Published in final edited form as:

Vaccine. 2018 May 31; 36(23): 3359–3367. doi:10.1016/j.vaccine.2018.04.047.

Vaccination with an Alkaline Extract of *Histoplasma capsulatum* Packaged in Glucan Particles Confers Protective Immunity in Mice

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Abstract

Infection with the dimorphic fungus, *Histoplasma capsulatum*, occurs world-wide, but North and South America are regions of high endemicity. Interventions to mitigate exposure and consequent disease are limited to remediating a habitat harboring the fungus. The development of a vaccine to prevent infection or lessen its severity is an important advance in disease prevention. Accordingly, we prepared an alkaline extract from the yeast phase of *Histoplasma* and encased it in glucan particles that act as an adjuvant and delivery vehicle. Immunization of C57BL/6 mice with this encapsulated extract decreased the number of CFUs in lungs and spleens at days 7 and 14 following intranasal infection. Moreover, this vaccine conferred protection against a lethal challenge with the fungus. Cytokine assessment in lungs at a time when the CFUs were similar between controls and vaccinated groups revealed increased quantities of interferon- γ and interleukin-17 in vaccine recipients. This finding was supported by increased generation of both Th1 and Th17 cells in lungs and draining lymph nodes of vaccinated mice compared to controls. Neutralization of interferon- γ or interleukin-17 blunted the effectiveness of vaccination. To identify the proteins comprising this extract, liquid chromatography tandem mass spectrometry was performed. Thus, an *H. capsulatum* alkaline extract packaged in glucan particles confers protection in an interferon- γ and interleukin-17-dependent manner. Discovery of a single protein

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Contributions: W.R.B., H.H., C.A.S., G.S.D., and A.A. performed experiments and created reagents. G.S.D., G.R.O., A.A., H.H., C.A.S., W.R.B. and S.M.L. designed experiments and wrote the manuscript.

Competing Interests: The authors report no conflicts of interest.

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or a few proteins in this admixture that mediate protective immunity would represent significant progress in efforts to prevent histoplasmosis.

Keywords

Lung; Fungus; T cells; Immunity; Rodent; Glucan Particles

Introduction

The mammalian pathogen, *Histoplasma capsulatum*, is a dimorphic fungus with a global distribution. It resides in the soil of numerous regions of the world but is highly endemic to the Midwestern and Southeastern US and Central and South America. Humans are coincidentally infected via the lungs upon disturbance of the soil. The spectrum of illness ranges from asymptomatic to a life-threatening progressive infection. It is estimated that 250,000 to 500,000 new infections occur annually in the US alone. Among those infected the majority will require medical intervention. However, approximately 10% of individuals develop serious infection that necessitates antifungal therapy [1].

The implementation of a targeted vaccination strategy could reduce the severity of disease if not completely prevent it. The at-risk populations include those with HIV infection, recipients of immunosuppressive agents such as glucocorticoids, individuals with inflammatory diseases that are treated with biologicals especially the tumor necrosis factor antagonists, and workers with frequent exposure to soil that is laden with fungal spores [2-6]. To date, there is no licensed vaccine for this fungal infection or for any of the medically important human fungal pathogens. We previously reported that *H. capsulatum* heat shock protein 60 is immunogenic for mouse and human cells and protected against challenges with yeast cells. Protection required CD4⁺ and CD8⁺ T cells [7-10]. However, a concern about the use of this immunogen as a vaccine candidate is its identity with the corresponding mammalian gene. Theoretically, this could induce an autoimmune reaction [7]. Moving forward other vaccine candidates are needed. While genetically manipulated yeast cells that exhibit impaired growth might confer excellent protection, the likelihood that such an agent could be deployed for human use is remote.

Given those considerations, we utilized an alkaline extract from *H. capsulatum* to generate a protective immunogen. This material was packaged in glucan particles (GPs) which combine antigen presenting cell targeting with potent adjuvant activity. Our data demonstrate that GPs containing the alkaline extract protected mice against sublethal and lethal challenges with *H. capsulatum*. The protective efficacy was associated with enhanced interferon (IFN)- γ and interleukin (IL)-17 generation by T cells, in particular CD4⁺ T cells. These results provide a fresh approach to creating a vaccine for *H. capsulatum*.

Materials and Methods

Mice

Male C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in isolator cages and maintained by the Department of Laboratory Animal

Medicine, University of Cincinnati or the University of Massachusetts Medical School. Both are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experiments were performed in accordance with the Animal Welfare Act guidelines of the National Institutes of Health, and all protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati and the University of Massachusetts Medical School. Infected animals were housed in an ABSL2.

Preparation of *H. capsulatum* and infection of mice

H. capsulatum strain G217B yeast cells were grown for 72 h at 37° C as described (15). Mice were inoculated intranasally with 2×10^6 yeasts or 1.5×10^7 yeasts in ~30 µl of Hank's Balanced Salt Solution (HyClone, Logan, UT).

Organ culture for *H. capsulatum*

Organs were homogenized in sterile HBSS and serially diluted and plated onto Mycosel™-agar plates containing 5% sheep blood and 5% glucose. Plates were incubated at 30° C for 7 days. The limit of detection was 10^2 colony forming units (CFU).

Preparation of alkaline extract

The extract was prepared similar to that for *C. neoformans* [11]. Yeast cells were grown to mid to late log phase in Ham's F12 medium, collected, washed three times in phosphate-buffered saline (PBS). Pellets were stored at -20°C. For alkaline extraction, the cell pellets were thawed at 22°C, suspended in 1.5 volumes of 0.1 M potassium hydroxide (KOH) and rotated for 10 min at 22°C. Particulate material was removed by centrifugation, as described above. For each ml 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. The precipitate was collected following centrifugation at $6,000 \times g$ for 10 min at 4°C, suspended in 15 ml 0.1 M ammonium acetate, then re-precipitated with 2 volumes of ethanol, and stored at -20°C. The precipitate was lyophilized. Subsequently, it was then suspended in 7.5 ml 0.01 M ammonium acetate and centrifuged at $6,000 \times g$ for 10 min at 4°C. The supernatant containing soluble alkaline extracted antigens was stored at -80°C. Protein concentration was assayed by the bicinchoninic acid assay.

Glucan particle vaccine

Saccharomyces cerevisiae cells were converted into GPs following a series of hot alkali, organic, and aqueous extraction steps, as described previously [12]. The final product consisted of a highly purified 3- to 4-µm-diameter yeast cell wall preparation devoid of cytoplasmic contents and outer mannoproteins and bounded by a porous, insoluble shell of β-glucans. The final GP vaccine consisted of *Histoplasma* alkaline extract, mouse serum albumin (Equitech-Bio, Kerrville, TX), and yeast RNA (yRNA; Sigma, St. Louis, MO) complexed within the glucan shells. Samples to be loaded into GPs were concentrated in 6M urea, 20 mM Tris-HCl, pH 7.9 at 10 mg/ml or 50 mg/ml by protein content. Antigens were loaded into GPs and complexed with MSA and yRNA as described elsewhere [12]. Vaccines were diluted in sterile 0.9% saline for injection. A control vaccine consisted of GPs loaded with MSA and yRNA but without *Histoplasma* alkaline extract. Vaccines were stored in 0.6-

ml aliquots at -80°C , thawed, and vortexed prior to use. Vaccines were also extracted and analyzed by SDS-PAGE to quantify antigen/MSA loading.

To vaccinate mice, they were given 10^8 particles i.n. (in two 50 μl aliquots) followed by subcutaneous injections of 10^8 particles 2 wk and then 4 wk later. In initial experiments mice were injected subcutaneously 3 times with 100 μl (10^8 particles) of GPs. This number of particles contained 50 μg of extract or GPs containing mouse serum albumin. Each injection was separated by 2 wks. Mice were challenged with *H. capsulatum* 2-4 weeks following the last injection. In another set of experiments, we injected 10^8 particles once i.n. followed by infection with 2×10^6 yeasts i.n. 2 weeks later.

Analysis of alkaline extracts by electrospray ionization Liquid Chromatography-Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS)

Alkaline extracts were processed for LC-MS/MS analysis as described [11]. Analysis and database searching was with Mascot (Matrix Science, London, UK; version 2.1.1.21) against the *H. capsulatum* database (GenBank, *Histoplasma capsulatum*, taxid 5037). Scaffold software (Proteome Software, Inc., Portland, OR; version 4.8.4) was used to validate the identity of peptides (trypsin digest products) and proteins. Probabilities were assigned by using the Protein Prophet algorithm at 70% for peptides and 90% for proteins that included at least 2 identified peptides. The relative amount (normalized iBAQ value) of each protein was determined so that protein abundance could be ranked. Protein sequences were analyzed using SignalP 4.1 for signal peptide cleavage (<http://www.cbs.dtu.dk/services/SignalP/>).

Flow cytometry analysis

Fluorescence-activated cell sorting (FACS) flow cytometry was performed as previously described with slight modifications [13]. 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 $600 \times g$ for 20 min. The lung leukocytes at the interface were collected and washed twice with PBS. For lymphoid tissue, spleens and lymph nodes were collected minced through a stainless steel mesh and red cells lysed. Lung leukocytes ($2-4 \times 10^5$) were incubated in 200 μl of RPMI + 10% fetal bovine serum in each well of a 96 well plate; lymphoid cells (2×10^6) were incubated in 500 μl of medium in each well of a 48 well plate. Cells were incubated with GPs at a ratio of 3 particles for every 10 leukocytes. For heat-killed yeast cells, they were incubated at a ratio of 3 yeast cells for every 10 leukocytes. Cells exposed to the indicated stimuli for 24 to 28 h. Brefeldin A (10 $\mu\text{g}/\text{ml}$) 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), and IL-17A (phycoerythrin conjugate) after fixation and permeabilization using the Cytotfix/Cytoperm fixation/permeabilization solution kit (BD Biosciences). Single-color controls for CD3, CD4, CD8, IFN- γ , and IL-17A were prepared using OneComp ebeads according to the manufacturer's protocol (eBioscience, San Diego, CA). Briefly, singlets were selected while debris and side scatter (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 and IL-17A on the CD3⁺ CD4⁺CD8⁻ and CD3⁺CD4⁻CD8⁺ gated populations were examined. FACS data were collected using an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Cytokine assays

Lung homogenates of mice infected with *H. capsulatum* were prepared as described [14]. They were clarified by centrifugation and cytokines were assayed using MagpixTM beads (EMD Millipore, Billerica MA).

Anti-cytokine antibody treatment

Antibody to IL-17 A/F (clone 50104) was purchased from Thermo Fisher Scientific. Anti-IFN- γ (clone XMG 1.2) was purchased from the National Cell Culture Center, Minneapolis, MN. Isotype controls were purchased from Leinco Technologies (St. Louis, MO). For studies with anti-IL-17, mice were treated with 100 μ g of rat IgG_{2a} or anti-IL-17 intraperitoneally on the day before infection. In experiments with anti-IFN- γ , mice received 1 mg rat IgG_{2b} or anti-IFN- γ the day before infection.

Statistical analyses

The log rank test was used to analyze differences in survival; analysis of variance was employed to analyze differences in cytokine production. If the data were not normally distributed, the Mann-Whitney test was used. Dunnet's correction was used for multiple comparisons.

Results

Protective efficacy of an *H. capsulatum* alkaline extract

Previous reports have documented that an alkaline extract from *Cryptococcus neoformans* confers protection against a pulmonary infection [11]. Using a similar approach, we prepared an extract from the yeast cells of *H. capsulatum* strain G217B and then encased the extract in a biopolymer matrix within GPs. Initially, mice were vaccinated subcutaneously with the extract three times and challenged with a sublethal (2×10^6 yeast cells) inoculum of the fungus. At days 7 after delivery of yeast cells intranasally (i.n.), the lungs and spleens were cultured for the presence of the fungus. This mode of injection of the antigen-laden GPs did not stimulate a protective immune response (Fig. 1 A and B).

We reasoned that immunization at the site of infection might promote the protective qualities of the alkaline extract. Hence, we altered our vaccination protocol to incorporate an initial inoculation into the lung by i.n. installation followed by two subcutaneous injections each separated by 2 weeks. Mice were then challenged with a sublethal dose of yeast cells and lungs and spleens cultured for the presence of *H. capsulatum*. This change in the route of inoculation resulted in a marked reduction in the number of CFUs recovered from lungs and spleens of vaccinated animals, both at 7 (Fig. 1C and D) and 14 (Fig. 1 E and F) days after infection. Since the approach using i.n. followed by subcutaneous injection conferred protective immunity, we explored whether a single i.n. injection would be beneficial. Mice

were given control GPs or GPs containing the alkaline extract once i.n.; 2 wk later they were challenged with *H. capsulatum* and fungal recovery assessed 1 wk later. One single i.n. injection did not promote protective immunity (Supplement Fig. 1).

Subsequently, we asked whether the impact of this vaccination strategy would protect mice from a lethal inoculum of the *H. capsulatum*. Mice were immunized with GPs containing the alkaline extract or control mouse serum albumin and 2 weeks later were challenged with 1.5×10^7 yeast cells i.n. Seventy-five percent of the alkaline extract recipients survived ($p < 0.005$) whereas none of the mice given mouse serum albumin encased in GPs lived beyond 30 days (Fig.1G).

Vaccine-induced effector T cell differentiation

We sought to categorize the cytokine profile of effector T cells following vaccination but before infection. Mice were injected intranasally (i.n.) followed by two subcutaneous injections of the alkaline extract or control mouse serum albumin vaccines in GPs. Two weeks later, leukocytes from lungs and draining lymph nodes were collected and re-stimulated ex vivo with the cognate antigen (3 GPs per 10 leukocytes) and analyzed by flow cytometry. Vaccination with the alkaline extract vaccine induced both Th17 and Th1 cells in the lungs and draining lymph nodes. In addition, a small but detectable population of IL-17A⁺IFN- γ ⁺ CD4⁺ T cells was observed. We also assayed the CD8⁺ T cell response to vaccination. Although not as robust as that seen for CD4⁺ T cells, a distinct population of IFN- γ ⁺ CD8⁺ T cells was observed. No IL-17A⁺ CD8⁺ T cells (Tc17) were evident in lungs or draining lymph nodes (Fig. 2). We quantified the number of cytokine positive cells and demonstrated that Th1 and Th17 cells were more numerous in vaccinated animals. Likewise, the number of Tc1 (IFN- γ ⁺) were greater in number in this group while Tc17 were fewer (Fig. 2). Representative flow cytometry plots are shown in Figure 3.

Cytokine responses in lungs following infection

We posited that vaccinated mice would manifest a quantitative change in several cytokines after infection with *H. capsulatum*. One of the difficulties in assessing this response is that different fungal burdens between groups may alter the quantity of cytokines. Hence, we adopted a strategy to examine cytokines when the fungal burdens between groups were similar. We analyzed the number of *H. capsulatum* CFUs at early time points since we already were aware that these values diverged at days 7 and 14. The number of fungi in lungs and spleens at days 1 and 3 post-infection did not differ between recipients of either the GPs containing mouse serum albumin or alkaline extract (Fig. 4 A and B). We restricted our analysis to those two days. At days 1 and 3, both IL-17A and IFN- γ were increased in the alkaline extract-vaccinated mice (Fig. 4 C and D). IL-4, granulocyte macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)- α were similar between groups on day 1 (Supplemental Table 1). On day 3, GM-CSF and IL-4 in the lungs of vaccinated mice exceeded that of controls. However, the values for IL-4 were quite low and unlikely to be biologically important. These results indicate that: 1) the accelerated clearance of *H. capsulatum* did not transpire until after day 3, and 2) the quantitative increase in IL-17 and IFN- γ developed very shortly after infection.

Impact of IL-17 and IFN- γ on vaccine-mediated immunity

To ascertain the impact of IL-17 and IFN- γ on the effector phase of vaccine-induced immunity, mice were administered anti-IL-17 or anti-IFN- γ antibodies after vaccination but before infection with *H. capsulatum*. CFUs were determined at day 7 of infection. Vaccinated mice administered the control antibody manifested an 80% decrease in CFUs (Fig. 5A and B). The fungal burden in controls and vaccinated mice was markedly higher upon neutralization of IFN- γ . The data are shown as log₁₀ CFUs because of their 100-fold difference between groups. In a separate experiment, we assessed the impact of IL-17. Vaccinated mice given control antibody exhibited a 50% decline in CFUs. This protection was nearly abolished by anti-IL-17 only in the lungs. A reversal of the effect of anti-IL-17 was not detected in spleens (Fig. 5D). Thus, protection mediated by the alkaline extract was dependent on both IFN- γ and IL-17.

LC-MS/MS analysis of the antigenic extract

To gain a better understanding of the complexity of this extract, we subjected it to LC-MS/MS analysis and ranked identified proteins by their relative abundance. We analyzed two independent extracts that protected mice and that were prepared 1 year apart. Ninety-one percent of the 1363 proteins identified were found in both extracts and had similar quantitative values. Table 1 displays the top 20 proteins in terms of quantity, and supplemental Table 2 provides an expanded list that includes 232 proteins with relative abundance within the range of 2-100. Among these, there was a great deal of heterogeneity in terms of function with the majority of proteins being cytoplasmic. Two of the 20 most abundant proteins, glucosidase (EEH11584) and allergen (EEH02677) are predicted to have a signal peptide and to be extracellular.

Discussion

Fungal infections remain a serious health threat to humans despite the introduction of new antifungal therapies. Drug resistance and impaired immunity are two factors that hamper successful resolution of infection. The creation of vaccines for the medically important fungi has been a long sought goal despite many hurdles given the complexity of the organisms. Two key elements that are necessary for creating a beneficial vaccine are the proper antigen or antigens and an immunogenic adjuvant. In this regard, GPs are an exciting and useful consideration as a combined antigen-presenting cell targeted delivery system and adjuvant. GP vaccines have been shown to be highly immunogenic, engaging dectin-1 and complement receptor 3 (CD11b), and stimulating strong IL-17 and IFN- γ responses [11, 13]. Hence, we undertook a study to capitalize on the utility of GPs for a vaccine for *H. capsulatum*. We also took advantage of recent data indicating that an alkaline extract from *C. neoformans* is protective when encased in GPs [11]. We produced an alkaline extract from *H. capsulatum* yeast cells in a manner identical to that of *C. neoformans*. As with the cryptococcal study, the alkaline extract from *H. capsulatum* when packaged in GPs, conferred vaccine-induced immunity. This extract not only reduced fungal burden by ~ 80% but improved survival in mice challenged with lethal inoculum. These data extend the utility of an alkaline extract to another fungal pathogen. This suggests that this material may be useful for vaccine antigen discovery for other fungal pathogens.

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GPs as a vaccine delivery vehicle are ideal for fungal infections which typically rely on IL-17 and IFN- γ for clearance [15-21]. These particles can be packaged with multiple antigens as well as other modifiers of immunity such as toll-like receptor agonists DNA or RNA to modulate the immune response. GPs will chiefly be recognized and internalized by antigen-presenting cells (APCs) since these are the cells that typically bear dectin-1 and complement receptor 3 [22-24]. Following engagement of these receptors, APCs must generate the proper cytokines that shape both Th1 and Th17 differentiation. In this regard, engagement of dectin-1 by β -glucan increases IL-23 and IL-12 p40 but not IL-12p35 or IL-12p70 [25-27]. This finding raises the issue of how dectin-1 signaling leads to the differentiation of Th1 cells. One clue is that exposure to β -glucan and IFN- γ results in Th1 cells [28]. Thus, dendritic cell release of IL-12p70 likely requires exposure to IFN- γ to prompt Th1 cell expansion, and the probable source will be an innate cell such as natural killer cells.

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IFN- γ and IL-17 are necessary for vaccine-induced immunity to fungal pathogens [19, 29-32]. These two cytokines are largely generated by CD4⁺ T cells. Surprisingly, we detected a Tc1 (IFN- γ ⁺ CD8⁺ T cells) response following immunization but before infection. This finding was somewhat unexpected given that antigenic protein loaded into GPs are most likely shuttled through the class II major histocompatibility complex pathway. However, the findings do suggest that the GP-antigen complex undergoes cross-presentation to trigger the expansion of Tc1 [33, 34].

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An unanticipated feature of this work is that the efficacy of the alkaline extract was dependent on the route of immunization. Initial attempts to vaccinate strictly using the subcutaneous route failed to alter the number of CFUs in lungs of spleens of mice. This finding differs from that observed with the alkaline extract derived from *C. neoformans* in which 3 subcutaneous injections provided protective immunity [11, 35]. However, when mice were given the *H. capsulatum* alkaline extract i.n. initially followed by subcutaneous injection, the results were strikingly different. The GPs containing the *Histoplasma* alkaline extract given in this manner reduced the burden in mice infected with a sublethal number of yeast cells and reduced mortality appreciably in mice given a lethal inoculum. The reason(s) why a onetime i.n. inoculation converts a vaccine candidate from non-functional to functional remains enigmatic. Nevertheless, the findings illuminate an important concept in vaccinology. While an adjuvant is central for efficacy especially for peptides or proteins, the route of delivery is another variable in determining efficacy. Thus, delivery of the vaccine candidate into the portal of entry of a pathogen may be one important way in which to enhance its effectiveness.

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To discern the vital soluble mediators of resistance, we analyzed lung homogenates for the presence of multiple cytokines. We chose to examine time points in which the number of CFUs between the controls and the experimental group were similar. This strategy reduces the confounding variable of the number of organisms in a given tissue. IFN- γ and IL-17 were elevated whereas TNF- α , GM-CSF and IL-4 were not. To decipher the importance of the former two cytokines, we vaccinated mice and subsequently administered blocking antibody. Neutralization of either IFN- γ or IL-17 sharply restricted the effectiveness of vaccination. We have previously reported the importance of IFN- γ upon immunization with

heat shock protein 60 [8, 32]. IL-17 has been implicated in vaccine-induced immunity associated with subcutaneous injection of live yeast cells [19, 31]. The results presented herein indicate that these two cytokines exert independent functions to confer protective immunity upon vaccination. Therefore, IFN- γ and IL-17 are not interdependent. The loss of one causes a collapse of protective immunity.

The top 20 proteins that were identified by LC-MS/MS did not cluster into any specific grouping other than a preponderance of cytoplasmic proteins. Two proteins, however, are predicted to have a signal peptide for conventional, extracellular secretion, a beta glucosidase and an allergen-like protein. Of these 20, only one also has been studied in models of histoplasmosis. *Histoplasma* cyclophilin A is found on the surface of *Histoplasma* yeast cells and is a ligand for the integrin $\alpha_5\beta_1$ (very late antigen-5) on human dendritic cells [36]. Among the 232 most abundant proteins identified in the extract (Table 2 supplement) were heat shock protein 60, heat shock protein 70, or H antigen, which were previously reported to mediate protection to *H. capsulatum* when delivered as a vaccine. Sec 31 which also mediates protection was not identified in the extracts [37-40]. It is not yet clear how many other proteins in the extract that while predicted to be cytoplasmic are also found on the cell surface and are potential vaccine candidates.

A comparative analysis of the 20 most abundant proteins in the alkaline extracts from *C. neoformans* and *H. capsulatum* revealed no shared protein homologs. The finding that the alkaline extracts were disparate is not surprising given as *H. capsulatum* is an ascomycete whereas *C. neoformans* is a basidiomycete. Sensitivity to alkaline extraction may vary as a result of differences in their cell wall organization and composition. How proteins are eluted from either fungus by alkaline treatment is not clear, although it is predicted to disrupt hydrogen bonding.

Conclusion

This preclinical study has identified an extract from *H. capsulatum* that confers protective immunity when packaged in GPs. The data also affirm the utility of GPs as a combined delivery vehicle and adjuvant. This finding serves as a platform to pursue the identity of a protein or proteins in that complex admixture that enhance protective immunity. In addition, the effectiveness of a protein or proteins as a vaccine may be improved upon by inclusion in GPs of additional compounds that modify immune responses to the agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (R01 AI 102618 and R01 025780) and the Veterans Administration (101 BX000717). The authors thank Dr. John Leczyk and the Mass Spectrometry Facility at the University of Massachusetts Medical School for performing the LC-MS/MS analyses.

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Highlights

- An alkaline extract from the fungus, *Histoplasma capsulatum*, packaged in glucan particles confers protection when used as a vaccine.
- This vaccine induces a vigorous Th1 and Th17 response in lungs and lymphoid tissue.
- Tc17 cells were modestly induced in lungs.
- The vaccine provokes production of both IL-17 and IFN- γ .
- IL-17 and IFN- γ are independently required for protection.

Figure. 1.

H. capsulatum alkaline extract in GPs promotes protective immunity. A and B. Box plot of CFUs of lungs and spleens of mice injected subcutaneously $\times 3$ with GPs containing 50 μ g of mouse serum albumin (GP control, GP MSA) or 50 μ g of alkaline extract. Mice assessed 7 days after infection. $n=12$. NS = not significant ($p > 0.05$). C-F. Box plot of lung and spleen CFUs of mice immunized initially with GPs i.n. followed by 2 subcutaneous injections. C and D are data from day 7 and E and F are from day 14. $N=12-18$. * = $p < 0.05$ ** = $p < 0.01$, and *** = $p < 0.005$. Mann-Whitney test used. G. Survival curve of mice challenged with 1.5×10^7 yeast cells i.n. 2 weeks after conclusion of vaccination. Pooled data from 2 independent experiments with a total $N=12/\text{group}$.

Figure. 2.

Numbers of IFN- γ , IL-17, or IFN- γ and IL-17 positive T cells in lungs and lymphoid tissue 2 weeks after vaccination with GPs. Stimulus refers to the in vitro inducer of cytokine synthesis. GP MSA= mouse serum albumin, GP Alk Ext = alkaline extract, and HK Hc = heat killed *H. capsulatum*. N=4. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$. p values derived from comparison to GP MSA

Figure. 3.

Proportions of IFN- γ , IL-17, or IFN- γ and IL-17 positive T cells in lungs and lymphoid tissue 2 weeks after vaccination with GPs. Representative flow plots of T cells stained with IFN- γ or IL-17. Cells processed as in the Material and Methods. N =4.

Figure 4.

H. capsulatum CFUs and cytokine responses at days 1 and 3 of infection. Mice were vaccinated as described in Material and Methods. Two weeks later, they were challenged with 2×10^6 yeast cells i.n. Panel A and B show the CFUs at days 1 and 3 after infection. Data represent mean CFUs $\pm$ SEM of 6 animals/group. Panels C and D manifest IFN- γ and IL-17 quantities in lung homogenates. Data represent mean $\pm$ SEM of 6. ** = $p < 0.01$. ND, not detected.

Figure. 5.

Effect of anti-cytokine antibody on vaccine effectiveness. Panels A and B contain CFUs in lungs and spleens of mice given control antibody or anti-IFN- γ . Data represent mean log₁₀ CFUs $\pm$ SEM of 6 mice/group. ** = $p < 0.01$. Data log transformed because of large splay among groups. One of 2 representative experiments shown. C and D demonstrate effect of anti-IL-17. Data represent mean $\pm$ SEM of CFUs of 6 mice/group. ** = $p < 0.01$. MSA = mouse serum albumin. Alk Ext = alkaline extract. NS = not significant.

Table 1
Proteomic analysis of Alkaline Extract from *H. capsulatum* yeast cells

Designation	Accession Number	Relative Abundance ^a
Non-histone chromosomal protein 6	EEH04805.1	100
Hypothetical protein	EGC46375.1	97
Cell cycle control protein	EGC46781.1	54
40 S Ribosomal Protein	EEH10971.1	42
HAD-superfamily hydrolase	EEH06199.1	42
Eukaryotic translation initiation factor 5A-2	EGC49667.1	40
Cell wall synthesis protein	EEH11584.1	38
Allergen Asp F4	EEH02677.1	34
Histone 2B	AAP69672.1	33
60 S Ribosomal	EEH09457.1	33
Superoxide dismutase 3	EEH07890.1	32
Hypothetical protein	EER44935.1	32
Cyclophilin A	EER42986.1	31
Hypothetical protein	EDN08691.1	31
Hypothetical protein	EEH03619.1	31
Hypothetical protein	EDN03314.1	29
Mitochondrial ATP synthase	EER40499.1	23
Cofilin	EER40488.1	22
GTP-nuclear binding protein GSP1/Ran	EER43989.1	22
Cytochrome c	EDN10135.1	21

^aRelative abundance of protein, averaged for two *H. capsulatum* extracts. The most abundant protein, non-histone chromosomal protein 6, was arbitrarily set at 100.