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Protection against Experimental Cryptococcosis following Vaccination with Glucan Particles Containing *Cryptococcus* Alkaline Extracts

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ABSTRACT A vaccine capable of protecting at-risk persons against infections due to *Cryptococcus neoformans* and *Cryptococcus gattii* could reduce the substantial global burden of human cryptococcosis. Vaccine development has been hampered though, by lack of knowledge as to which antigens are immunoprotective and the need for an effective vaccine delivery system. We made alkaline extracts from mutant cryptococcal strains that lacked capsule or chitosan. The extracts were then packaged into glucan particles (GPs), which are purified *Saccharomyces cerevisiae* cell walls composed primarily of β-1,3-glucans. Subcutaneous vaccination with the GP-based vaccines provided significant protection against subsequent pulmonary infection with highly virulent strains of *C. neoformans* and *C. gattii*. The alkaline extract derived from the acapsular strain was analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS), and the most abundant proteins were identified. Separation of the alkaline extract by size exclusion chromatography revealed fractions that conferred protection when loaded in GP-based vaccines. Robust Th1- and Th17-biased CD4$^+$ T cell recall responses were observed in the lungs of vaccinated and infected mice. Thus, our preclinical studies have indicated promising cryptococcal vaccine candidates in alkaline extracts delivered in GPs. Ongoing studies are directed at identifying the individual components of the extracts that confer protection and thus would be promising candidates for a human vaccine.

IMPORTANCE The encapsulated yeast *Cryptococcus neoformans* and its closely related sister species, *Cryptococcus gattii*, are major causes of morbidity and mortality, particularly in immunocompromised persons. This study reports on the preclinical development of vaccines to protect at-risk populations from cryptococcosis. Antigens were extracted from *Cryptococcus* by treatment with an alkaline solution. The extracted antigens were then packaged into glucan particles, which are hollow yeast cell walls composed mainly of β-glucans. The glucan particle-based vaccines elicited robust T cell immune responses and protected mice from otherwise-lethal challenge with virulent strains of *C. neoformans* and *C. gattii*. The technology used for antigen extraction and subsequent loading into the glucan particle delivery system is relatively simple and can be applied to vaccine development against other pathogens.

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Virtually all cases of cryptococcosis are caused by *Cryptococcus neoformans* and the closely related species *Cryptococcus gattii* (1, 2). *C. neoformans* and *C. gattii* are unique among medically important fungi in that their major virulence factor is a capsule composed primarily of glucuronoxylomannan (GXM) (3). *C. neoformans* has a worldwide distribution, with human exposure mainly occurring following inhalation of airborne organisms. The global burden of cryptococcal meningitis in people with AIDS has been estimated to be about 1 million cases annually, with a 60% mortality rate (4). Other immunosuppressed persons are also at high risk, e.g., about 1 to 5% of solid organ transplant recipients will develop cryptococcosis in their lifetimes (5). *C. gat-*tii has historically been found in tropical and subtropical regions, but hypervirulent strains have emerged on Vancouver Island, Canada, and spread to the mainland, including the U.S. Pacific Northwest (2, 6, 7). This habitat expansion has been theorized to be due to climate change, raising concerns about further spread (2, 8).

Despite the prevalence of cryptococcosis, there are no licensed cryptococcal vaccines. Populations that could be targeted for vaccination include the following: (i) HIV-infected persons; (ii) persons on medications which suppress T cells (particularly transplant recipients); (iii) persons living in regions where *C. gattii* is endemic; and (iv) persons with other high-risk diseases (e.g., sar-

coidiosis, lymphoma). Preclinical approaches to developing cryptococcal vaccines have included immunization with protein-GXM conjugates and peptide mimetics of GXM designed to elicit antibody-mediated protection, as well as live and killed *C. neoformans* strains (9–15). Other work has focused on the identification of *C. neoformans* antigens that stimulate protective T cell responses. Much of this effort has focused on mannoproteins (MPs; defined by their affinity for the mannose-binding lectin concanavalin A), which comprise a major antigenic fraction recognized by T cells of mice and humans (16–18). The extensive mannosylation of MPs promotes uptake by mannose receptors on antigen-presenting cells, which leads to adaptive immune responses (18–21). However, vaccination with MPs admixed via the Ribi adjuvant system (Abcam; which biases toward antibody responses) resulted in only modestly prolonged survival of *C. neoformans*-challenged mice (22). Moreover, vaccination with the flowthrough fraction (defined as the antigens which did not bind concanavalin A) also elicited modest protection. These data suggest *Cryptococcus* vaccines designed to elicit protective T cell responses will require novel adjuvant and/or antigen compositions in order to be successful.

Glucan particles (GPs) are highly purified, hollow, porous cell wall shells manufactured by treating baker's yeast (*Saccharomyces cerevisiae*) with a series of alkaline, acid, and solvent extractions (23–26). They are composed primarily of β-1,3-glucan and are devoid of proteins, lipids, and mannans. GPs are recognized by the C-type lectin receptor Dectin-1, but also potently activate the alternative pathway of complement (27, 28). *In vivo*, phagocytosis of GPs is mediated by both complement receptors and Dectin-1 (29). GPs stimulate dendritic cells (DCs) to produce cytokines associated with beneficial responses in vaccine models of protection (27). Importantly, polymer-complexed "payload" classes, including proteins, small interfering RNA, DNA, and other small molecules can be constructed within the hollow GPs, enabling the cellular delivery of a wide variety of "payload" classes (23, 29–31). Immunization of mice with GPs loaded with the model antigen ovalbumin (OVA) results in potent and long-lasting antigen-specific antibody and Th1/Th17-biased CD4$^+$ T cell responses, even when using low doses of ovalbumin (23, 24). Moreover, beneficial effects have been noted in experimental Ascomycota infections, including aspergillosis and coccidioidomycosis, following vaccination of mice with fungal antigens in GPs (32, 33).

In the present study, we tested GPs as a vaccine delivery system in murine models of cryptococcosis. As a source of antigens, the GPs were loaded with soluble extracts obtained following mild alkali treatment of cryptococcal strains. Mice that received extracts encased in GPs mounted antigen-specific CD4$^+$ T cell recall responses and were protected from subsequent lethal challenges with *C. neoformans* and *C. gattii*.

RESULTS

Establishment of a C57BL/6 mouse model of cryptococcosis using *C. neoformans* strains derived from H99 for vaccination and challenge studies. A robust test of vaccine efficacy should demonstrate protection of genetically susceptible hosts against challenge with fully virulent pathogens. Therefore, when setting up our cryptococcosis vaccine model, we chose to use the C57BL/6 mouse strain. This inbred strain has been extensively studied and has been found to be particularly sensitive to infection by *C. neoformans* (34, 35). *C. neoformans* Kn99α (here referred to as Kn99), a

hypervirulent α mating-type strain which has been backcrossed 10 times to the well-studied clinical H99 strain (36), was selected for the fungal challenges. To mimic natural infection, mice were inoculated intranasally under conditions promoting pulmonary aspiration. As expected, C57BL/6 mice rapidly succumbed to intranasal challenge doses of 10^3, 10^4, or 10^5 CFU Kn99 (Fig. 1A). Median survival was extended by only a few days as the challenge dose decreased. Even at the lowest challenge dose of 10^3 CFU, all mice succumbed. For the subsequent vaccination studies, we used 10^5 CFU of Kn99 as the challenge dose.

Acapsular strains of *C. neoformans* are not only hypovirulent, but also, when used as a live vaccine delivered subcutaneously, they confer some protection against subsequent challenge with fully encapsulated cryptococcal isolates (11). Consistent with these findings, mice that received a subcutaneous vaccination with live acapsular strain cap59 were partially protected from lethal challenge with Kn99 (Fig. 1B). Protection, albeit not as robust, was also observed following vaccination with the live *cda1Δ cda2Δ cda3Δ* mutant strain (cda123 strain), an attenuated strain which lacks cell wall chitosan (37–39). Strains cap59 and cda123 were derived from wild-type strains H99 and Kn99, respectively (39, 40). Two weeks after fungal challenge, CFU counts in lungs were significantly lower in the mice vaccinated with cap59 (Fig. 1C). In contrast, at this time point few mice had CFU in their brain tissue (Fig. 1D).

Extraction of antigens from strains cap59 and cda123. The vaccination studies with the live attenuated strains suggested that these strains contained antigens that conferred protection. A mild alkaline extraction technique, described in Materials and Methods, was developed to test whether protective antigens could be recovered from the two mutant fungal strains for use as subunit vaccines. Typically, a purified extract from 500 ml of culture contained about 7.5 mg of protein. The endotoxin concentration ranged from 0.5 to 18 endotoxin units (EU)/mg of protein in four lots of the cap59 extract and 0.02 to 0.05 EU/mg of protein in two extracts of cda123. The alkaline extracts contained numerous protein and carbohydrate bands when resolved by SDS-PAGE (Fig. 2). Despite being derived from the same genetic background, many of the bands were unique to only one of the strains. As expected, the carbohydrate stain periodic acid-Schiff (PAS) demonstrated that the extracts from the cda123 strain, but not the cap59 strain, had a very-high-molecular mass band consistent with the GXM component of the capsule.

The cap59 extracts were then subjected to LC-MS/MS. Proteins were identified using the *C. neoformans* var. *grubii* database and analyzed for their relative abundance. Phosphatidylglycerol transfer protein was the most abundant protein, followed closely by superoxide dismutase. Proteins whose relative abundance was at least 5% that of phosphatidylglycerol transfer protein are listed in Table 1. Importantly, a whole transcriptome shotgun sequencing (RNA-Seq)-based analysis of *C. neoformans* isolates obtained from the cerebrospinal fluid of two patients with cryptococcal meningitis (41) showed mRNA encoding all 23 proteins. Several had high RNA-Seq read numbers, suggesting that these extracted proteins are likely produced during infection.

Protection of mice vaccinated with cap59 and cda123 strain extracts in GPs. Soluble alkaline extracts from cap59 and cda123 were packaged into GPs (here designated GP-cap59 and GP-cda123). Mice were then vaccinated subcutaneously (three times, at 2-week intervals, with 10 μg of protein/dose) and challenged
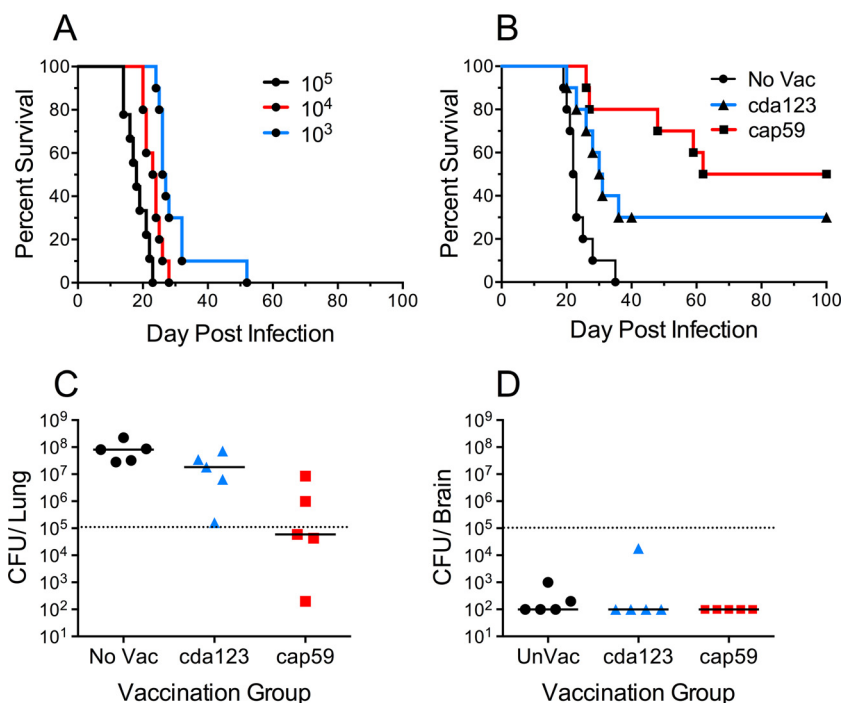
FIG 1 Protection of C57BL/6 mice by vaccination with live cap59 and cda123 strains. (A) Naive C57BL/6 mice were inoculated intranasally with 10^3, 10^4, or 10^5 CFU of strain Kn99, and their survival was monitored. $P < 0.01$ comparing animals that received 10^4 versus 10^3 or 10^5 CFU; $P < 0.0001$ comparing animals that received 10^3 versus 10^5 CFU. (B) An intranasal challenge with 10^5 CFU of Kn99 was used to monitor survival of mice that were vaccinated three times with 10^6 CFU of live *C. neoformans* strain cap59 or cda123. Survival curves are from two independent experiments, each with 5 mice per group. Unvaccinated (No Vac) mice served as controls. $P < 0.01$ and $P < 0.0001$ comparing No Vac animals versus animals vaccinated with cda123 or cap59, respectively. (C and D) CFU in the lungs and brains of unvaccinated and vaccinated mice 2 weeks postchallenge with 10^5 CFU of Kn99. Mice with no CFU were assigned a value of 100 CFU, which was the lower limit of detection of the assay. $P < 0.05$ comparing lung CFU of unvaccinated versus cap59-vaccinated mice.

with *C. neoformans* Kn99. Control mice that were left unvaccinated or were vaccinated with mouse serum albumin in GPs (GP-MSA) all died within 27 days (Fig. 3A). However, the mice that received GP-cap59 or GP-cap123 vaccination had significantly prolonged survival; 60% of the vaccinated mice were still alive

FIG 2 SDS-PAGE results for cap59 and cda123 extracts. Alkaline extracts were prepared from *C. neoformans* strains cap59 and cda123. Each lane was loaded with 50 μg of protein. The left lanes show molecular mass size markers (in kilodaltons). (A) Gels were stained for protein with Coomassie blue. (B) Replicate lanes from the same gel were stained for carbohydrate with PAS. The arrow identifies the region expected for GXM.

when the experiment was terminated at 50 days. CFU counts from lungs of the euthanized survivors were determined (Fig. 3B). While CFU in most mice were below the limit of detection, lungs from two mice had CFU greater than 10^6.

Chitin deacetylases (CDAs), particularly CDA2, have been shown to be targets of the T cell response (42, 43). Moreover, CDA2 was among the top antigens identified in the cap59 extracts (Table 1). Therefore, to avoid losing potentially protective CDA antigens and to circumvent potential inhibitory effects of GXM (44), we elected to focus on the alkaline extracts from the cap59 strain. To determine the reproducibility of the vaccination system, five distinct lots of GP-cap59 were made and separately tested. Overall, each lot behaved similarly and, taken in aggregate, significant protection compared to GP-MSA controls was seen (Fig. 3C and D). As in Fig. 3B, within the 50-day period following infection with 10^5 CFU of Kn99, the median fungal load in the lungs of survivors was reduced more than 100-fold (Fig. 3D); in several mice, CFU were below the detection limit.

***Ex vivo* CD4+ T cell responses following vaccination.** In previous studies, we demonstrated that vaccination of mice with GPs containing the model antigen ovalbumin (GP-OVA) induced potent CD4+ T cell responses, even when relatively low doses of antigen were used as *ex vivo* stimuli (23, 24). This raised the possibility that *ex vivo* assays could be used to determine the antigen components of cap59 responsible for T cell stimulation. CD4+ T cells, purified from mice that had been vaccinated with GP-cap59, were stimulated with a range of concentrations of GP-cap59, and
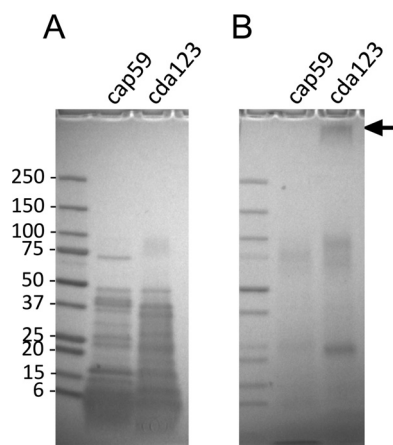
TABLE 1 LC-MS/MS analysis of protein in *C. neoformans* cap59 extracts

CNAG designation	Description	Abundance[a]	Signal peptide[b]	No. of RNA-Seq reads[c]	Homology[d]
CNAG_07442	Phosphatidylglycerol transfer protein	100	Yes	25,623	0.39
CNAG_01019	Superoxide dismutase [Cu-Zn]	97	No	70,771	5E−62
CNAG_06432	Acetate kinase	50	No	18,367	None
CNAG_00581	Saccharopepsin	27	Yes	97,106	2E−107
CNAG_01230	Chitin deacetylase 2/ MP98	24	Yes	5,835	None
CNAG_04625	Cerevisin	23	Yes	115,412	3E−27
CNAG_00919	Carboxypeptidase D	19	Yes	934	6E−19
CNAG_00450	3-Isopropylmalate dehydrogenase	16	No	1,129	9E−30
CNAG_00734	Dihydroorotase, homodimeric type	16	No	1,814	1.5
CNAG_03232	Lactamase	14	No	23,885	3E−04
CNAG_05097	YjeF family protein	13	No	8,699	1E−72
CNAG_01896	Alcohol dehydrogenase (NADP)	11	No	87,413	2E−71
CNAG_00264	Nuclease I	11	Yes	51,905	None
CNAG_01348	Cyanate hydratase	9	No	8,774	2.2
CNAG_04981	Catalase	7	No	101,133	2E−102
CNAG_03223	Hypothetical protein	6	Yes	48,826	None
CNAG_00575	Catalase	6	No	21,202	3E−81
CNAG_01498	Arylsulfatase	6	Yes	2,938	3E−43
CNAG_01137	Aconitate hydratase	6	No	19,654	0
CNAG_04443	Hypothetical protein	5	No	19,418	8E−10
CNAG_04269	Leucyl aminopeptidase	5	Yes	15,297	0.62
CNAG_07745	Alcohol dehydrogenase, propanol-preferring	5	No	46,734	3E−12

[a] The relative abundance of protein, averaged for two cap59 extracts. The most abundant protein, phosphatidylglycerol transfer protein, was arbitrarily set at 100.

[b] Determined using SignalP 4.0.

[c] Average numbers of normalized RNA-Seq reads recorded for two strains of *Cryptococcus* isolated directly from infected human patients (GEO accession number GSE51573) (41).

[d] Homology to the human protein with the highest similarity (BLASTp analysis); the smaller the value, the greater the similarity.

lymphoproliferation (LP) was determined. Significant *ex vivo* responses were seen at concentrations of cap59 as low as 1 ng/well (Fig. 4).

Fractions of cap59 extracts responsible for T cell stimulation and protection. cap59 extracts were separated by size exclusion chromatography into 1-ml fractions. Each fraction was then assayed for protein and carbohydrate concentrations as well as the capacity to stimulate *ex vivo* CD4+ T cell lymphoproliferation (Fig. 5A). Lymphoproliferative responses were strongest with the higher-molecular-size fractions. Moreover, lymphoproliferative responses correlated with carbohydrate concentrations to a much greater extent than to protein concentrations.

We next sought to determine which fractions were responsible for protection in mice. Due to the small amount of antigen in each fraction, we combined fractions into pools of 4 fractions and concentrated them 30-fold. These were then loaded into GPs and used to vaccinate mice. Only five mice per group could be studied, on account of the limited amount of material obtained. Nevertheless, the results were striking, with 4/5 mice in the group containing pooled fractions 8 to 11 surviving (Fig. 5B). In contrast, except for one survivor in the fractions 4 to 7 group, all the other mice succumbed to infection. Interestingly, survival did not directly correlate with *ex vivo* lymphoproliferation.

Immune recall responses in the pulmonary compartment. Natural exposure to *C. neoformans* most commonly occurs following inhalation, and the lungs are a common site of clinical infection with this fungus. We postulated that vaccination with GP-cap59 would stimulate robust lung recall responses following pulmonary challenge with *C. neoformans*. To test this, four groups of mice were studied: unvaccinated uninfected, vaccinated uninfected, unvaccinated infected, and vaccinated infected. Lungs were harvested 5 days after infection. Readouts were the numbers of lung leukocytes, CD4+ T cells, and CD8+ T cells, as well as

cytokine skewing after *ex vivo* stimulation with GP-cap59. As expected, infection was associated with an influx of leukocytes into the lung, which was more robust in the mice that had been vaccinated (Fig. 6A). CD4+ and CD8+ T cells also increased following infection, although the absolute numbers were similar regardless of whether the mice had been vaccinated (Fig. 6B).

Vaccination did profoundly affect the quality of the pulmonary T cell response to infection, as assessed by intracellular cytokine staining for the Th1, Th2, and Th17 markers gamma interferon (IFN-γ), interleukin-4 (IL-4), and IL-17a, respectively. Mice that were vaccinated and infected had a large influx of CD4+ T cells that stained positive for IFN-γ or IL-17a (Fig. 6C). CD4+ T cells that expressed both IFN-γ and IL-17a were also observed; however, there was only a slight increase in cells staining for IL-4. Cytokine staining of CD8+ T cells from the vaccinated and infected mouse lungs revealed a somewhat different pattern (Fig. 6D). First, the total number of CD8+ T cells that stained for any of the three cytokines assayed was considerably lower. Second, the number of CD8+ T cells that stained for IL-4 was not significantly different from the number staining for IFN-γ or IL-17a.

Protection against *C. gattii* infections. In the final set of experiments, we examined whether a GP-cap59 vaccine would protect against challenge with *C. gattii*. An alkaline extract was made from the *C. gattii* cap59 (acapsular) strain (45) and packaged into GPs. Mice received a prime and 2 boosts of GP-cap59 and then were challenged with 10^5 wild-type *C. gattii* strain R265 cells (46). Controls included unvaccinated mice and mice that received GP-MSA. Mice vaccinated with GP-cap59 had significantly prolonged survival compared with the control groups (Fig. 7A). Four vaccinated mice survived the 70-day observation period; however, following euthanasia, we were able to detect *C. gattii* CFU in each of those mice (Fig. 7B).
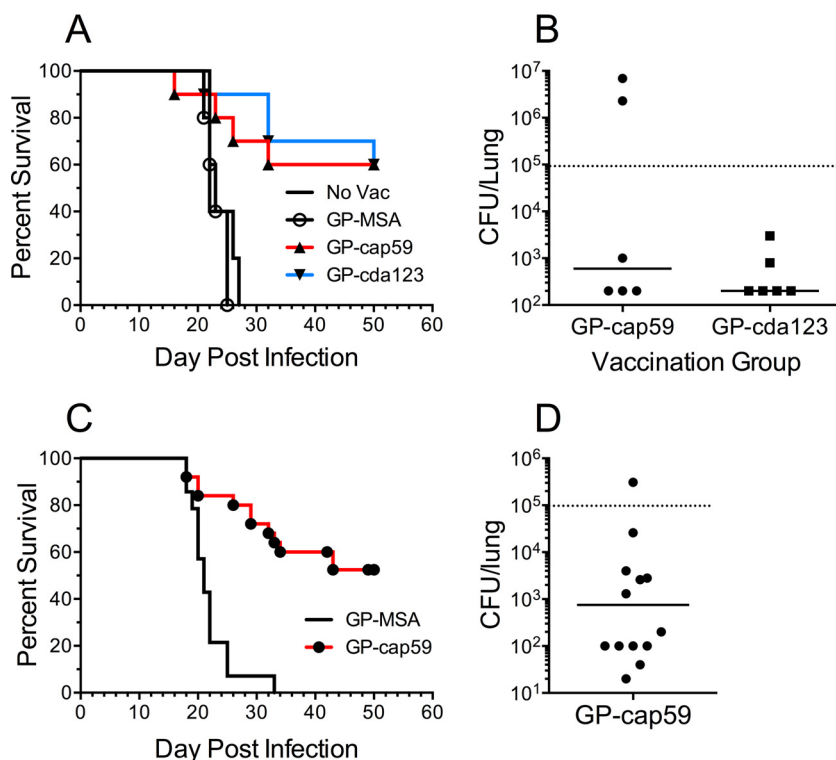
FIG 3 Survival curves following vaccination with GP-cap59 and GP-cda123. (A) Mice were left unvaccinated (No Vac) or vaccinated three times with GP-MSA, GP-cap59, or GP-cda123. Mice were then challenged intranasally with 10^5 CFU of Kn99. Each experiment had five mice per group. The results with GP-cap59 and GP-cda123 are combined from two independent experiments, one with "No Vac" and one with GP-MSA as the control. $P < 0.01$ comparing No Vac or GP-MSA with GP-cap59 or GP-cda123. (B) The mice that survived 50 days were euthanized and the CFU of Kn99 in the lungs were measured. (C) Five distinct lots of GP-cap59 vaccine were manufactured and tested as described for panel A. Each experiment had five mice per group. The cumulative results of the five studies are shown. $P < 0.0001$ comparing GP-MSA with GP-cap59. (D) CFU of Kn99 in lungs of mice surviving 50 days (shown in panel C). The lower limit of detection of the fungal load in the lung was 200 CFU (B) or 20 CFU (D). Samples below detection were assigned 200 CFU (B) or 20 CFU (D). The dotted lines denote the inoculum of the challenge dose.

DISCUSSION

Vaccination is one of the greatest public health successes, greatly reducing and in some cases eliminating many deadly diseases (47). Development of a vaccine to protect vulnerable people against
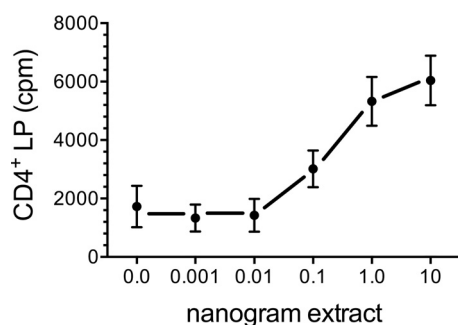

FIG 4 Amount of cap59 extract in GPs needed to induce an *ex vivo* CD4 T cell response. CD4$^+$ T cells were purified from the lymph nodes and spleen of mice that had been vaccinated three times with GP-cap59. The cap59 extract, quantified by protein concentration, was loaded in 10-fold decrements into GPs. GPs in the "0.0" group contained MSA without cap59 extract. The T cells (10^5/well) were added to wells containing 10^4 mitomycin C-treated BMDCs and 10^5 GPs containing the indicated amount of cap59 extract. Lymphoproliferation was assayed by [^{3}H]thymidine incorporation. Data are means ± standard errors of the means of results from three independent experiments, each performed in triplicate. $P < 0.01$ and $P < 0.001$ comparing 0.0 versus 1.0 and 10 ng cap59 extract, respectively.

cryptococcosis is needed, given the prevalence of this disease and its associated morbidity and mortality (2, 4). Here, we provide proof of principle for a vaccination approach that packages fungal antigens in a GP delivery system. Similar protection against a lethal challenge with a *C. neoformans* strain was observed in mice that received subcutaneous vaccinations of GPs containing alkaline extracts from either of two different mutant *C. neoformans* strains. Similarly, an alkaline extract derived from a *C. gattii* strain protected against subsequent lethal challenge with *C. gattii*. Protection did not appear to be due to the stimulatory effects that β-glucans have on innate and trained immunity (48, 49), as control mice that received immunizations with GPs containing only MSA had 100% mortality.

The cryptococcal strains used to make the alkaline extracts either lacked capsule entirely (cap59) or, in the case of the cda123 strain, the cells were loosely encapsulated (39). The lack of capsule facilitated loading of the cap59 extracts into GPs, as the extracts were less viscous than those from cda123. It is recognized that the cda123 strain is deficient in three proteins that could contribute to protection. One of the three deleted chitin deacetylases, CDA2 (also known as MP98), has been shown to be a major target of the T cell response in mice (42, 43). For these reasons, our focus was on the cap59 extracts.

The protection we observed was with highly virulent cryptococcal strains and use of a mouse strain, C57BL/6, that is highly susceptible to cryptococcosis (35, 50). Nevertheless, while protec-
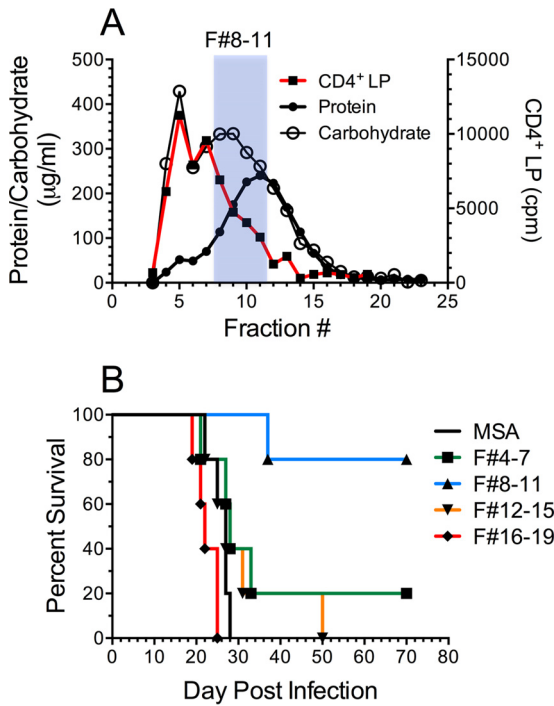
FIG 5 Immunostimulatory and protective fractions of the cap59 extract following size exclusion chromatography. (A) The cap59 extract was separated on a Sephadex G-100 column. One-milliliter fractions were collected and assayed for protein and carbohydrate. A 260-μl portion of each fraction was then loaded into GPs and used as a stimulus of CD4$^+$ T cell LP (as in Fig. 4). The shaded area indicates the pool of fractions 8 to 11 that best protected mice as a GP vaccine. (B) Pools of four sequential fractions were loaded into GPs and used to vaccinate mice. The fraction numbers (F#) of the pools are indicted in shaded column. GPs loaded only with MSA served as the control GP vaccine. Mice (5/group) were vaccinated three times and challenged with 10^5 CFU of Kn99. $P < 0.01$ comparing F#8 to 11 with MSA.

tion was significant, it fell short of 100%, and sterilizing immunity was seen in only some of the survivors. Ongoing efforts are focused on improving vaccine efficacy. This includes identifying which of the many antigens in the alkaline extracts elicit the most robust protection and thus should be considered for inclusion in future subunit vaccines. The mild alkaline extraction technique we used is predicted to remove non-covalently linked cell wall proteins without lysing the cells. However, many of the proteins identified in the extracts do not have signal sequences, suggesting that the extraction procedure selectively recovers proteins located inside the cell membrane or in extracellular vesicles (51). While the most abundant proteins in our extracts are expressed by cryptococci in infected humans (41), the possibility of additional protective antigens not found in the extracts must be entertained. Thus, while alkaline extraction is a relatively simple technique for generating antigens from *Cryptococcus*, in future candidate vaccines we will also test proteins identified as immunogenic (18, 52–56) and not found in abundance in the alkaline extracts.

The arm(s) of the immune system responsible for GP vaccine-mediated protection remains to be determined. Interestingly, when the alkaline extracts were fractioned by size exclusion chromatography, the fractions that stimulated the greatest CD4$^+$ lymphoproliferative responses were not those that afforded the greatest protection. However, a caveat for interpretation of these results
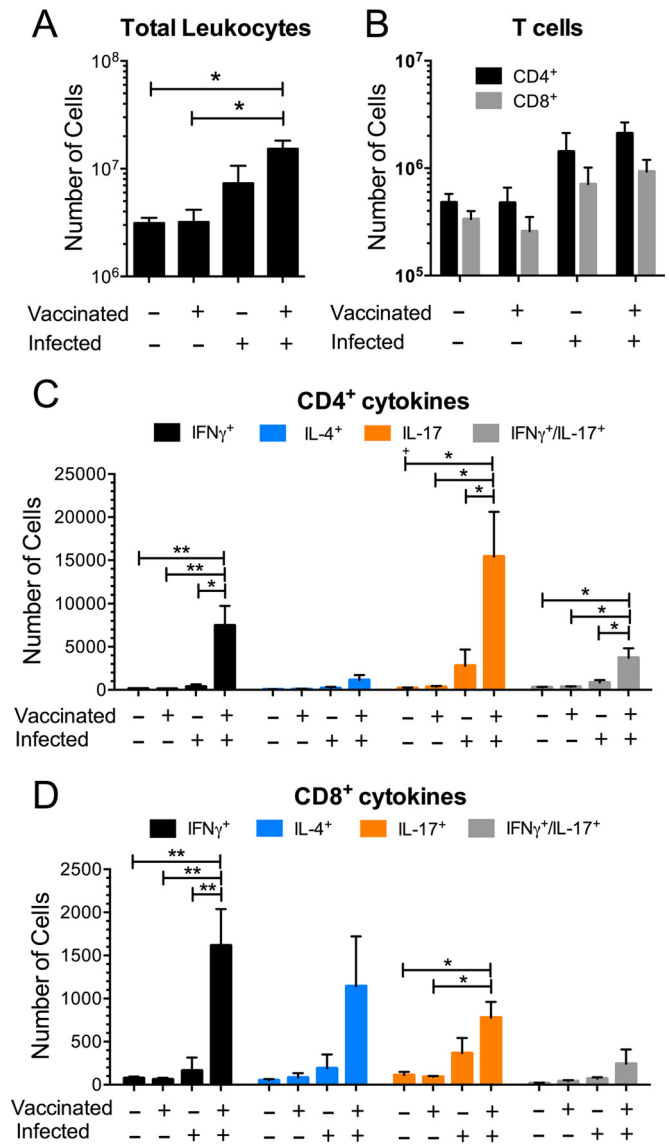
FIG 6 Analysis of leukocytes from lungs of vaccinated and/or infected mice. In each of three studies, two mice were vaccinated with GP-cap59 three times at 2-week intervals, and two mice were unvaccinated. Two or more weeks following the third vaccination, a naive and a vaccinated mouse were infected with 10^5 CFU of KN99 and a naive and a vaccinated mouse were not uninfected. Five days postinfection, the lungs were collected and single-cell suspensions were prepared. (A) The leukocytes at the interface of a 67% and 40% Percoll gradient were collected and counted. Purified leukocytes were stimulated with GP-cap59 *ex vivo* (at a ratio of 3 GPs per 10 leukocytes) for 24 h, and brefeldin A was added for the last 5 h of the incubation, as described in Materials and Methods. Then, the cells were collected, stained for CD3, CD4, CD8, IFN-γ, IL-4, and IL-17a, and analyzed by polychromatic FACS. (B) The numbers of CD4$^+$ (CD3$^+$ CD4$^+$ CD8$^-$) and CD8$^+$ (CD3$^+$ CD4$^-$ CD8$^+$) T cells were calculated by multiplying the percentage of each population times the total leukocyte counts. (C) The numbers of CD4$^+$ T cells producing IFN-γ, IL-4, IL-17a, or IFN-γ/IL-17a were similarly calculated. (D) As described for panel C, the numbers of CD8$^+$ T cells producing cytokines were calculated. Results shown are means ± standard errors of the means of three independently performed experiments with four mice for each experiment. One-way ANOVA with Tukey multiple comparison correction was performed to compare cell numbers among four groups (unvaccinated uninfected, vaccinated uninfected, unvaccinated infected, and vaccinated infected). *, $P < 0.05$; **, $P < 0.01$.
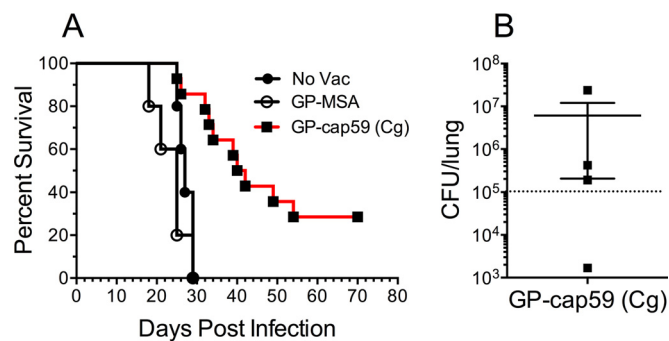
FIG 7 Protection against challenge with *C. gattii* provided by the GP-cap59 (*C. gattii*) vaccine. An alkaline extract from *C. gattii* strain cap59 was loaded into GPs (GP-cap59 [Cg], containing 10 µg extract protein per vaccine dose). Mice were left unvaccinated (No Vac) or vaccinated three times with GP-MSA or GP-cap59. Mice were then inoculated intranasally with 10^5 CFU of *C. gattii* strain R265. (A) Survival curves combined from two independent experiments. The first experiment had two groups of five mice, one of which received GP-cap59 and the other no vaccination. The second experiment had two groups. One group contained nine mice which received GP-cap59. The other group had five mice which received GP-MSA. $P < 0.0001$ comparing GP-cap59 with either control group. (B) *C. gattii* CFU in the lung of each mouse ($n = 4$) that survived 70 days. The dotted line denotes the inoculum of the challenge dose.

is that the fractions were loaded into GPs based on volume rather than protein content. Interestingly, the protective fractions had a ratio of carbohydrate to protein characteristic of mannoproteins (18).

Mice that were vaccinated with GP-cap59 and then challenged with live *C. neoformans* responded with a robust recall T cell response in the lung. As was observed when immunizing with GP-OVA (23, 24), the CD4$^+$ T cell response was Th1 and Th17 skewed, as determined by cellular IFN-γ and IL-17a production. In addition, a small but significant fraction of the CD4$^+$ T cells expressed both IFN-γ and IL-17a. In contrast, significant numbers of CD4$^+$ Th2 cells, defined by IL-4 expression, were not observed. In mouse models of cryptococcosis, protective responses are strongly associated with Th1-type cytokines, with a lesser role for Th17-type cytokines (57, 58). Th2-type cytokines are associated with exacerbation of disease.

Compared with the lung recall CD4$^+$ T cell response, the CD8$^+$ T cell response was considerably less robust, and Tc1, Tc2, and Tc17 cells were observed in approximately equal numbers. This does not preclude a role for GP-based cryptococcal vaccines in HIV-infected persons; using a live attenuated *Blastomyces* sp. strain, the Klein Lab has shown that while CD8$^+$ T cells are only weakly primed in CD4$^+$ T cell-sufficient hosts, robust CD8$^+$ T cell responses occur in CD4-deficient mice (59, 60). Ongoing experiments are directed at determining whether GP-based vaccines can similarly protect mice depleted of CD4$^+$ T cells. In addition, while we hypothesize that T cells are responsible for the protection conferred by our GP-based cryptococcal vaccines, the contribution of antibodies needs to be explored.

The ideal cryptococcal vaccine should protect against both *C. neoformans* and *C. gattii*, even in the setting of immunocompromise. However, in regions where *C. gattii* is endemic in the environment, there may be a role for a vaccine that specifically targets infections with this species. This may turn out to be particularly so if habitat expansion of *C. gattii* continues (2, 8). While

the vast majority of persons who acquire symptomatic infection with *C. neoformans* are severely immunocompromised prior to exposure, those who acquire *C. gattii* infections often have no known or only mild immunodeficiency (61). Notably, while alkaline extracts packaged in GPs protected against both cryptococcal species, there was a trend toward enhanced protection against *C. neoformans* compared with *C. gattii*, despite preparing species-specific extracts. In a related approach, Chaturvedi et al. made cell wall and cytoplasmic protein preparations from *C. gattii* (62). Intranasal vaccination with these preparations resulted in a decreased fungal burden and prolonged survival following challenge with *C. gattii*.

While the full translational potential of GP-based vaccines can only be addressed with clinical studies, related β-glucan-containing preparations have been tested in humans. Notably, β-glucan preparations derived from fungi have a record of safety in both preclinical and human trials (63–65). Moreover, heat-killed *S. cerevisiae* genetically engineered to express antigens has undergone clinical trials in immunotherapeutic vaccines (66, 67). In phase I trials, no major toxicities, clinically significant laboratory abnormalities, or serious adverse events were noted, even at the highest doses tested (66). Importantly, in both human and animal studies, strong antigen-specific helper and cytotoxic T lymphocyte responses were elicited (66, 68–70). Due to their greater purity, including a lack of endogenous baker's yeast proteins, we predict GPs will be even safer than heat-killed *S. cerevisiae*.

MATERIALS AND METHODS

Chemicals and cell culture media. Chemical reagents were purchased from Thermo, Fisher Scientific (Pittsburg, PA), unless stated otherwise. RPMI 1640 medium was obtained from Invitrogen Life Technologies (Carlsbad, CA). R10 medium contained RPMI 1640 with 10% fetal bovine serum (FBS; Tissue Culture BioLogicals, Tulare, CA), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (Invitrogen), and 55 µM 2-mercaptoethanol (Invitrogen). Incubations with mouse cells were performed at 37°C in humidified air supplemented with 5% CO_2.

Strains of *Cryptococcus* spp. Three strains of *C. neoformans* var. *grubii* were used in these studies: Kn99 (wild type) (71), cap59 (acapsular) (40), and *cda1Δ cda2Δ cda3Δ* (cda123; a chitosan-deficient mutant strain) (39). Strains of *C. gattii* included R265 (wild type) (46) and cap59 (acapsular, derived from *C. gattii* wild-type strain NIH444) (45). R265 is the major genotype strain from the Vancouver Island outbreak. NIH444 was originally isolated in Seattle and is identical to R265 across all 30 tested loci (72). Each strain was maintained as a glycerol stock at −80°C and was initially cultured on YPD agar (yeast extract [Difco], Bacto peptone [Becton, Dickinson, Sparks, MD], and dextrose [Sigma, St. Louis, MO]), which served as inoculum for liquid cultures of YPD or YNB medium (yeast nitrogen base [Becton, Dickinson]). To prepare *Cryptococcus* for *in vivo* challenge studies, strains Kn99 and R265 were cultured in liquid YPD for 18 h at 30°C with shaking. Yeast cells were then harvested by centrifugation, washed once with phosphate-buffered saline (PBS), and finally suspended in PBS.

Alkaline extraction of cryptococcal cell wall antigens. Strains of *C. neoformans* were cultured in 500 ml of 2× YNB, 1% glucose from a starting optical density at 600 nm (OD_{600}) of 0.1. Flasks were shaken for 2 to 3 days at 30°C, followed by 1 day at 37°C. Yeast cells, typically having reached an OD_{600} of 9.0 to 11.0 in that time, were collected by centrifugation at $1,600 \times g$, 22°C, for 5 min. Cells were suspended in PBS, split between two 50-ml tubes, and then centrifuged again. The yield of 500 ml of culture was about 5 to 6 ml of packed cells in each of the 50-ml tubes; PBS-washed cell pellets were stored at −20°C. For alkaline extraction, the cell pellets were thawed at 22°C. Cells were rapidly suspended in 1.5 vol-

umes of 0.1 M potassium hydroxide (KOH) and rotated for 10 min at 22°C, then removed by centrifugation, as described above. For each milliliter of supernatant, 25 μl 1 M sodium acetate buffer, pH 4.5, was added to neutralize the KOH, followed by mixing with 1.5 volumes of isopropanol and storage at −20°C overnight. The precipitate was collected following centrifugation at 6,000 × g for 10 min at 4°C, suspended in 15 ml 0.1 M ammonium acetate, then reprecipitated with 2 volumes of ethanol, and kept overnight at −20°C. Centrifugation was repeated, and the precipitate was dried by lyophilization. It was then suspended in 7.5 ml 0.01 M ammonium acetate and clarified by centrifugation at 6,000 × g for 10 min at 4°C. The supernatant containing soluble alkaline extracted antigens was saved and stored at −80°C. The protein concentration was measured in a bicinchoninic acid (BCA) assay, and carbohydrate was determined by the phenol-sulfuric acid method of Dubois (73). Endotoxin levels of the extracts were measured using the *Limulus* amebocyte lysate (LAL) pyrochrome reagent in Glucashield buffer according to the manufacturer's instructions (Associates of Cape Cod, Falmouth, MA). SDS-PAGE was used to separate antigens in extracts. Gels were stained with either Coomassie stain (RapidStain; G-Biosciences, St. Louis, MO) for protein or PAS (Schiff's reagent; Electron Microscopy Sciences, Hatfield, PA) for carbohydrates.

Analysis of cap59 extracts by electrospray ionization LC-MS/MS. Samples were processed for analysis by LC-MS/MS as previously described (74). Briefly, for each sample, 50 μg protein was electrophoresed into a 10% Mini-Protean Tris-glycine extended (TGX)-PAGE gel (Bio-Rad Laboratories) in Tris-glycine–SDS buffer, stained with Coomassie, destained with water, and removed as a gel slice. Gel slices were cut into 1- by 1-mm pieces, reduced in a 45 mM solution of 1,4-dithiothreitol (DTT) at 50°C for 30 min, alkylated in 100 mM iodoacetamide for 30 min, and digested with 2 ng/μl of sequencing-grade trypsin (Sigma) in 0.01% ProteaseMAX surfactant (Promega)–50 mM ammonium bicarbonate at 37°C for 18 h. The supernatant of each sample was then placed in a 0.5-ml microcentrifuge tube. The gel slices were further extracted with 80:20 acetonitrile–1% formic acid, combined with the supernatants of each sample, and completely dried in a SpeedVac.

Tryptic peptide digests were reconstituted in 25 μl 5% acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid and separated using a NanoAcquity ultraperfomance liquid chromatography system (Waters Corporation, Milford, MA). In brief, a 3.0-μl injection was loaded in 5% acetonitrile containing 0.1% formic acid at 4.0 μl/min for 4.0 min onto a 100-μm-inner-diameter (i.d.) fused-silica precolumn packed with 2 cm of 5-μm (200-Å) Magic C_{18}AQ resin (Bruker-Michrom, Auburn, CA) and eluted using a gradient at 300 nl/min onto a 75-μm-i.d. analytical column packed with 25 cm of 3-μm (100-Å) Magic C_{18}AQ particles to a gravity-pulled tip. The solvents were A (water–0.1% formic acid) and B (acetonitrile–0.1% formic acid). A linear gradient was developed from 5% solvent A to 35% solvent B in 60 min. Ions were introduced by positive electrospray ionization via liquid junction into a Q Exactive hybrid mass spectrometer (Thermo Scientific). Mass spectra were acquired over m/z 300 to 1750 at 70,000 resolution (m/z 200), and data-dependent acquisition selected the 10 most abundant precursor ions for tandem mass spectrometry by higher-energy collisional dissociation fragmentation using an isolation width of 1.6 Da, collision energy of 27, and a resolution of 17,500.

Raw data files were peak processed with Proteome Discoverer (version 1.3; Thermo Scientific) or Mascot Distiller (version 2.4; Matrix Science, Inc., Boston, MA) prior to database searching with the Mascot server (version 2.4) against the *C. neoformans* var. *grubii* database (National Center for Biotechnology Information). Search parameters included both trypsin specificity with two missed cleavages and no enzymatic specificity. The variable modifications of oxidized methionine, pyroglutamic acid for N-terminal glutamine, deamidation of asparagine and glutamine, N-terminal acetylation of the protein, and a fixed modification for carbamidomethyl cysteine were considered. The mass tolerances were 10 ppm for the precursor and 0.05 Da for the fragments. Search results were then loaded into the Scaffold viewer (Proteome Software, Inc., Portland, OR) for peptide/protein validation. MS data were analyzed with Mascot software using two search criteria: a conventional search based on tryptic digestion sites and an expanded search in which no digestion enzyme was specified. Proteins were identified with Scaffold software (Proteome Software, Inc.), and probabilities were assigned by using the Protein Prophet algorithm; identifications were accepted if they could be established at >95% probability for at least two peptides. The Mascot Distiller average quantitation method was used, in which the precursor intensities of the three most abundant peptides are used to compute a protein's relative abundance in a sample.

GP vaccines. *Saccharomyces cerevisiae* cells were converted into GPs following a series of hot alkali, organic, and aqueous extraction steps, as described previously (23, 24, 27, 31). The final product consisted of a highly purified 3- to 4-μm-diameter yeast cell wall preparation devoid of cytoplasmic content and bounded by a porous, insoluble shell of β-glucans. The final GP vaccine consisted of *Cryptococcus* alkaline extract, mouse serum albumin (Equitech-Bio, Kerrville, TX), and yeast RNA (yRNA; Sigma) complexed within the glucan shells. Samples to be loaded into GPs were concentrated by lyophilization and dissolved in water at 5 to 10 mg/ml by protein content. Antigens were loaded into GPs and complexed with MSA and yRNA as described elsewhere (23, 24, 27). Vaccines were diluted in sterile 0.9% saline for injection to deliver the indicated amount (ranging from 0.01 to 10 μg) of antigen in 200-μg GPs (approximately 10^8 particles) per 0.1-ml dose. A control vaccine consisted of GPs loaded with MSA and yRNA but without cryptococcal alkaline extract. Vaccines were stored in 0.6-ml aliquots at −80°C and briefly vortexed prior to use. Vaccine formulations were quality controlled by manual counting of the number of GPs per milliliter by using a hematocytometer and microscopically assessing for intact GPs with a phase-distinct protein-yRNA complex. Vaccines were also extracted and analyzed by 10% SDS-PAGE to quantify antigen/MSA loading.

GP antigen fraction vaccines. *Cryptococcus* extracts were size fractionated by Sephadex G-100 gel filtration. One milligram (based on protein content) in 0.5 ml of 0.1 M ammonium acetate, 20 mM Tris-HCl, pH 7.4, was separated on a 1.5- by 7.0-cm column equilibrated with the same buffer as the sample. One-milliliter fractions were collected and analyzed for protein and carbohydrate. A 260-μl portion from each fraction was lyophilized and suspended in 26 μl water, and then 5-μl aliquots of each 10× concentrated fraction were loaded into 1-mg GPs and complexed with MSA and yRNA, as described above. The loaded GPs were each suspended in 0.5 ml 0.9% saline and frozen for later use as stimuli for LP assays. The fractions were also tested in GP-based vaccines. Pools of four sequential fractions containing 0.6 ml of one fraction were lyophilized, suspended in 20 μl of water, and loaded into 4-mg GPs, as described above. Vaccines were diluted to 2 mg/ml in sterile 0.9% saline for injection. Aliquots containing 0.6 ml of vaccine were frozen until use in vaccination studies.

Mice. Six-week-old female C57BL/6 mice were obtained from either Charles River Laboratories (Kingston, NY) or The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free environment in the animal facility at the University of Massachusetts Medical School, All animal procedures were carried out under a protocol approved by the University of Massachusetts Medical School Institutional Use and Care of Animals Committee.

Vaccination and challenge studies. The GP-based vaccines and the live *Cryptococcus* vaccines (0.1 ml dose) were administered three times at 2-week intervals as a subcutaneous injection at the midline of the abdomen, as described elsewhere (23, 24). Two weeks after the third vaccination, the mice were challenged with *C. neoformans* strain Kn99 or *C. gattii* strain R265. Mice were anesthetized with 2% isoflurane (Piramal Health Care, Andrah Pradesh, India) in a laboratory animal anesthesia system (VetEquip, Livermore, CA) and inoculated intranasally with 50 μl of fungal suspension, so as to deliver 10^3, 10^4, or 10^5 CFU of *Cryptococcus*. For survival studies, mice were monitored twice daily and euthanized if they

developed advanced signs of disease, including ataxia, listlessness, and failure to groom. At the termination of the study, surviving mice were euthanized, and lungs were removed and homogenized in 4 ml PBS containing penicillin and streptomycin. Undiluted and diluted homogenates were plated on Sabouraud dextrose agar, Emmons, and incubated at 30°C for 2 to 3 days, at which time CFU of *Cryptococcus* were enumerated. For other studies, mice were sacrificed 2 weeks after fungal challenge, at which time lung and brain CFU were counted.

Lymphoproliferation. An LP assay was performed essentially as described elsewhere (23, 24). Briefly, 2 or more weeks after the third vaccination, mice were euthanized and spleens and inguinal lymph nodes were collected. Single-cell suspensions were prepared, and CD4$^+$ T cells were isolated over magnetic bead columns by negative selection according to the manufacturer's protocol (Miltenyi Biotec, San Diego, CA). Purified CD4$^+$ T cells (10^5/well) were incubated with the indicated stimuli in round-bottom 96-well plates containing 200 μl R10 medium. Mitomycin C-treated murine bone marrow-derived dendritic cells (BMDCs; 10^4/well, generated as described elsewhere [23]) served as antigen-presenting cells. After a 3-day incubation, [^{3}H]thymidine (1 μCi/well; PerkinElmer, Boston, MA) was added, and the plates were incubated for an additional 24 h. Cells were then collected on filter paper (Wallac, Turku, Finland) by using a harvester (Brandel, Gaithersburg, MD), and [^{3}H]thymidine incorporation was measured with a beta counter (1450 MicroBeta; Wallac). Each condition was analyzed in triplicate.

Polychromatic fluorescence-activated flow cytometry. Fluorescence-activated cell sorting (FACS) flow cytometry was performed as previously described with slight modifications (24). Briefly, single-cell suspensions were prepared from mouse lungs by using a lung dissociation kit (Miltenyi Biotec) according to the manufacturer's protocol. The cell pellets were resuspended in 40% Percoll in PBS, layered on top of 67% Percoll in RPMI, and centrifuged at 600 × g for 20 min. The lung leukocytes at the interface were collected and washed twice with PBS. Cells were then counted on a hemocytometer and incubated with the indicated stimuli for 24 to 28 h. Brefeldin A (10 μg/ml) was added for the last 5 h of incubation. Following incubation with Fc-blocking antibodies (BD Biosciences, San Jose, CA), the cells were stained for surface antigens (CD3 conjugated to fluorescein isothiocyanate, CD4 conjugated to V500), and CD8 conjugated to V450) and intracellular cytokines IFN-γ (Alexa 700 conjugate), IL-4 (allophycocyanin conjugate), and IL-17A (phycoerythrin conjugate) after fixation and permeabilization using the Cytofix/Cytoperm fixation/permeabilization solution kit (BD Biosciences). Single-color controls for CD3, CD4, CD8, IFN-γ, IL-4, and IL-17A were prepared using OneComp ebeads according to the manufacturer's protocol (eBiosciences). FACS data were collected using an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Briefly, singlets were selected while debris and SSC^{hi} cells were removed. Then, the CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^+$ cell populations were selected from the CD3$^+$ population. Finally, the expression levels of IFN-γ, IL-4, and IL-17A on the CD3$^+$ CD4$^+$ CD8$^-$ and CD3$^+$ CD4$^-$ CD8$^+$ gated populations were examined.

Statistics. Data were analyzed using GraphPad Prism, version 6.0 (GraphPad Software, La Jolla, CA). Kaplan-Meier survival curves were analyzed for significance using the log rank test. For the *ex vivo* experiments, comparisons were made using a one-way analysis of variance (ANOVA) with the Tukey multiple correction.

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