes and macrophages in the inflammatory infiltrates of B-cell-deficient and C57BL/6J mice (Fig. 6C). However, analysis of the granulocyte composition revealed significant differences in the proportions of eosinophils and neutrophils in the inflammatory responses of B-cell-deficient and C57BL/6J mice. There were higher percentages of eosinophils in IgG1-treated B-cell-deficient mice than in IgG1-treated C57BL/6J mice ($P = 0.005$) (Fig. 6D). In contrast, no differences were seen in the granulocyte compositions of B-cell-deficient and C57BL/6J control treatment mice. C57BL/6J mice exhibited higher percentages of lymphocytes and neutrophils than B-cell-deficient mice ($P = 0.041$ and 0.048, respectively).

DISCUSSION

In this study, we used B-cell-deficient mice to reevaluate the role of B cells in the defense against pulmonary cryptococcal infection and found that B-cell-deficient mice were more susceptible than C57BL/6J mice to *C. neoformans* infection by either the pulmonary or the i.v. route. Although we noted greater mortality in B-cell-deficient mice infected by the pulmonary route, we found it difficult to obtain results with sta-

TABLE 2. Protein cytokine levels in B-cell-deficient and C57BL/6J mice 14 days after infection with *C. neoformans*[a]

Cytokine	Mouse background	Mean cytokine level ± SD (pg/ml) in mice treated with:			
		CN/PBS[b]	CN/IgG1[b]	PBS/PBS	PBS/IgG1
IL-2	μMT$^{-/-}$	708.0 ± 290.5	730.3 ± 45.5	941.3 ± 52.0	1,100.8 ± 14.4
	C57BL/6J	956.2 ± 196.7	919.4 ± 102.8	949.8 ± 39.9	807.8 ± 130.5
IL-4	μMT$^{-/-}$	823.4 ± 283.2[c]	1,081.3 ± 240.1	1,129.2 ± 29.3	1,341.0 ± 8.06
	C57BL/6J	1,493.9 ± 173.9[c,d]	1,505.5 ± 113.8	985.4 ± 7.92	884.4 ± 161.4[d]
IL-6	μMT$^{-/-}$	1,113.4 ± 390.1	1,356.7 ± 292.3	1,051.0 ± 80.6	1,004.8 ± 59.2
	C57BL/6J	988.3 ± 144.9[d]	1,169.4 ± 129.9[d]	705.9 ± 106.1[d]	550.5 ± 206.4[d]
IL-10	μMT$^{-/-}$	1,429.2 ± 372.6[e,f]	1,732.1 ± 358.2	2,355.3 ± 69.9[f]	2,135.4 ± 88.9
	C57BL/6J	1,897.4 ± 162.7[e,g]	2,210.8 ± 286.2[g]	2,104.8 ± 126.6	1,737.6 ± 585.2
IL-12	μMT$^{-/-}$	2,870.5 ± 1,124.4[h]	3,064.2 ± 380.0[f]	3,552.5 ± 148.5	4,762.5 ± 116.7[h]
	C57BL/6J	4,387.5 ± 1,355.1[i]	5,732.3 ± 1,449.9[i]	3,340.0 ± 186.7	2,736.0 ± 690.1[i]
TNF-α	μMT$^{-/-}$	833.8 ± 495.1	1,082.8 ± 31.6	1,091.7 ± 15.1	1,190.5 ± 163.4
	C57BL/6J	934.0 ± 204.5	1,044.8 ± 56.4	1,020.7 ± 24.5	1,022.7 ± 163.1

[a] μMT$^{-/-}$ and C57BL/6J mice received 1 mg of IgG1 or PBS 24 h prior to infection with 10^6 *C. neoformans* cells. Mice were killed 14 days later. Statistical analysis was performed with the Student *t* test and one-way ANOVA. Six mice were in each group except the sham-infected groups, for which three mice were used.
[b] Sham-infected mice were given PBS at the time of infection.
[c] Compared with values for C57BL/6J control-treated mice, *P* was < 0.001.
[d] Compared with values for C57BL/6J sham-infected mice, *P* was < 0.05.
[e] Compared with values for μMT$^{-/-}$ control-treated mice, *P* was equal to 0.02.
[f] Compared with values for μMT$^{-/-}$ sham-infected mice, *P* was 0.02.
[g] Compared with values for C57BL/6J MAb 2H1-treated mice, *P* was 0.056.
[h] Compared with values for μMT$^{-/-}$ sham-infected mice, *P* was 0.01.
[i] Compared with values for C57BL/6J MAb 2H1-treated mice, *P* was 0.03.
[j] Compared with values for C57BL/6J sham-infected mice, *P* was 0.056.

tistical significance by using this model because these mice are relatively resistant to *C. neoformans*, and few death events occurred even after waiting for more than 1 year for several experiments. However, B-cell-deficient mice infected i.v. lived for significantly fewer days than C57BL/6J mice. These results differ from those from an earlier study that found no difference in the susceptibilities of mice depleted of B cells and control mice (41). The discrepancy between our results and the results of the earlier study may reflect incomplete depletion of B cells with Ab and/or uncontrolled effects of antigen-Ab complexes formed during the depletion process. Furthermore, lung fungal burdens in B-cell-deficient mice were higher than those in C57BL/6J mice, thereby contributing to shortened survival.

We considered various explanations for the enhanced susceptibility of B-cell-deficient mice independent of the immunoglobulin-secreting function of B cells. B cells are known to produce several cytokines, including IL-6, IL-10, TNF-α, transforming growth factor β1 (39, 47), IL-8, MIP-1α, and MCP-1 (40, 49), and could conceivably protect against *C. neoformans* infection by modulating the inflammatory response. We found greater levels of IFN-γ, MIP-1α, and MCP-1 in the lung tissues of *C. neoformans*-infected B-cell-deficient mice than in C57BL/6J mice. In addition, we measured higher levels of IL-4 and IL-12 in C57BL/6J mice than those in B-cell-deficient mice. IL-12 is critical for effective responses against *C. neoformans* (33), whereas IL-4 is not classically associated with protection against *C. neoformans* infection but could have a role in reducing tissue damage from inflammatory responses and is necessary for Ab-mediated protection (5). IL-4 can promote B-cell isotype switching to IgE production (54). In our study, we noted an increase in serum IgE levels in infected C57BL/6J mice, consistent with a high expression of IL-4 and a Th2-polarized response.

The higher levels of proinflammatory cytokines and the more exuberant inflammatory responses in the lungs of B-cell-deficient mice than in C57BL/6J mice suggest a regulatory defect that might translate into greater tissue damage through an inappropriately vigorous Th1 response. Similar findings were reported for NOS2-deficient mice infected with *C. neoformans* (48). Studies of other systems support the idea of the presence of this regulatory defect for enhanced susceptibility to *C. neoformans* infection. B-cell-deficient mice infected with *Plasmodium chabaudi chabaudi* (35) or *Trichuris muris* (6) demonstrated increased production of IFN-γ, suggesting a polarized Th1 response. T cells from B-cell-deficient mice infected with *Leishmania major* and *Chlamydia trachomatis* manifested higher IFN-γ production than C57BL/6J mice (30, 51), whereas B-cell reconstitution of the B-cell-deficient mice suppressed IFN-γ production of T cells during the course of a *Leishmania* infection (30). *Francisella tularensis* infection in B-cell-deficient mice produced marked neutrophilia in the spleen, suggesting that B cells contribute to protection by modulating the inflammatory response (7, 20). Lungs from C57BL/6J mice treated with and without Ab and infected with *C. neoformans* had similar lung weights and similar numbers of CD4, CD8, and B lymphocytes and total numbers of inflammatory cells. However, we noted that the numbers of CD8 lymphocytes decreased in MAb-treated B-cell-deficient and C57BL/6J mice. Since CD8 lymphocytes can down-regulate inflammatory responses, this observation suggests that another mechanism with passive Ab may modify the outcome of infection. Collectively, these data suggest a B-cell-mediated regu-

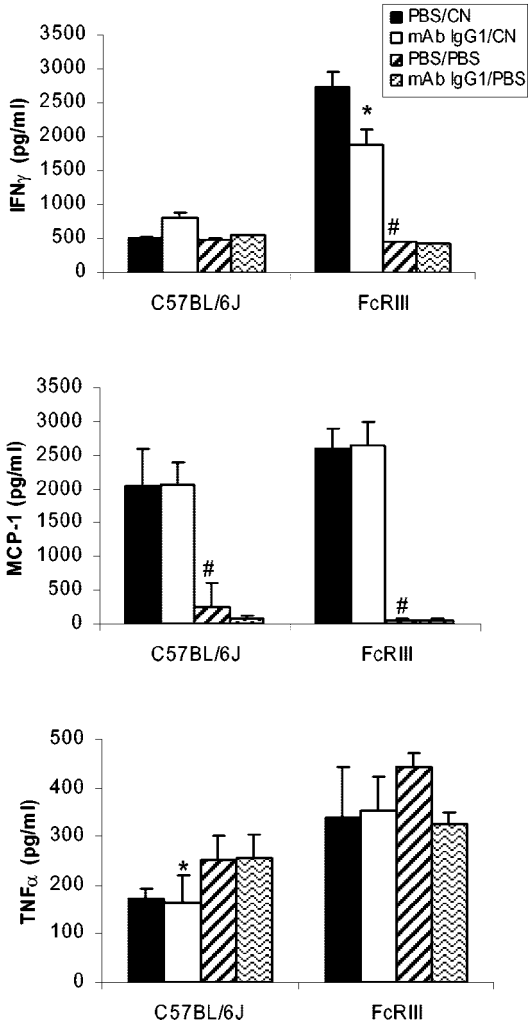

FIG. 4. Differences in levels of cytokine and chemokine expression in lungs of infected C57BL/6J and FcRγIII-deficient (FcRIII) mice. Bars denote mean protein concentrations. There were five C57BL/6J and four FcRγIII-deficient mice in the groups treated with PBS and *C. neoformans* (PBS/CN), MAb 18B7 and *C. neoformans* (MAb 18B7/CN), PBS only (PBS/PBS), and MAb 187B7 and PBS (MAb 18B7/PBS). There was a trend towards increased IFN-γ levels in IgG1-treated C57BL/6J mice, but this did not reach significance ($P = 0.277$). *, value is significantly different from that obtained under conditions with IgG1. Error bars denote standard deviations.

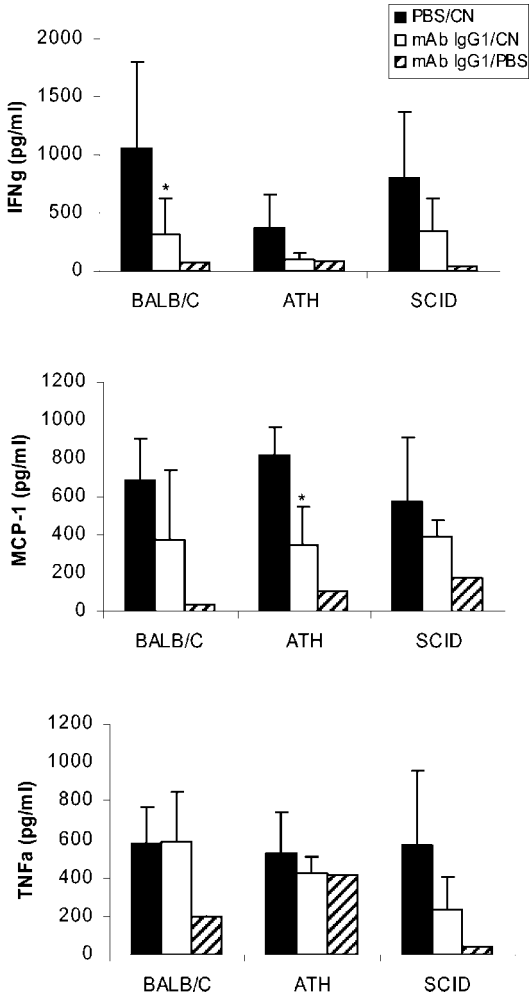

FIG. 5. Levels of cytokine and chemokine expression in the lungs of *C. neoformans*-infected BALB/c, athymic (ATH), and SCID mice. Cytokine and chemokine levels were measured by ELISA at day 14 postinfection. Bars denote mean protein concentrations. There were five mice in each group. #, value is significantly different from that measured for sham-infected mice. Error bars denote standard deviations. TNFa, TNF-α; IFNg, IFN-γ.

lation of T-cell responses against various pathogens, including *C. neoformans*.

Histological studies revealed a more exuberant inflammatory response in the lungs of B-cell-deficient mice than that observed in the lungs of C57BL/6J mice. However, given that there were no differences in the lung weights or total numbers of inflammatory cells in the lungs of B-cell-deficient and C57BL/6J mice, the differences in histological appearance must reflect differences in the organizations of the tissue cellular inflammatory responses. *C. neoformans*-infected B-cell-deficient mice had a higher percentage of eosinophils among inflammatory cells than C57BL/6J mice. The marked infiltration of eosinophils in B-cell-deficient mice relative to the infiltration in C57BL/6J mice is consistent with the higher levels of MCP-1 and MIP-1α. Since eosinophils can release polycationic proteins and generate superoxide and leukotrienes (28) that are toxic to several pathogens (22), as well as to host tissues (16), it is possible that the marked eosinophilia may contribute to lung damage. In fact, eosinophils can damage lung tissue by a novel mechanism that involves brominating proteins (59).

Passive administration of MAb prolonged the survival of C57BL/6J mice infected with *C. neoformans* despite having no effect on the numbers of CFU in organs, a result similar to that observed previously for A/J mice (23). A dissociation between survival and CFU has been noted during murine infection with *C. neoformans* (21) as well as *C. albicans* (9) and *M. tuberculosis* (53). The prolongation of survival in the absence of a fungal burden reduction strongly suggests that the deaths of these mice were not a direct consequence of increasing the microbial load. In fact, the observation that passive Ab was associated with both prolonged survival and reductions in the levels of

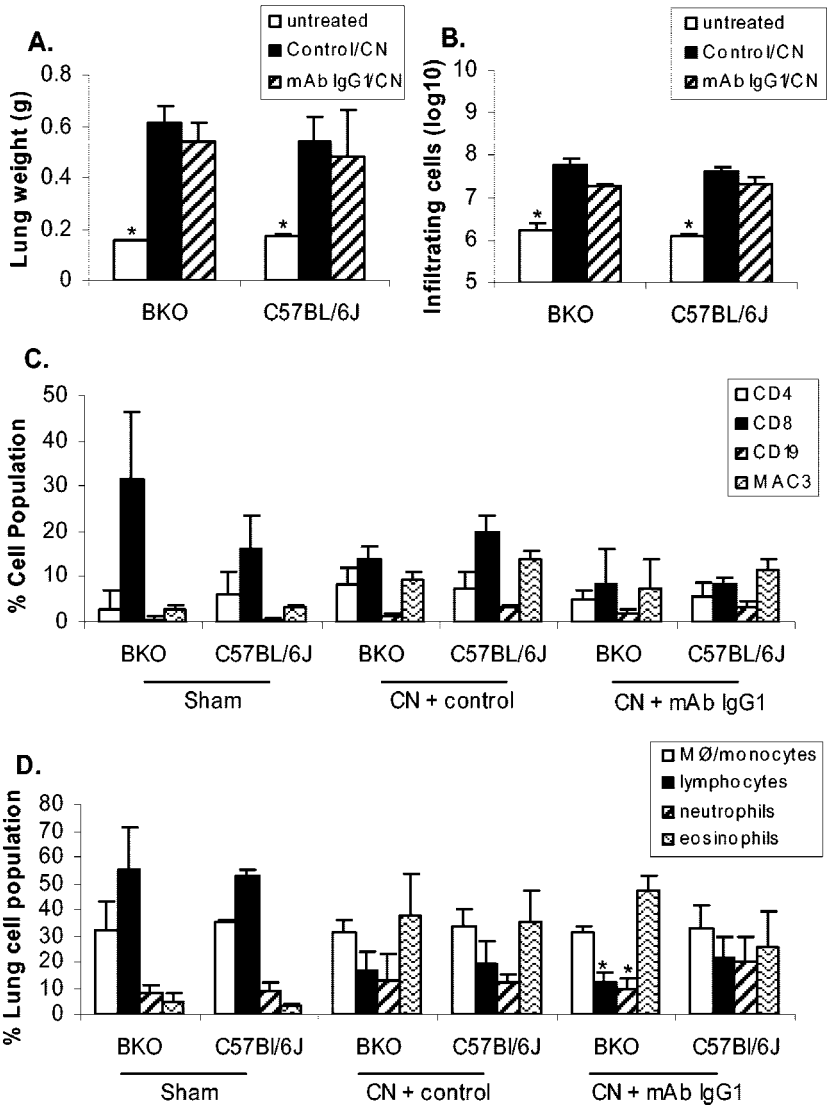

FIG. 6. Characterization of inflammatory responses of *C. neoformans* (CN)-infected B-cell-deficient and C57BL/6J mice 14 days postinfection. (A) Lung weights of infected B-cell-deficient and C57BL/6J mice. Bars denote mean organ weights. *, values for infected B-cell-deficient and C57BL/6J mice are significantly different ($P < 0.05$). (B) Total numbers of cells in the lungs of infected B-cell-deficient and C57BL/6J mice. *, values for infected B-cell-deficient and C57BL/6J mice are significantly different ($P < 0.05$). Bars denote mean numbers of lung cells. (C) FACS analysis of pulmonary B cells, T cells, and macrophages. Bars denote mean percentages. (D) Morphological analysis of pulmonary macrophages (MØ)/monocytes, lymphocytes, neutrophils, and eosinophils from infected B-cell-deficient and C57BL/6J mice. *, value is significantly different from that for C57BL/6J mice ($P < 0.05$); #, value is significantly different from that for B-cell-deficient mice ($P < 0.05$). There were three mice each in the sham-treated groups and six mice each in the control and MAb 2H1 groups. Error bars denote standard deviations (in all panels). BKO, B-cell-deficient mice.

some proinflammatory cytokines suggests that death in this system may ensue from chronic host damage mediated by unrelenting inflammation in the setting of persistent fungal burdens. Hence, the protection associated with passive Ab administration could reflect a down-regulation of inflammation with reduced tissue damage without necessarily reducing the fungal burden. Ab-mediated regulation of inflammatory responses has also been observed for mice infected with *Streptococcus pneumoniae* (10). In fact, there is a large body of literature that suggests an anti-inflammatory role for IgG, and we have recently proposed that a major role of IgG is to down-regulate T-cell and inflammatory responses (14). The results reported in this paper fit within this scenario, since passive Abs may not be expected to effect subtle regulation of inflammatory responses in hosts with impaired components of the immune system, such as B-cell-deficient, athymic, and SCID mice.

In an effort to better understand the changes in cytokine expression levels observed in B-cell-deficient and C57BL/6J mice, we carried out additional experiments with FcRγIII-deficient, athymic, and SCID mice. Similar to B-cell-deficient mice, FcRγIII-deficient mice manifested marked elevations of IFN-γ and MCP-1 relative to levels in C57BL/6J mice. IgG1 administration to FcRγIII-deficient mice had a modest effect

on IFN-γ levels but no significant effect on MCP-1 levels. Hence, the responses of FcRγIII-deficient mice to infection and IgG1 administration were similar to those of B-cell-deficient mice. Since B-cell-deficient mice lack IgG but have FcRγIII and FcRγIII-deficient mice lack the main receptor for IgG1, this result is internally consistent with and strongly supports the view that a major function of Ab action is to modulate inflammatory responses (14). Athymic and SCID mice were available only in a BALB/c background and, consequently, not directly comparable to the C57BL/6J mice. Nevertheless, they provide a useful reference point for comparing the effects of infection and Ab administration on the inflammatory response in another mouse strain. Similar to those of C57BL/6J mice and B-cell-deficient mice, these strains responded to infection and IgG1 administration with reductions of some cytokines and not others. For example, in all three strains, IgG1 administration resulted in reduced average levels of IFN-γ and MCP-1, consistent with the powerful immunomodulatory role of IgG1. The emerging picture is that when infection is the stimulus for increased IFN-γ levels in hosts with defective regulation of this cytokine, such as NOS2-deficient (48), B-cell-deficient, and SCID mice, passive Ab is not effective at mediating protection. IFN-γ appears to be beneficial or harmful, depending on its level in the tissues of B-cell-deficient and C57BL/6J mice, which may have a critical role in determining the outcome of infection. However, IFN-γ is likely to be only one component of this effect, since other studies have shown that passive Ab also affects IL-4, IL-10, and chemokines (24, 52).

The observation that passive Ab had no effect on fungal burdens in B-cell-deficient mice was surprising because it is different from what has occurred with other pathogens and with passive Ab in B-cell-deficient mice. For example, passive Ab administration protects against *Borrelia burgdorferi* (15), *Salmonella enterica* (38), vesicular stomatitis virus (55), and herpes simplex virus (4) in B-cell-deficient mice. Our study is different from those studies in that we used IgG1, which has been shown to be protective against *C. neoformans* infection, whereas other studies used immune sera. Since the selection of epitopes presented to T cells depends on the Fc receptors, it is conceivable that IgG1 cannot protect B-cell-deficient mice because of defective antigen presentation (3).

In summary, our study demonstrated that B-cell-deficient mice were more susceptible to *C. neoformans* infection, indicating a role for B cells in protection against *C. neoformans* through a mechanism that probably involves direct and/or indirect effects on the cell-mediated response. Unlike in C57BL/6J mice, passive Ab administration with IgG1 does not affect the course of *C. neoformans* infection in B-cell-deficient mice. The inability of Ab to protect B-cell-deficient mice was surprising, given precedents in other systems, and it implies the existence of complex links between humoral and cellular immune effector mechanisms.

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