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A Proteomic-based Approach for the Identification of Immunodominant *Cryptococcus neoformans* Proteins

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Abstract

Cryptococcus neoformans is an opportunistic fungal pathogen that can cause life-threatening meningoencephalitis in immune compromised patients. Previous, studies in our laboratory have shown that prior exposure to an IFN- γ -producing *C. neoformans* strain (H99 γ) elicits protective immunity against a second pulmonary *C. neoformans* challenge. Here, we characterized the antibody response produced in mice protected against experimental pulmonary *C. neoformans* infection compared to non-protected mice. Moreover, we evaluated the efficacy of using serum antibody from protected mice to detect immunodominant *C. neoformans* proteins. Protected mice were shown to produce significantly more *C. neoformans*-specific antibodies following a second experimental pulmonary cryptococcal challenge compared to non-protected mice. Immunoblot analysis of *C. neoformans* proteins resolved by 2-DE using serum from non-protected mice failed to show any reactivity. In contrast, serum from protected mice was reactive with several cryptococcal protein spots. Analysis of these spots by capillary HPLC-ESI-MS/MS identified several cryptococcal proteins shown to be associated with the pathogenesis of cryptococcosis. Our studies demonstrate that mice immunized with *C. neoformans* strain H99 γ produce antibodies that are immune reactive against specific cryptococcal proteins that may provide a basis for the development of immune based therapies that induce protective anti-cryptococcal immune responses.

Keywords

Cryptococcus neoformans; Cryptococcosis; ImmunoProteomics; Surface proteins; Vaccination

1 Introduction

Cryptococcus neoformans, the etiological agent of cryptococcosis, is an encapsulated fungal pathogen that frequently infects the central nervous system (CNS) of immune compromised individuals causing life-threatening meningoencephalitis [1]. Cryptococcal meningoencephalitis is the most common disseminated fungal infection in AIDS patients and those who are successfully treated for AIDS-associated cryptococcal meningitis oftentimes require life-long maintenance anti-fungal therapy [2] due to a high relapse rate [3]. Immune reconstitution due to highly active antiretroviral therapy (HAART) has been associated with a marked decrease in AIDS-associated cryptococcosis [4-5]. However, the use of HAART to treat HIV infection has also been associated with the development of *C.*

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neoformans-related immune reconstitution inflammatory syndrome (IRIS) which is also life-threatening [6-8]. Nonetheless, the clinical relevance of invasive cryptococcal disease continues to gain prominence due to the increasing population of immune compromised patients (i.e. HIV infected individuals [9-13], individuals receiving corticosteroid therapy [14-16], lymphoproliferative disorders [17-20], and organ transplant recipients [21]). Studies have shown that 2.8% of organ transplant recipients can develop cryptococcal infections resulting in an overall death rate of 42% [21]. In addition, the acute mortality rate is between 10-25% in medically-advanced countries [22] and at least one third of patients with cryptococcal meningitis who receive appropriate therapy will undergo mycologic and/or clinical failure [23-24]. Currently, there are no standardized vaccines available for the prevention of fungal diseases in humans underlying an urgent need for additional therapeutics to combat fungal infections.

Cell-mediated immunity (CMI) by T helper (Th) 1-type CD4⁺ T cells is the predominant host defense mechanism against *C. neoformans* infections [25-31]. Consequently, there has been great interest in identifying the cryptococcal antigens that elicit protective CMI responses to cryptococcal infection. Vaccination of mice with a *C. neoformans* culture filtrate antigen (CneF) in complete Freund's adjuvant (CFA) has been shown to induce delayed-type hypersensitivity (DTH) responses and limited protection in mice against a subsequent cryptococcal challenge [32]. Fractionation of CneF demonstrated that it is composed of glucuronoxylomannan (GXM) which is inhibitory to T cell proliferation [33] and a mannoprotein (MP) fraction which is largely responsible for the stimulation of anti-cryptococcal CMI responses observed in mice [34]. Since this initial observation, several MPs have been identified that stimulate T cells responses and mediate partial-protection in mice against experimental cryptococcal infection [35,36 and [37]. Other immunogenic proteins include a protein identified from culture supernatants, designated *DHA1*, [38] and a cryptococcal polysaccharide deacetylase that was shown to prolong survival and decrease fungal burden in mice [39]. The value of these cryptococcal proteins as vaccine candidates for the prevention and/or treatment of *C. neoformans* infections or relapses in immune suppressed patients have yet to be validated on a definitive basis. However, it certainly appears that these and other yet to be identified cryptococcal proteins have the potential to improve the management of cryptococcosis.

We have recently demonstrated that an experimental pulmonary infection of mice with a *C. neoformans* strain that was genetically modified to produce IFN- γ results in the induction of Th1-type cell mediated immune responses and resolution of the acute infection [40]. Moreover, prior challenge with this IFN- γ -producing *C. neoformans* strain and not heat-killed *C. neoformans* yeast results in complete protection against a second pulmonary challenge with a pathogenic *C. neoformans* strain. We are thus able to utilize this model system as a tool to elucidate the mechanisms that confer protective host immune responses against *C. neoformans* infections. Consequently, the studies presented herein showed that serum from mice protected against a second experimental pulmonary challenge with a pathogenic cryptococcal strain could be used to identify immune dominant cryptococcal proteins. These results suggest other putative targets for the development of anti-fungal drugs or vaccines.

2 Materials and Methods

2.1 Strains and media

C. neoformans strains H99 (serotype A, Mat α) and H99 γ (an interferon-gamma producing *C. neoformans* strain derived from H99 [40]) were recovered from 15% glycerol stocks stored at -80°C prior to use in the experiments described herein. The strains were maintained on yeast-extract-peptone-dextrose (YPD) media (1% yeast extract, 2% peptone,

2% dextrose, and 2% Bacto agar). Yeast cells were grown for 18-20 h at 30°C with shaking in YPD broth (Bectin, Dickinson and Company, Sparks, MD), collected by centrifugation, washed three times with sterile phosphate-buffered saline (PBS), and viable yeast quantified using trypan blue dye exclusion in a hemacytometer.

2.2 Murine Model

Female BALB/c (H-2^d) mice, 4 to 6 weeks of age (National Cancer Institute/Charles River Laboratories), were used throughout these studies. Mice were housed at The University of Texas at San Antonio Small Animal Laboratory Vivarium and handled according to guidelines approved by the Institutional Animal Care and Use Committee. Pulmonary *C. neoformans* infections were initiated by nasal inhalation as previously described [41-42]. Briefly, anesthetized mice received an initial inoculum of 1×10^4 CFU of *C. neoformans* strain H99γ or heat-killed *C. neoformans* strain H99 yeasts in 50 μl of sterile PBS and allowed 100 days to resolve the infection. Subsequently, the immunized mice received a second experimental pulmonary infection with 1×10^4 CFU of *C. neoformans* strain H99 in 50 μl of sterile PBS. The inocula used for immunizations and rechallenge were verified by quantitative culture on YPD agar. The mice were fed ad libitum and were monitored by inspection twice daily. Mice were euthanized on days 3, 7 or 14 post secondary inoculation and lung tissues excised using aseptic technique, homogenized in 1 ml of sterile PBS, followed by culture of 1, 10 dilutions on YPD agar supplemented with chloramphenicol (Mediatech, Inc., Herndon, VA). CFU were enumerated following incubation at 30°C for 48 h. Alternatively, mice intended for survival analysis were monitored by inspection twice daily and euthanized on day 100 post secondary inoculation or if they appeared to be in pain or moribund using CO₂ inhalation.

2.3 Protein Extraction

Extraction of cryptococcal cell surface proteins was accomplished using a modified protocol previously used to extract cell surface proteins from *Candida albicans* [43]. Briefly, *C. neoformans* strain H99 yeast was incubated overnight in liquid YPD medium at 30°C. The yeast was collected by centrifugation and subsequently washed twice in sterile PBS, suspended in ammonium carbonate, (NH₄)₂CO₃, buffer containing 1% (v/v) β-ME, and incubated for 45 min at 37°C with gentle agitation. After treatment, the cells were collected by centrifugation and the supernatant fluid sterile filtered. The supernatant was then desalted and concentrated by centrifugation through an Amicon Ultrafree-15 (Millipore Corporation, Billerica, MA) centrifugal filter device. Protein content was estimated using a RC DC Protein Assay Kit (Bio-Rad, Hercules, CA). Subsequently, the proteins were further concentrated and non-protein contaminants removed using the ReadyPrep 2-D Cleanup Kit (Bio-Rad) according to manufacturer's instructions. The resulting protein pellet was resuspended in rehydration/sample buffer (Bio-Rad; 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% w/v Bio-lite 3/10 ampholytes, and trace Bromophenol blue).

2.4 Total and *C. neoformans*-specific immunoglobulin isotype typing

Immunoglobulin isotypes were determined using the components and reagents of a commercially available kit (Zymed Laboratories; San Francisco, CA) according to manufacturer's instructions. Briefly, the wells of microtiter plates (Corning Incorporated, Corning, NY) were coated with capture antibody designed to bind IgG, IgA, or, IgM (Zymed Laboratories) or 0.5 μg of *C. neoformans* β-ME cell surface proteins in phosphate buffer saline (PBS) overnight at 4°C. The plates were then washed twice with PBS containing 0.05% Tween 20 (PBST) and blocked with PBS containing 1% BSA for 1 hour at room temperature. Sera were tested at 1:100 dilutions in PBST with 1% BSA. Normal Rabbit sera and PBST buffer with 1% BSA alone were added to separate wells and served as negative controls. Plates were washed with PBST and incubated with rabbit antibodies

specific for the following mouse immunoglobulin subclasses: IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM. After incubation, wells were washed with PBST and incubated with a goat anti-rabbit IgG diluted 1:250 in PBST with 1% BSA. Plates were then washed and developed with o-phenyldiamine substrate. Color development was stopped by addition of 100 μ l per well of 1M H₂SO₄, and the plate read at 405 nm using a BioTek Elx 808 absorbance microplate reader with Gen5 v1.04.5 software (BioTek Instruments, Winooski, VT).

2.5 2-DE

IPG strips (ReadyStrip IPG, pH 4-7, Bio-Rad) were rehydrated in 200 μ l of rehydration/sample buffer (Bio-Rad) containing 300 μ g of the *C. neoformans* β -ME protein preparation. IEF was carried out using the PROTEAN IEF power supply (Bio-Rad) under the following conditions: Step 1, 250 V for 20 min.; Step 2, ramped to 8000 V over 2.5 h, and Step 3, 8000 for a total of 30,000 V/h. Strips were then placed into equilibration buffer (Bio-Rad; 6 M urea, 2% SDS, 375 mM Tris-HCl pH 8.8, 20% glycerol, 2% DTT) for 15 min. Disulfide groups were subsequently blocked for 10 min with equilibration buffer of the same composition but using 2.5% w/v iodacetamide instead of DTT. Equilibrated IPG strips were then drained and placed on the top of 12.5 % SDS-PAGE Criterion Precast Gels (Bio-Rad) and fixed using hot ReadyPrep Overlay agarose (Bio-Rad). The separation of proteins in the second dimension was carried out for 55 min at 200 V in Tris/glycine/SDS (TGS) running buffer (Bio-Rad) using Criterion electrophoresis equipment (Bio-Rad). Proteins in the gels were stained using Sypro Ruby Red protein gel stain (Molecular Probes Inc., Eugene, OR) or alternatively transferred to PVDF membranes for immunoblot analysis.

2.6 Immunoblot analysis

Separated proteins were transferred to Hybond-P PVDF membranes (GE Healthcare, Buckinghamshire, UK) using a Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) according to manufacturer's instructions. The membranes were subsequently blocked using 5% non-fat milk in 20 mM Tris containing 500 mM NaCl (Tris-buffered saline) and Tween 20 (TBS-T) for 1 hr at room temperature. The blocking solution was then discarded and the membranes incubated overnight at 4°C with a 1:200 dilution of immune sera collected on day 14 post secondary challenge from mice immunized with heat-killed wild-type *C. neoformans* strain H99 or serum collected on days 14 or 100 post secondary challenge from mice immunized with *C. neoformans* strain H99 γ . The membranes were then washed six times in TBS-T and antibody binding detected by the addition of goat anti-mouse IgG HRP-conjugated antibody (Pierce Biotechnology Inc., Rockford, IL) diluted 1:1000 in TBS-T containing 5% non-fat milk for 1 hr at room temperature. After six washes in TBS-T, the membranes were briefly incubated with SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology Inc.) and protein spots detected using a ChemiDoc XRS Camera and Quantity One 1-D analysis software (Bio-Rad).

2.7 Excision, trypsinization, and identification of protein spots by LC/MS/MS

Spots excision and identification was accomplished at the Institutional Mass Spectrometry Laboratory, The University of Texas Health Science Center at San Antonio. Spots of interest were excised from the gel and digested *in situ* with trypsin (Promega, Madison, WI; modified). The digests were analyzed by capillary HPLC-electrospray ionization tandem mass spectra (HPLC-ESI-MS/MS) using a Thermo Fisher LTQ linear ion trap mass spectrometer fitted with a New Objective PicoView 550 nanospray interface. On-line HPLC separation of the digests was accomplished with an Eksigent NanoLC micro HPLC, column, PicoFrit™ (New Objective; 75 μ m i.d.) packed to 10 cm with C18 adsorbent (Vydac; 218MS 5 μ m, 300 Å); mobile phase A, 0.5% acetic acid (HAc)/0.005% trifluoroacetic acid (TFA); mobile phase B, 90% acetonitrile/0.5% HAc/0.005% TFA; gradient 2 to 42% B in

30 min; flow rate, 0.4 μ l/min. MS conditions were: ESI voltage, 2.9 kV; isolation window for MS/MS, 3; relative collision energy, 35%; scan strategy, survey scan followed by acquisition of data dependent collision-induced dissociation (CID) spectra of the seven most intense ions in the survey scan above a set threshold. The uninterpreted CID spectra were searched against published databases available at the websites of the Stanford University Technology Center *C. neoformans* Genome Project (<http://www.sequence.stanford.edu/group/C.neoformans/index.html>), the University of Oklahoma's Advanced Center for Genome Technology (<http://www.genome.ou.edu/cneo.html>), the *C. neoformans* H99 Sequencing Project, Duke Center for Genome Technology (<http://cneo.genetics.duke.edu/>) and the NCBI database by means of Mascot (Matrix Science, London, UK). Methionine oxidation was considered as a variable modification for all searches. Cross correlation of the Mascot results with X! Tandem and determination of protein identity probabilities were accomplished by Scaffold™ (Proteome Software). A 95% confidence level threshold was used for MASCOT protein scores.

2.8 Statistical analysis

The unpaired Student's *t* test (two-tailed) and log-rank test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA) to analyze fungal burden and survival data, respectively. Significant differences were defined as $P < 0.05$.

3 Results

3.1 Assessment of protective immunity

BALB/c mice were given an intranasal inoculation with 1×10^4 CFU of *C. neoformans* strain H99 γ , or 1×10^4 CFU heat-killed *C. neoformans* strain H99 yeasts and allowed 100 days to resolve the infection. Subsequently, the mice were challenged with 1×10^4 CFU of the wild-type *C. neoformans* strain H99 and evaluated for their capacity to resolve the secondary infection over a span of 100 days post secondary challenge or, alternatively, the pulmonary fungal burden quantified on days 3, 7, and 14 post inoculation. Figure 1A demonstrates that on day 100 post secondary inoculation, all mice immunized with *C. neoformans* strain H99 γ survived a second intranasal challenge with the wild-type *C. neoformans* strain H99. In contrast, 100% mortality with a median survival time of 25 days was observed in mice immunized with heat-killed *C. neoformans* yeast and subsequently challenged with *C. neoformans* strain H99 ($P < 0.001$ compared to *C. neoformans* strain H99 γ immunized mice). Brain and pulmonary tissue cultures from mice immunized with *C. neoformans* strain H99 γ were sterile at day 100 post secondary challenge with wild-type *C. neoformans* strain H99. The pulmonary fungal burden was significantly lower on days 7 and 14 post secondary inoculation with wild-type *C. neoformans* strain H99 in mice immunized with *C. neoformans* strain H99 γ , compared to mice immunized with heat-killed *C. neoformans* strain H99 (Figure 1B).

3.2 Antibody response in serum of immunized mice

Serum obtained from mice immunized with *C. neoformans* strain H99 γ , or heat-killed *C. neoformans* strain H99 yeast on day 14 following receiving a second experimental pulmonary challenge with *C. neoformans* strain H99 were tested for the relative distribution of total immunoglobulin subclasses: IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, IgM, and IgA by ELISA. Also, the relative distribution of *C. neoformans*-specific antibodies was determined by using a β -mercaptoethanol (β -ME) cell surface protein extract prepared from *C. neoformans* strain H99 as the antigen for capture of *C. neoformans*-specific serum antibodies. Mice immunized with *C. neoformans* strain H99 γ , or heat-killed *C. neoformans* strain H99 yeast will be

designated as protected and non-protected mice, respectively, for clarity. We should note that these experiments do not allow for an accurate quantification of the various immunoglobulin subclasses. Nonetheless, we did not observe any significant differences in all total immunoglobulin subclasses tested in protected mice compared to non-protected mice (Figure 2A). In contrast, significantly more *C. neoformans*-specific IgG₁, IgG_{2a}, IgG_{2b}, IgM, and IgA were detected in serum obtained from protected mice compared to non-protected mice following receiving a second experimental *C. neoformans* pulmonary challenge (Figure 2B). No significant difference in *C. neoformans*-specific IgG₃ was observed in protected compared to non-protected mice (Figure 2B).

3.3 Detection and identification of *C. neoformans* immunodominant protein spots using immune sera from vaccinated mice

A β -ME cell surface protein extract prepared from *C. neoformans* strain H99 was separated by 2-DE and analyzed for reactivity to serum by immunoblotting. Initial experiments using pI 3-10 IPG strips and 12.5% acrylamide gels showed that the majority of protein spots resolved in the gels were in the 4-7 pI range. Thus, most experiments and subsequent analyses were accomplished using pI 4-7 IPG strips and 12.5% acrylamide gels for improved separation and resolution of protein spots.

After running, the gels were stained for total protein with Sypro Ruby Red or alternatively transferred to PVDF membranes for immunoblot analysis using immune sera collected on days 14 and 100 post secondary challenge from protected mice or day 14 from non-protected mice. The immunoblot analysis was used as a basis to identify potentially immunogenic cryptococcal proteins. Protein spot selection was determined following performing three separate 2-DE and immunoblot experiments using serum derived from three separate experiments in order to ensure reproducibility of the results. Figure 3A shows that the Sypro Ruby Red stained proteins predominantly appeared as discrete, well resolved spots. Immunoblot analysis showed that no significant reactivity was observed using sera from non-protected mice given a second experimental pulmonary *C. neoformans* challenge (Figure 3B). However, immunoblot analysis using serum from protected mice given a second cryptococcal challenge detected a total of twenty-four distinct protein spots (Figures 3B and 3C). Each immunoreactive protein spot was subsequently excised from a companion Sypro Ruby Red stained acrylamide gel that was ran concurrent to the blotted gel (Figure 3A) and analyzed by HPLC-ESI-MS/MS. Overall, analysis of the immunoreactive protein spots led to the identification of fourteen separate cryptococcal proteins. A summary of the identified immunoreactive protein spots is provided in Table 1.

4 Discussion

To date, there is no immune therapy or vaccine strategy approved for the prevention of cryptococcosis. Accordingly, some attention has been invested into the identification of cryptococcal proteins or antibodies that elicit protection against *C. neoformans* infections. Previous studies and results presented herein show that an experimental pulmonary infection with *C. neoformans* strain H99 γ in mice results in the induction of Th1-type CD4⁺ CMI responses, resolution of the acute infection, and complete protection against a second pulmonary challenge with a pathogenic *C. neoformans* strain [40]. Subsequently, we sought to use serum antibody produced in mice protected against experimental pulmonary *C. neoformans* infection compared to susceptible mice to identify immune dominant proteins that may contribute to the development of protective immunity.

Our studies show that antibody of all isotypes tested were present and no significant differences in total antibody distribution were observed in mice given a second pulmonary *C. neoformans* infection following immunization with *C. neoformans* strain H99 γ or heat-

killed *C. neoformans* strain H99 yeast. However, mice immunized with *C. neoformans* strain H99 γ produced significantly more *C. neoformans*-specific antibodies of every isotype, except IgG3, compared to mice immunized with heat-killed *C. neoformans* in response to a second pulmonary cryptococcal challenge. Comparisons of variable-region-identical mAbs to the glucuronoxylomannan (GXM) component of the *C. neoformans* polysaccharide capsule of the murine IgG1, IgG2a, IgG2b, and IgG3 isotypes have consistently shown that all IgG isotypes, except IgG3, protect mice against *C. neoformans* infection [44-46]. Thus, it is conceivable that protected mice may produce less *C. neoformans* specific IgG3 antibodies if this isotype is indeed less protective. Nonetheless, the anti-cryptococcal antibody distribution observed in protected mice during our analysis concurs with that shown in previous experimental models of cryptococcosis. However, recent studies showing that the efficacy of various immunoglobulin isotypes against *C. neoformans* may vary between humans and mice [47] underscores the need for continued investigation

We postulated that the serum antibody generated in mice protected against pulmonary *C. neoformans* infection may be used to identify immunodominant cryptococcal proteins with the potential to induce protective anti-cryptococcal immune responses. Similar studies have used immune sera from infected mice, AIDS patients and/or individuals with cryptococcosis to identify immune dominant cryptococcal proteins [48-49]. Previous investigations predominantly evaluated cryptococcal proteins derived from a cryptococcal culture filtrate preparation known as CneF or various fractions derived from it (reviewed in [50]) using 1-DE. We instead chose to isolate cell surface associated cryptococcal proteins using a modified protocol previously used to extract cell wall associated proteins from *Candida albicans* [43] and employ 2-DE as a method to increase the resolution of proteins within the protein extract. Our analysis demonstrated that serum obtained from protected mice given a second experimental pulmonary infection with the fully pathogenic strain reacted with specific *C. neoformans* proteins; an observation that was not observed using serum from non-protected mice. Our analysis led to the identification of a total of twenty-four cell surface protein spots representing fourteen distinct proteins. HPLC-ESI-MS/MS analysis revealed that some of the spots, although well resolved, contained multiple proteins that are also included in Table 1. This is not unusual in that the power of these analyses allows for the identification of individual proteins from within complex mixtures [51]. The immunodominant proteins identified included proteins involved in stress responses, carbohydrate metabolism, signal transduction, amino acid biosynthesis, protein synthesis, and some yet to be characterized proteins. Not surprisingly, a number of the proteins identified in our analysis have previously been implicated in the virulence component of *C. neoformans* and other fungal pathogens. A brief description of some of these proteins and their putative impact on the pathogenesis of *C. neoformans* follows.

Heat shock protein (hsp)-70 and hsp90 were specifically identified on our analysis. A serial analysis of gene expression (SAGE) of *C. neoformans* cells recovered from the central nervous system of rabbits given an experimental *C. neoformans* infection demonstrated that hsp70 and hsp90 were highly expressed *in vivo* [52]. Similar observations by Kakeya et. al. also demonstrated that hsp70 is highly abundant and immunogenic *in vivo* during pulmonary cryptococcosis [53-54]. Mycograb, a recombinant antibody that targets an epitope within the hsp90 of *Candida albicans* that is conserved with the corresponding protein in *C. neoformans*, has been demonstrated to act in synergy with amphotericin B against multiple *Candida* species and *C. neoformans* clinical isolates [55-56].

Two enzymes involved in carbohydrate metabolism that were identified in our analysis include transaldolase, a member of the pentose-phosphate shunt pathway, and phosphopyruvate hydratase (alternatively named enolase) a member of the glycolytic and gluconeogenesis pathways. Transaldolase expression has been shown to increase within *C.*

neoformans in response to nitric oxide stress [57] and in an experimental rabbit model of cryptococcal meningitis [52]. Enolase has been localized to the cell wall and cytoplasm of *C. albicans* [58-59] and identified as part of a milieu of vesicle proteins recognized by sera from cryptococcosis patients [49]. SAGE of cryptococcal cells derived from the CNS of rabbits with experimental cryptococcosis has also demonstrated an increase in enolase expression [52].

One fungal-specific target identified in our analysis is saccharopine dehydrogenase (NAD⁺, L-lysine-forming), an enzyme involved in the biosynthesis of the essential amino acid lysine. Lysine is synthesized in fungi, including pathogenic fungi, via the aminoadipate pathway [60]. The aminoadipate pathway is unique to fungi and auxotrophs are nonpathogenic [61-62] suggesting that enzymes of this pathway may serve as good candidates for anti-fungal drug development. Accordingly, disruption of the gene encoding saccharopine dehydrogenase in *C. neoformans* resulted in attenuation of virulence in a murine inhalational model of cryptococcosis [63].

Serum from mice demonstrably protected against experimental pulmonary cryptococcosis also reacted with 14-3-3 protein; a protein identified alongside several virulence-related proteins within *C. neoformans* extracellular vesicles [49]. 14-3-3 gene expression was also shown to be up-regulated *in vivo* using an experimental model of cryptococcal meningitis [52] and in the CSF of patients with cryptococcal meningitis [64]. 14-3-3 proteins are evolutionarily conserved and regulate signal transduction [65], cell cycle regulation [66], the activation of catecholamine and serotonin neurotransmitter biosynthesis [67-68] and stimulate catecholamine secretion [69]. Studies have indicated that the 14-3-3 gene in *C. albicans*, *BMH1*, is essential for growth [70]. Therefore, 14-3-3 gene expression in *C. neoformans* may also serve as an attractive anti-fungal drug target.

Interestingly, we did not observe any “classical” cell surface proteins, including mannoproteins, among the immune dominant proteins detected in our analysis. We were particularly surprised by the absence of mannoproteins among the immune dominant proteins found in our study. Most of the mannoprotein that is not secreted by *C. neoformans* is found in its inner cell wall and not on the outer cell wall and thus surface exposed and the rather “mild” treatment with β -ME may not reach these *C. neoformans* proteins as with *C. albicans* [71]. Thus, our cell surface extract may not contain a significant amount of mannoproteins, or at least, any that are immune dominant in our model. Additionally, highly glycosylated high molecular weight mannoproteins are perhaps impeded from entering our gels due to their high sugar content and poly-dispersed nature. This is an unfortunate technical issue that, although typical in these studies [59,72-73], may also explain the absence of mannoproteins in our analysis.

Many of the immune dominant proteins identified in our analysis have traditionally been associated with a cytoplasmic function. However, it is now widely accepted that several cytosolic proteins are also associated with the cell wall of fungi [72,74-76]. The proteins enolase, hsp70, hsp90, and 14-3-3 protein, for example, have each been detected in fungal cell walls and cell surface fractions [72,74,77]. Several theories have been proposed for the non-conventional export of proteins lacking an N-terminal signal peptide to the cell surface [49,72,74,78]. In addition, studies have demonstrated that *C. neoformans* produces vesicles that fuse with the cell wall prior to secretion of several virulence related molecules (i.e., chaperone, cytoplasmic, nuclear, and mitochondrial proteins) [49]. Thus, both canonical and non-canonical protein export mechanisms could serve as a means to deliver molecules that promote virulence, suppress host immunity, or, more importantly for our studies, stimulate host immune responses. Moreover, highly conserved antigens such as hsps and glycolytic enzymes that are shared among several fungal species could serve as common targets for the

induction of protective immune responses against different fungal species as previously suggested [79].

Altogether, our studies show that mice immunized with *C. neoformans* strain H99 γ are protected against a subsequent pulmonary challenge with a fully pathogenic *C. neoformans* strain and produce significantly more *C. neoformans*-specific antibodies compared to mice immunized with heat-killed cryptococci. In addition, the distribution of the *C. neoformans*-specific antibodies is predominantly of a protective isotype phenotype; although a more in-depth analysis is needed to examine the protective nature of the antibody response. Nonetheless, using a proteomic approach, we were able to identify several immunodominant cryptococcal proteins using sera from mice demonstrably protected against pulmonary cryptococcosis. Our results are in agreement with previous studies evaluating gene and protein expression by *C. neoformans* either *in vivo* or under conditions analogous to that observed *in vivo* [49,52,57]. These results are unique in that we are able to use an experimental model in which complete protection against pulmonary cryptococcosis is achieved together with proteomic techniques to identify putative immunodominant cryptococcal proteins. The proteins identified in our analysis may provide additional targets for the development of novel anti-fungal drugs and subunit vaccines that are urgently needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Protection against experimental pulmonary cryptococcosis following immunization. BALB/c mice received an initial inoculum of 1×10^4 CFU of heat-killed *C. neoformans* yeasts or *C. neoformans* strain H99 γ in 50 μ l of sterile PBS, allowed 100 days to resolve the infection, and subsequently given a second challenge with 1×10^4 CFU of *C. neoformans* strain H99 in 50 μ l of sterile PBS. Survival of secondary infected mice was monitored twice daily and mice that appeared moribund or not maintaining normal habits (grooming) were sacrificed by CO₂ inhalation (A). Survival data shown are cumulative of 1 experiment each using 10-12 mice per experimental group. Alternatively, lungs were excised at days 3, 7, and 14 post secondary inoculation and the cryptococcal burden quantified (B). Pulmonary fungal burden data are cumulative of two experiments using 5-10 mice per time point. Separate mice were used for each time point. Results are expressed as mean Log CFU per milliliter $\pm$ standard errors of the means. Asterisks indicate where significant decreases ($P < 0.01$) were observed compared to mice immunized with heat-killed *C. neoformans* strain H99.

Figure 2.

Evaluation of total and *C. neoformans*-specific Ab isotypes in mice during experimental pulmonary cryptococcosis. BALB/c mice received an initial inoculum of 1×10^4 CFU of heat-killed *C. neoformans* yeasts or *C. neoformans* strain H99γ in 50 μl of sterile PBS, allowed 100 days to resolve the infection, and subsequently given a second challenge with 1×10^4 CFU of *C. neoformans* strain H99 in 50 μl of sterile PBS. Serum was obtained from mice immunized with *C. neoformans* strain H99γ on days 14 and 100 post secondary inoculation, and from mice immunized with heat-killed *C. neoformans* strain H99 on day 14 post secondary inoculation. The isotype of total (A) and *C. neoformans*-specific (B) serum antibodies were determined. The results shown are cumulative of two experiments using 10 mice per time point. Asterisks indicate where significant increases ($P < 0.05$) were observed compared to mice immunized with heat-killed *C. neoformans* strain H99.

Figure 3. 2-DE profile and Immunoblot analysis of *C. neoformans* β -ME cell surface protein extract. Protein components were separated in the first dimension using a pI 4-7 IPG strip and in the second dimension using a 12% polyacrylamide gel. Following 2-DE, the proteins were stained with Sypro Ruby Red (A) or transferred to PVDF membranes and incubated with pooled serum taken on day 14 post secondary infection of mice immunized using heat-killed *C. neoformans* (B) or pooled serum taken on day 14 (C) and day 100 (D) post secondary infection of mice immunized using *C. neoformans* strain H99 γ . Numbered spots represent immunogenic proteins whose corresponding identities are given in Table 1.

Immunodominant *C. neoformans* proteins identified by HPLC-ESI-MS/MS after 2-DE

Spot no.	Protein I.D.	MW (Da)	Accession Number	Identification Probability (%)	Unique Peptides	Sequence Coverage (%)
1	Transaldolase	35,321	XP_567910	100	13	33
1	UMP-CMP kinase	30,435	XP_571305	100	5	21
2	Transaldolase	35,321	XP_567910	100	10	30
3	Transaldolase	35,321	XP_567910	100	10	30
4	Transaldolase	35,321	XP_567910	100	11	32
4	Hypothetical protein	38,004	XP_567255	100	8	23
5	Transaldolase	35,321	XP_567910	100	10	28
6	Phosphopyruvate hydratase (enolase)	47,657	XP_569379	100	12	32
7	Phosphopyruvate hydratase (enolase)	47,657	XP_569379	100	13	33
8	Phosphopyruvate hydratase (enolase)	47,657	XP_569379	100	12	30
9	Phosphopyruvate hydratase (enolase)	47,657	XP_569379	100	6	15
9	Saccharopine dehydrogenase	43,480	XP_570078	100	7	23
10	Phosphopyruvate hydratase (enolase)	47,657	XP_569379	100	7	22
11	Phosphopyruvate hydratase (enolase)	47,657	XP_569379	100	9	29
11	Translation initiation factor	45,144	XP_567112	100	4	10
12	Heat shock protein 70	69,355	XP_569509	100	13	27
13	Heat shock protein 70	69,355	XP_569509	100	22	36
14	Heat shock protein 70	69,355	XP_569509	100	14	29
15	Hypothetical protein	74,463	XP_570402	100	8	13
16	Hypothetical protein	74,463	XP_570402	100	9	17
17	Hypothetical protein	74,463	XP_570402	100	11	17
18	Hypothetical protein	74,463	XP_570402	100	8	14
19	Aldehyde reductase i	37,331	XP_567710	100	11	29
20	Aldehyde reductase i	37,331	XP_567710	100	13	32
20	Cytoplasm protein	38,975	XP_568064	100	3	14
20	D-lactaldehyde dehydrogenase	38,183	XP_570921	100	5	16
21	2-oxoglutarate metabolism related protein	49,199	XP_569105	100	4	8
22	2-oxoglutarate metabolism related protein	49,199	XP_569105	100	5	10
23	Heat shock protein 90	78,276	AAN76525	100	14	21

Spot no.	Protein I.D.	MW (Da)	Accession Number	Identification Probability (%)	Unique Peptides	Sequence Coverage (%)
24	14-3-3 protein	28,995	XP_567951	100	6	30