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A cell wall protein-based vaccine candidate induce protective immune response against *Sporothrix schenckii* infection

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ABSTRACT

Sporotrichosis is a subcutaneous mycosis caused by several closely related thermo-dimorphic fungi of the *Sporothrix schenckii* species complex, affecting humans and other mammals. In the last few years, new strategies have been proposed for controlling sporotrichosis owning to concerns about its growing incidence in humans, cats, and dogs in Brazil, as well as the toxicity and limited efficacy of conventional antifungal drugs. In this study, we assessed the immunogenicity and protective properties of two aluminum hydroxide (AH)-adsorbed *S. schenckii* cell wall protein (ssCWP)-based vaccine formulations in a mouse model of systemic *S. schenckii* infection. Fractioning by SDS-PAGE revealed nine protein bands, two of which were functionally characterized: a 44 kDa peptide hydrolase and a 47 kDa enolase, which was predicted to be an adhesin. Sera from immunized mice recognized the 47 kDa enolase and another unidentified 71 kDa protein, whereas serum from *S. schenckii*-infected mice recognized both these proteins plus another unidentified 9.4 kDa protein. Furthermore, opsonization with the anti-ssCWP sera led to markedly increased phagocytosis and was able to strongly inhibit the fungus' adhesion to fibroblasts. Immunization with the higher-dose AH-adjuvanted formulation led to increased *ex vivo* release of IL-12, IFN-γ, IL-4, and IL-17, whereas only IL-12 and IFN-γ were induced by the higher-dose non-adjuvanted formulation. Lastly, passive transference of the higher-dose AH-adjuvanted formulation's anti-ssCWP serum was able to afford *in vivo* protection in a subsequent challenge with *S. schenckii*, becoming a viable vaccine candidate for further testing.

1. Introduction

Diseases caused by opportunistic fungi are an increasing health problem, especially for immunocompromised individuals. Sporotrichosis, also known as "rose gardener's disease", is a subcutaneous mycosis caused by several closely related thermo-dimorphic fungi of the *Sporothrix schenckii* species complex, including *Sporothrix brasiliensis*, *Sporothrix globosa, Sporothrix mexicana, Sporothrix luriei* and *S. schenckii sensu stricto* (Oliveira et al., 2014). The disease is commonly found in tropical and subtropical regions, but isolated cases and outbreaks have been reported worldwide (Rodrigues et al., 2013). *S. schenckii* is a ubiquitous envi-

Abbreviations: ssCWP, *S. schenckii* cell wall protein; AH, aluminum hydroxide; gp70, glycoprotein of 70 kD; PMSF, phenylmethyl sulfonyl fluoride; EDTA, Ethylenediamine tetraacetic acid; BCA, bicinchoninic acid; DTT, dithiothreitol; FA, formic acid; ACN, acetonitrile; FBS, fetal bovine serum; NIS, serum from non-immunized mice; IS, serum from *S. schenckii*-infected mice; MFI, median fluorescence intensity; ADCC, antibody-dependent cellular cytotoxicity.

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ronmental saprophyte that can be isolated from soil and plant debris, normally causing infection by its traumatic inoculation from contaminated material (Barros et al., 2011). In Brazil, sporotrichosis has become an important zoonosis, with cats being the main source of infection and transmitting agent to humans and other animals (Rodrigues et al., 2013). Cat-transmitted sporotrichosis represents 91% of all cases of human sporotrichosis in Brazil (Freitas et al., 2010), which in great part due to the presence of a large number of viable yeasts on the surface of feline ulcerated lesions or by inoculation of the fungus following cat scratches (Cruz, 2013). Sporotrichosis' most frequent clinical manifestations are the localized or regional lymphocutaneous forms, while the disseminated form has been mainly reported among immunocompromised individuals (Barros et al., 2011).

The usual sporotrichosis treatment requires long periods of antifungal drug administration accompanied by frequent relapses in immunocompromised patients (Kauffman et al., 2007). This therapy is often associated with sometimes severe adverse effects and frequent fungal resistance (Rodrigues et al., 2014). Therefore, vaccination has been proposed as a viable alternative for both therapeutic and prophylactic purposes (Almeida, 2012; Lacerda et al., 2011). For decades, a variety of cell wall proteins (CWPs) from many different pathogenic fungi have been evaluated in mouse models of vaccination for assessment of their immunogenicity, safety and protection-affording potential (Edwards, 2012). Several authors have reported the development of a specific immune response and increased resistance to subsequent infection following either a previous infection or active immunization with *S. schenckii* CWPs (ssCWPs) or whole cells (Charoenvit and Taylor, 1979; Tachibana et al., 1999).

Studies performed in our lab have shown that immunization with dendritic cells stimulated with *S. schenckii* yeasts or their exoantigen was able to induce a Th1 and Th17 mixed response *in vitro*(Verdan et al., 2012), the latter of which has been since associated with control of the *S. schenckii* infection *in vivo* (Ferreira et al., 2015). Also, Nascimento and Almeida (2005) reported induction of a specific humoral response against the 70 kDa glycoprotein (gp70), a key immunodominant antigen of the *S. schenckii* cell wall, in a mouse model of infection. This antigen's relevance was confirmed by studies in which transference of anti-gp70 mAbs was able to convey protection against highly virulent *S. schenckii* and *S. brasiliensis* strains, thus providing definitive evidence for the role of antibodies in the protective immunity against *S. schenckii* (de Almeida et al., 2015). Although very few clinical trials have been performed in humans, a growing number of antifungal vaccine candidates are being evaluated in preclinical studies, as part of the renewed interest in the potential use of vaccines, replacing or associated with chemotherapy, to reduce antifungal drugs use and consequently limit drug resistance and toxicity (Portuondo et al., 2015).

In this study, we assessed the immunogenicity and protective properties of two AH-adsorbed ssCWP-based vaccine formulations in a mouse model of systemic *S. schenckii* infection. Our results showed the vaccine formulations to be immunogenic and able to promote protection-affording antibody production upon immunization.

2. Materials and methods

2.1. Animals

Male 5–7 week-old BALB/c mice were purchased from "Centro Multidisciplinar para Investigação Biológica na Área da Ciência de Animais de Laboratório" (CEMIB), UNICAMP University (Brazil) and maintained under standard laboratory care as previously described (Goncalves et al., 2015). This work was approved by the Institutional Ethics Committee for Animal Use in Research (Protocol CEUA/FCF/CAR no. 30/2012) and was in accordance with the National Institutes of Health Animal Care guidelines.

2.2. Microorganism and culture conditions

S. schenckii ATCC 16345 was kindly provided by the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. The isolate was maintained by regular passage in mice and grown on Mycosel™ (BD Biosciences) agar tubes at 25 °C. Mycelial-to-yeast phase conversion was accomplished as previously described (Ferreira et al., 2015).

2.3. Extraction of the ssCWPs

Extraction of the ssCWPs was performed as previously described (Castro et al., 2013), with minor modifications. Briefly, yeast cells collected from logarithmically growing cultures were incubated with the dithiothreitol (DTT)-based protein extraction buffer (2 mM DTT, 1 mM phenylmethyl sulfonyl fluoride, and 5 mM EDTA in This/HCl buffer) for 2 h at 4 °C under mild agitation. The ssCWP-containing supernatant was collected, dialyzed against distilled water, filtered through a 0.22 μm nitrocellulose membrane (Millipore) and then concentrated using the Amicon Ultra 15 MWCO concentrator (Millipore). The proteins were then precipitated by overnight incubation with 10% (w/v) trichloroacetic acid in acetone at 4 °C and the resulting pellets were washed in ice-cold acetone, dried in a SpeedVac® and reconstituted in phosphate buffered saline, pH 7.2–7.4 (hereafter referred to as PBS only). Protein concentration was measured by the BCA assay (Pierce).

2.4. Yeast viability assay

The effect of DTT protein extraction on *S. schenckii*'s viability was assessed using the LIVE/DEAD® yeast viability kit (Molecular Probes) in accordance with the manufacturer's instructions. Live DTT-untreated or heat-killed *S. schenckii* yeasts were used as the negative or positive controls, respectively. Samples were visualized on the BH50 fluorescence microscope (Olympus) using the fluorescein and DAPI filters for the FUN1 and Calcofluor White M2R stains, respectively.

2.5. One-dimensional sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE) and MALDI-TOF mass spectrometry analysis

Samples containing 50 μg of the ssCWPs were separated by SDS-PAGE (10%) as described by Laemmli (1970). Protein spots excised from silver-stained gels were reduced, alkylated, and subjected to tryptic digestion with trypsin (Promega). Next, tryptic peptides were extracted and submitted to mass spectrometry (MS) analysis; results were collected with the AB Sciex Maldi TOF/TOF series explorer version 4.1.0 and data was analyzed in the Protein Pilot Software using the MASCOT search engine. The obtained sequences were compared to those in the SwissProt databanks (http://expasy.org/sprot). Additionally, prediction of adhesin-like proteins was performed using the FungalRV database.

2.6. Adsorption studies

Aluminum hydroxide (AH) gel (Invivogen) containing 10 mg/mL of Al^{3+} was used in the present work. The equilibrium time for adsorption of the ssCWPs on AH was determined by mixing 1 mg of the ssCWPs with an amount of AH corresponding to 1 mg of AL^{3+} to a final volume of 1 mL in PBS. The suspensions were kept under agitation on a rotary spinner at room temperature (RT) and pH 7.4 for 10–100 min. The amount of adsorbed protein

was determined in each time interval by subtracting the amount of protein remaining in the supernatant after adsorption from that initially added by the BCA assay. In order to assess protein fragmentation during adsorption, the supernatants above were subjected to SDS-PAGE as described on item 2.5. Subsequently, the adsorption isotherm was determined by incubating a fixed 1 mg of Al^{3+}/mL concentration with increasing protein concentrations (i.e., 0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1.0 mg/mL, and 1.25 mg/mL in PBS) for 40 min (the optimum adsorption time) at RT. The amount of adsorbed protein was determined as before.

2.7. Vaccine formulation and immunization protocol

AH-ssCWP formulations were prepared with 10 μg or 100 μg of the ssCWPs formulated with an AH equivalent of 100 μg of AL^{3+} (termed AH+CWP10 and AH + CWP100, respectively) as described on item 2.6, using an adsorption time of 40 min. Groups of 5 BALB/c mice were subcutaneously (s.c.) immunized twice in a 2-week interval (at days 0 and 14) with AH+CWP10 or AH+CWP100, or with 10 μg or 100 μg of the ssCWPs alone in PBS (termed CWP10 and CWP100, respectively), or with PBS alone as the negative control. One week after the second immunization, mice were euthanized in a CO_2 chamber and bled by heart puncture. The resulting sera were heat-inactivated at 56 °C for 30 min, aliquoted and stored at -20 °C until use.

2.8. Cytokine induction assay

Thioglycollate-elicited peritoneal macrophages and total splenocytes were harvested from mice immunized as described on item 2.7, prepared in RPMI-1640 complete medium (RPMIc; defined as the base RPMI-1640 medium containing 0.02 mM 2β-mercaptoethanol, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine, and 5% fetal bovine serum [FBS]) as described elsewhere (Goncalves et al., 2015) and cultured for 24 h at 37 °C and 5% CO_2 in the presence of the ssCWPs. Final concentrations were 2.5×10^6 cells/mL and 40 μg of ssCWPs/mL in RPMIc; concanavalin A (0.25 μg/mL) or *E. coli* O111B lipopolysaccharide (10 μg/mL) were used as positive controls for the macrophages' or splenocytes' cultures, respectively; RPMIc alone was used as the negative control. The following supernatant-accumulated cytokines were measured by ELISA (eBioscience) according to the manufacturer's instructions: IL-12, IFN-γ, IL-4, and IL-17.

2.9. Quantification of ssCWP-specific IgG, IgG1, and IgG2a by ELISA

Quantification of serum ssCWP-specific IgG, IgG1 and IgG2a was carried out by ELISA. Briefly, 96-well ELISA plates (Costar) were coated with 100 μL/well of a 40 μg/mL ssCWP solution in 0.1 M bicarbonate buffer, pH 9.6 and incubated overnight at 4 °C. The plate was washed in PBS containing 0.1% Tween 20 (washing buffer) and then blocked with PBS containing 10% FBS (blocking buffer) at 37 °C for 1 h. Next, dilutions of the anti-ssCWP sera in blocking buffer were added to each well and incubated at 37 °C for 1 h as before. After washing once with washing buffer, 100 μl/well of peroxidase-conjugated anti-mouse IgG (1/500) (Sigma) in blocking buffer was added and incubated again at 37 °C for 1 h. Alternatively, for determination of the IgG1 and IgG2a subclasses, ELISA plates coated as before were first incubated with an unconjugated rabbit anti-mouse IgG1 or IgG2a (Bio-Rad) at 37 °C for 1 h and then with a peroxidase-conjugated goat anti-rabbit IgG (Sigma) overnight at 4 °C. After exhaustive washes, the reaction was developed with tetramethylbenzidine during 30 min at RT and then stopped with 50 μL/well of 1 N H_2SO_4. Absorbance was read at 450 nm (Multiskan ascent, Labsystem).

2.10. Flow cytometry

A 100 μL suspension containing 10^6 *S. schenckii* yeasts were incubated for 1 h at 37 °C with the various anti-ssCWP sera, or with serum from *S. schenckii*-infected mice (IS), all in a 1/20 dilution. Serum from non-immunized mice (NIS) was used as a non-specific binding control. Yeast suspensions were washed, resuspended in 100 μL of PBS and then incubated for 1 h at 37 °C with a FITC-conjugated rabbit anti-mouse IgG antibody (Sigma–Aldrich) in a 1/50 dilution. After washing, samples were acquired with the BD Accuri C6 flow cytometer (BD Biosciences). The acquisition threshold was set to 50,000 on FSC-H for debri exclusion and at least 60,000 events were effectively included in each analysis. Binding of serum antibodies to the yeasts' cell surface was assessed through the median fluorescence intensity (MFI) on the FL1 channel using the flow cytometer's proprietary software.

2.11. Immunoblotting

SDS-PAGE-fractionated proteins were transferred to a nitrocellulose membrane overnight at 4 °C. Next, the membrane-cut strips were saturated with 5% dried skim milk in PBS for 4 h at 37 °C. After washing with PBS, each strip was incubated overnight at RT with the various anti-ssCWP sera or with NIS as negative control. A secondary peroxidase-conjugated goat anti-mouse IgG antibody, diluted 1/500, was added after further washing, and immunoreactive proteins were visualized by adding 3,3′-diaminobenzidine plus hydrogen peroxide.

2.12. Yeast opsonization

S. schenckii yeasts were opsonized for 1 h at 37 °C with a pool of the various anti-ssCWP sera, or with NIS as the negative control, all in a 1/100 dilution. After exhaustive washes with PBS to remove unbound antibodies, yeast suspensions were adjusted to 4×10^7/mL or 2.5×10^7/mL in PBS for use in the opsonophagocytosis or fungus-fibroblast adhesion-inhibiting assays, respectively.

2.13. Opsonophagocytosis assay

The opsonophagocytosis assay was based on a modification of a previously described method (Negrini et al., 2013). Thioglycollate-elicited peritoneal macrophages were adjusted to 5×10^6 cells/mL in RPMIc and then plated (500 μL/well) in a 1.8 cm^2/well LabTek® slide (Nunc) and incubated overnight at 37 °C and 5% CO_2. After discarding the supernatant, adherent cells were washed once with PBS and incubated with opsonized (as on item 2.12) or PBS-treated 2×10^7 *S. schenckii* yeasts in 500 μL of PBS for 2 h at 37 °C. The slides were stained with Giemsa and 200 macrophages were counted on an optical microscope under 100× magnification to determine the phagocytic index (PI), calculated as the percentage of phagocytosing cells multiplied by the mean number of internalized yeasts.

2.14. Inhibition of fungal adhesion to fibroblasts

A previously published adhesion assay (Mendes-Giannini et al., 2004) was adapted to determine whether immunization-induced antibodies were able to inhibit fungal adhesion to fibroblasts. Briefly, mouse L929 fibroblasts were adjusted at 2.5×10^5 cells/mL in Dulbecco's modified Eagle's medium (DMEM) and cultured in 6-well plates with square coverslips-covered bottoms, at 37 °C for 24 h. Next, opsonized (as on item 2.12) or PBS-treated 2.5×10^7 yeasts were added to each well in a 1:100 fibroblast-to-yeast ratio

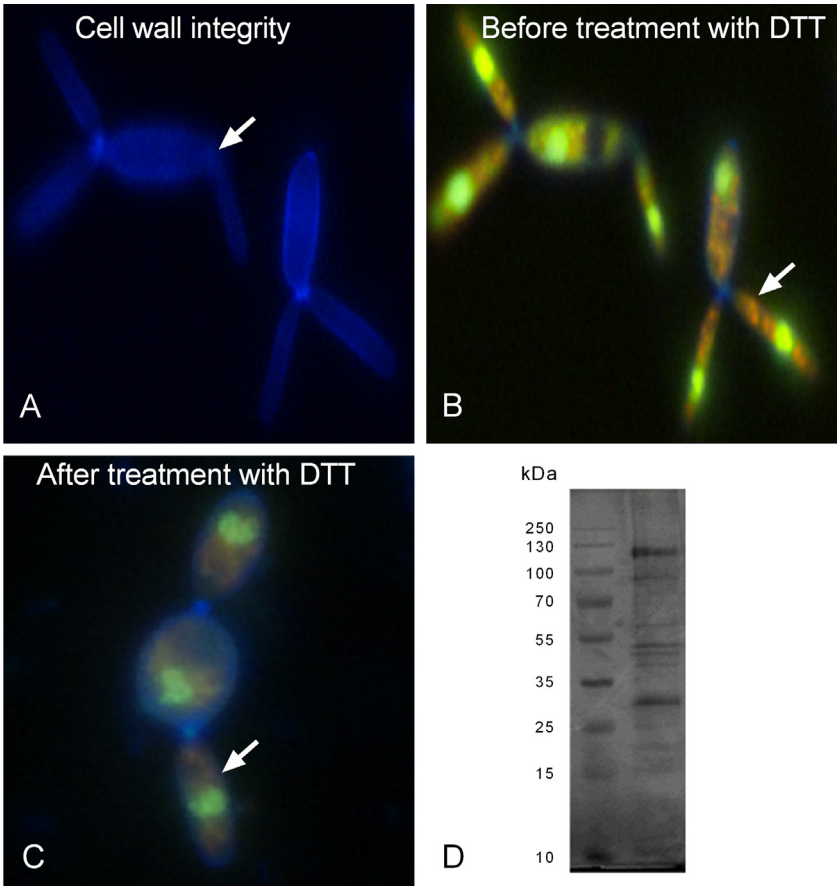


Fig. 1. Yeast viability and cell wall integrity were not affected by extraction of the ssCWPs with DTT. (A) Calcofluor white-stained *S. schenckii* yeast showing cell wall integrity (white arrows). (B and C) FUN1®-stained yeast cells showing the orange intravacuolar fluorescence (white arrows) before (B) and after (C) DTT treatment. (D) SDS-PAGE profile of the DTT-extracted ssCWPs. Protein bands were visualized by silver staining. Lane 1: molecular weight standard. Lane 2: protein extract.

and incubated at 37 °C for 3 h. After discarding the supernatants, the coverslips-adhered cells were washed with PBS, fixed with methanol, and then stained with crystal violet. A total of 500 fibroblasts were counted on an optical microscope under 100× magnification to determine the adhesion index, calculated as the percentage of fibroblasts containing attached yeasts multiplied by the mean number of attached yeasts per fibroblast.

2.15. Passive transference

Groups of 7 mice were intraperitoneally (i.p.) injected with 100 μL of a 1/2 dilution of the CWP100 or AH + CWP100 sera in PBS, or with NIS or PBS alone as negative controls two hours prior to infection with 10^6 *S. schenckii* yeasts in 200 μL of PBS through the i.p. route. Protection was assessed by determining the number of colony forming units (CFUs) recovered from mice's spleen and liver on day 5 post-infection, when the peak of systemic fungal burden is expected to occur in this model (Ferreira et al., 2015). This was done by plating an adequate dilution of the organs' macerate on Mycosel™ agar plates. CFUs were counted after 3-day incubation at RT.

2.16. Statistical analysis

Data were analyzed in GraphPad Prism 5 using one-way analysis of variance (ANOVA) and the Tukey–Kramer method for comparison between groups. Confidence interval was set at 95% for all tests.

3. Results

3.1. SDS-PAGE profile and identification of the ssCWPs by MALDI-TOF/TOF

Extraction with DTT did not affect the yeasts' integrity, as revealed by the presence of an orange fluorescence characteristic of live cells when using the LIVE/DEAD® yeast viability kit, thus yielding an extract predominantly composed of proteins from the yeasts' cell wall (Fig. 1A–C). Fractioning by SDS-PAGE revealed nine bands ranging from 15 kDa and 130 kDa (Fig. 1D). MALDI-TOF/TOF mass spectrometry analysis showed, according to the SwissProt databanks, two functionally-characterized proteins: A peptide hydrolase from *S. schenckii* ATCC 58,251 and an enolase from *S. schenckii* ATCC 58,251, *S. schenckii* 1099-18 and *S. brasiliensis* ATCC 5110, with MWs of 44 kDa and 47 kDa, respectively (Supplemental Table 1). Among these, only enolase was predicted to be an adhesin upon submission of the protein sequences to the FungalRV database.

3.2. Adsorption isotherms

Next, we determined the optimal incubation time for adsorption of the ssCWPs on AH. Our results revealed that adsorption of the ssCWPs on AH reached equilibrium after 40 min (Fig. 2A), with a maximum adsorptive capacity of 474.85 μg of ssCWP per mg of AH. Also, protein fragmentation was not caused by the adsorption process, as judged by the absence of additional lower MW bands when compared to the protein extract prior to adsorption, which

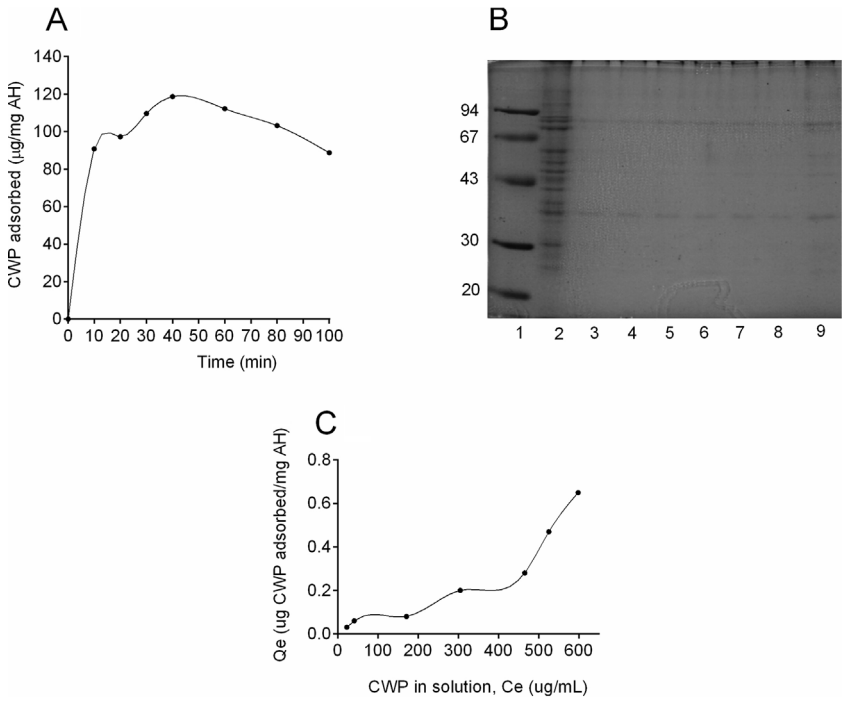


Fig. 2. Adsorption of the ssCWPs on AH reached equilibrium after 40 min and followed a non-linear isotherm. (A) A mixture of the ssCWPs with AH was left to react for the indicated times. (B) SDS-PAGE profile of the ssCWPs obtained before and after adsorption on AH. Lane 1: molecular weight standard. Lane 2: ssCWPs before adsorption. Lanes 3–9: Supernatants obtained after 10, 20, 30, 40, 60, 80, and 100 min of adsorption, respectively. (C) Adsorption isotherm of the ssCWPs on AH. Increasing concentrations of the ssCWPs were added to a fixed concentration of 1 mg/mL of Al^{3+}. Qe (amount of ssCWPs adsorbed (µg) per mg of AH at equilibrium). Ce (equilibrium concentration).

could affect the specificity of the following antibody response and, consequently, the immunization's effectiveness (Fig. 2B). Furthermore, adsorption of the ssCWPs on AH increased according to the starting protein concentration in a complex and non-linear way unlike a Langmuir isotherm (Fig. 2C), indicating that adsorption of the proteins occurred in several layers on AH.

3.3. Ex vivo *cytokine release*

Our next step was to assess the type of cytokine response triggered by immunization with the vaccine formulations being tested. Both IL-12 and IFN-γ were induced only by the higher-dose formulations (CWP100 and AH + CWP100) (Fig. 3A and B). Also, adsorption on AH was not able to trigger induction of neither IL-12 nor IFN-γ by the lower-dose non-adjuvanted formulation (CWP10), suggesting that these cytokines' initial induction is mostly dependent on the amount of ssCWPs rather than presence of the adjuvant. On the other hand, induction of IL-4 and IL-17 is, at least partially, dependent on AH, as treatment with both AH-adjuvanted, but not the non-adjuvanted formulations, was able to increase these cytokines' *ex vivo* release (Fig. 3C and D). Furthermore, contrary to the induction of IL-12 and IFN-γ, the amount of ss CWPs does not seem to play a role in the AH-adjuvanted formulations' ability to induce IL-4 or IL-17, given these cytokines' similar release by cells from AH + CWP10- and AH + CWP100-immunized animals.

3.4. *Antibody induction and immunoreactivity of sera*

One of the key features of any vaccine formulation is its ability to induce an antigen-specific antibody response against the desired target. As shown here, immunization with all formulations was able to induce production of ssCWP-specific total IgG and IgG1 antibodies, whereas only the two AH-adjuvanted formulations, although only slightly in the case of AH + CWP10, led to IgG2a antibody production (Fig. 4A–C). Furthermore, flow cytometry analysis showed that the ssCWP-specific antibodies present in all sera were able to bind non-permeabilized *S. schenckii* yeasts, providing further confirmation of the DTT-extracted proteins' cell surface origin (Fig. 4D–F). It is noteworthy that the amount of anti-ssCWP antibodies present in serum from AH + CWP100-immunized mice was similar to that obtained by the actual infection, as measured by their median fluorescence intensity (MFI), highlighting this formulation's efficiency in triggering antibody production. Additionally, we sought to determine which proteins, among the numerous ones present in our ssCWP extract, were being recognized by immunoblotting the anti-ssCWP sera against the ssCWP's one-dimensional SDS-PAGE product. Sera from CWP100-, AH + CWP10-, and AH + CWP100-immunized mice recognized the 47 kDa enolase band and another unidentified 71 kDa protein, whereas serum from *S. schenckii*-infected mice recognized both these proteins plus another unidentified 9.4 kDa protein. However, serum from CWP10-immunized mice was not able to detect any of the tested proteins (Fig. 4H). In the future, determining these immunodominant proteins' role in the establishment of the *S. schenckii* infection will allow development of a specifically-targeted, homogenous, and large-scale production-suitable recombinant vaccine.

3.5. *Opsonophagocytosis and adhesion assays*

Effective fungal vaccines are generally able to induce antibodies that promote phagocytosis and inhibit the fungus' adhesion onto host cells. In light of this, we assessed the ability of peritoneal macrophages obtained from previously immunized mice to phagocytose *S. schenckii* yeasts in the presence or absence of opsonization by the various anti-ssCWP sera. In the absence of opsonization, phagocytosis was extremely rare across all groups (Fig. 5A and B). By contrast, phagocytosis was markedly increased when the yeasts were previously opsonized with any of the sera, and particularly high when using the AH + CWP100 serum (Fig. 5C and D). When assessed for their yeast-fibroblast adhesion-inhibiting ability, all

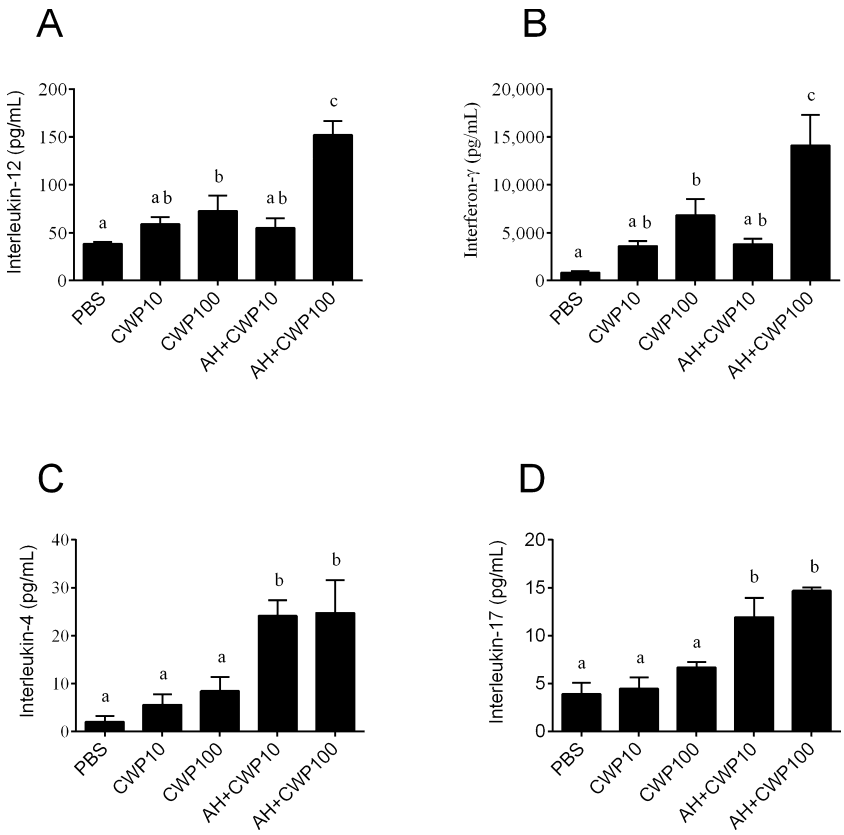


Fig. 3. Immunization with AH+CWP100 was able to induce a mixed Th1/Th2/Th17 cytokine profile in mice. Thioglycollate-elicited peritoneal macrophages and total splenocytes were harvested from immunized mice and cultured in the presence of the ssCWPs. Supernatant-accumulated cytokines were measured by ELISA. (A) *Ex vivo* release of IL-12 by peritoneal macrophages. (B–D) *Ex vivo* release of INF-γ, IL-4, and IL-17 by splenocytes. Results are presented as the mean ± SD of 5 mice from one of two separate experiments. Statistical significance was determined by one-way ANOVA using Tukey's multiple comparisons test and a 95% confidence interval. Different letters represent significant differences between treatments ($p < 0.05$).

anti-ssCWP sera, but especially the two AH-adjuvanted sera and the AH + CWP100 serum in particular, showed significant inhibitory activity (Fig. 5E and F).

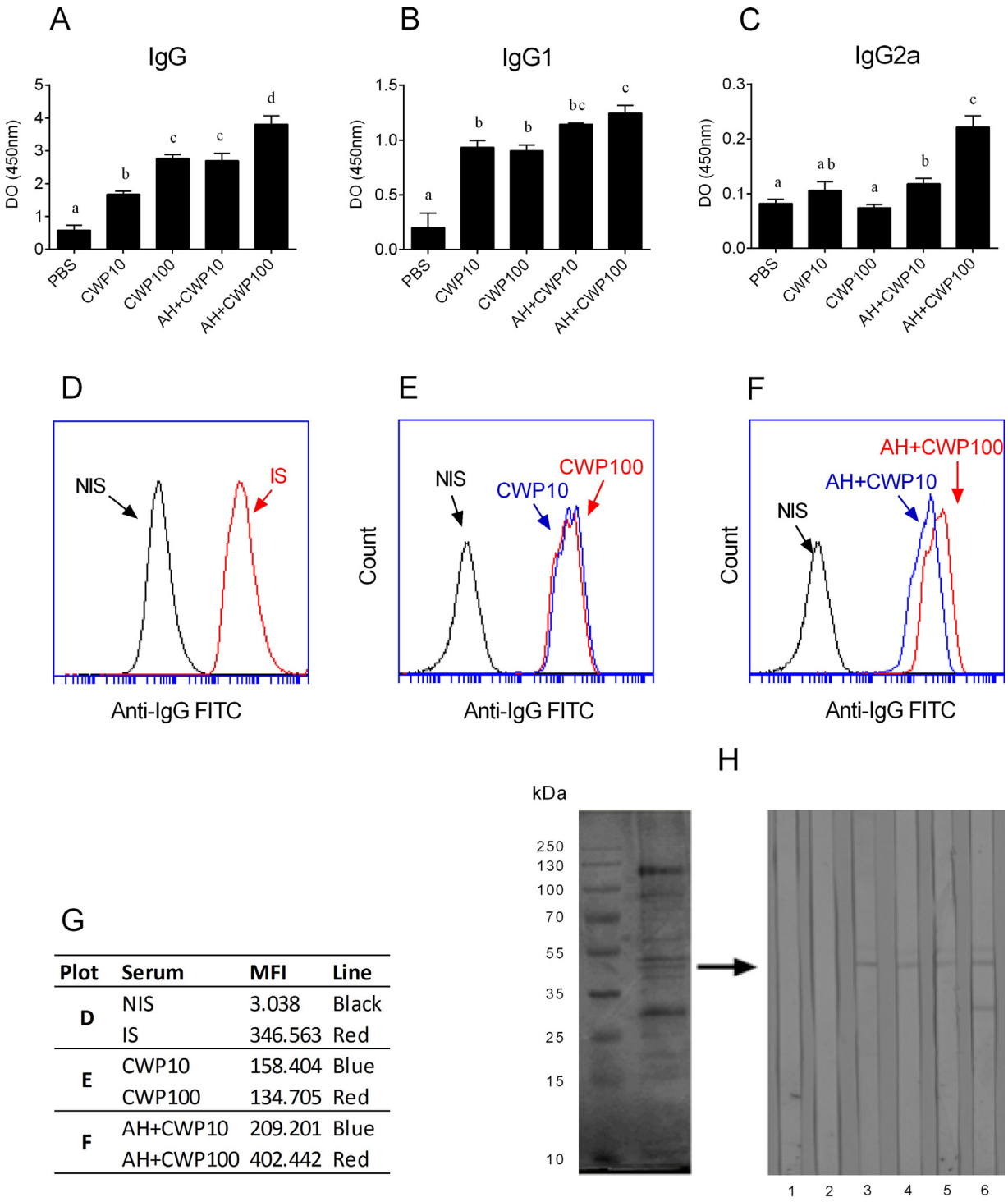
3.6. Passive transference assay

Our final step was to assess whether or not the formulations being tested were able to afford protection *in vivo*. Mice were passively transferred with sera from AH + CWP100- or CWP100-treated mice and challenged with *S. schenckii* two hours later. Protection was assessed on day 5 post-infection, when the peak of systemic fungal burden is expected to occur in this model (Ferreira et al., 2015). Passive transference of the AH + CWP100, but not of the CWP100 serum, was able to afford protection in the subsequent challenge with *S. schenckii*, as shown by the reduced number of CFUs recovered from the spleen and liver of AH + CWP100-treated mice (Fig. 6).

4. Discussion

In the last few years, new strategies have been proposed for controlling sporotrichosis owning to concerns about its growing incidence in humans, cats, and dogs in Brazil, as well as the toxicity and limited efficacy of conventional antifungal drugs (Barros et al., 2011; Rodrigues et al., 2013). The search for an anti-*Sporothrix* vaccine is a clear example of such trend. In this study, we focused on the CWPs from *S. schenckii*'s yeast-phase, which is this fungus' pathogenic form. We expected that, by using the same protein extraction method employed by Castro et al. (2013) for other *S. schenckii* strains, we would be able to identify the gp70 adhesin, which is the major immunogenic component characterized to date and a cell wall component of different species within the *S. schenckii* complex (Rodrigues et al., 2015). However, as recently shown by de Almeida et al. (2015), this glycoprotein's expression differ among the species of the *S. schenckii* complex, possibly reflecting their virulence status. We identified two other proteins instead, a peptide hydrolase and an enolase, the latter of which was predicted as an adhesin by the FungalRV database. Despite enolase being an enzyme of the glycolytic pathway in different organisms (Marcos et al., 2014), its presence was also shown on the cell wall of *Candida albicans* (Eroles et al., 1997), *Aspergillus fumigatus* (Denikus et al., 2005), and *Paracoccidiodes brasiliensis* (Nogueira et al., 2010), favoring the union of the fungi to fibronectin, laminin, and cells of the intestinal epithelium (Donofrio et al., 2009; Nogueira et al., 2010; Silva et al., 2014). Its protection-inducing potential was shown in a murine model of systemic candidiasis, where C57BL/6 mice immunized with recombinant enolase were effectively protected against infection with different *C. albicans* strains (Li et al., 2011). In our study, recognition of this protein by sera from both immunized and *S. schenckii*-infected animals allows us to speculate that it could be a valuable target in future therapies against sporotrichosis.

Generally, the particulate nature of AH-adsorbed antigens facilitates their uptake by antigen-presenting cells and thus enhances stimulation of the immune response (Ghimire, 2015). AH's surface is positively charged at the pH range normally used for vaccine formulation (pH 6.0–7.5), favoring adsorption of lower isoelectric point (pI) proteins which are negatively charged at such range (HogenEsch, 2012). Adsorption of the ssCWPs on AH occurred in several layers, as indicated by the non-linear adsorption isotherm, what could be attributed to their different degrees of affinity to



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Plot	Serum	MFI	Line
D	NIS	3.038	Black
	IS	346.563	Red
E	CWP10	158.404	Blue
	CWP100	134.705	Red
F	AH+CWP10	209.201	Blue
	AH+CWP100	402.442	Red

Fig. 4. Immunization with both AH-adjuvanted formulations induced production of anti-cell wall IgG1 and IgG2a antibodies. Sera collected from mice immunized with the indicated formulations was used to quantify ssCWP-specific IgG (A), IgG1 (B), and IgG2a (C) antibodies by ELISA and also to assess each serum's capacity to bind the *S. schenckii* yeast's surface by flow cytometry (D–G). For the latter, a *S. schenckii* yeast suspension was previously incubated with the anti-ssCWP sera or with sera from *S. schenckii*-infected mice (IS). Serum from non-immunized mice (NIS) was used as a non-specific binding control. (D–F) Representative histograms for each set of comparisons. (G) Median fluorescence intensity (MFI) values for each condition. (H) SDS-PAGE profile and immunoblotting of the ssCWPs with the anti-ssCWP sera. The arrow indicates the 47 kDa protein band. Lane 1: PBS. Lane 2: CWP10. Lane 3: CWP100. Lane 4: AH+CWP10. Lane 5: AH+CWP100. Lane 6: IS. Results are presented as the mean ± SD of 5 mice from one of two separate experiments. Statistical significance was determined by one-way ANOVA using Tukey's multiple comparisons test and a 95% confidence interval. Different letters represent significant differences between each pair of means ($p < 0.05$).

AH's surface. Furthermore, other individual characteristics of the ssCWP extract components could also help explain the desorption process occurring after 40 min, provably caused by competitive protein exchanges owning to the interplay between van der Waals effects, electrostatic interactions, and steric repulsions on AH's surface in a short-time interval (Lindblad and Schonberg, 2010; Matheis et al., 2001). Moreover, the degree of ssCWPs adsorption on AH had both qualitative and quantitative effects in the enhancement and modulation of the immune response, as seen by the changes in antibody production and cytokine profile resulting from

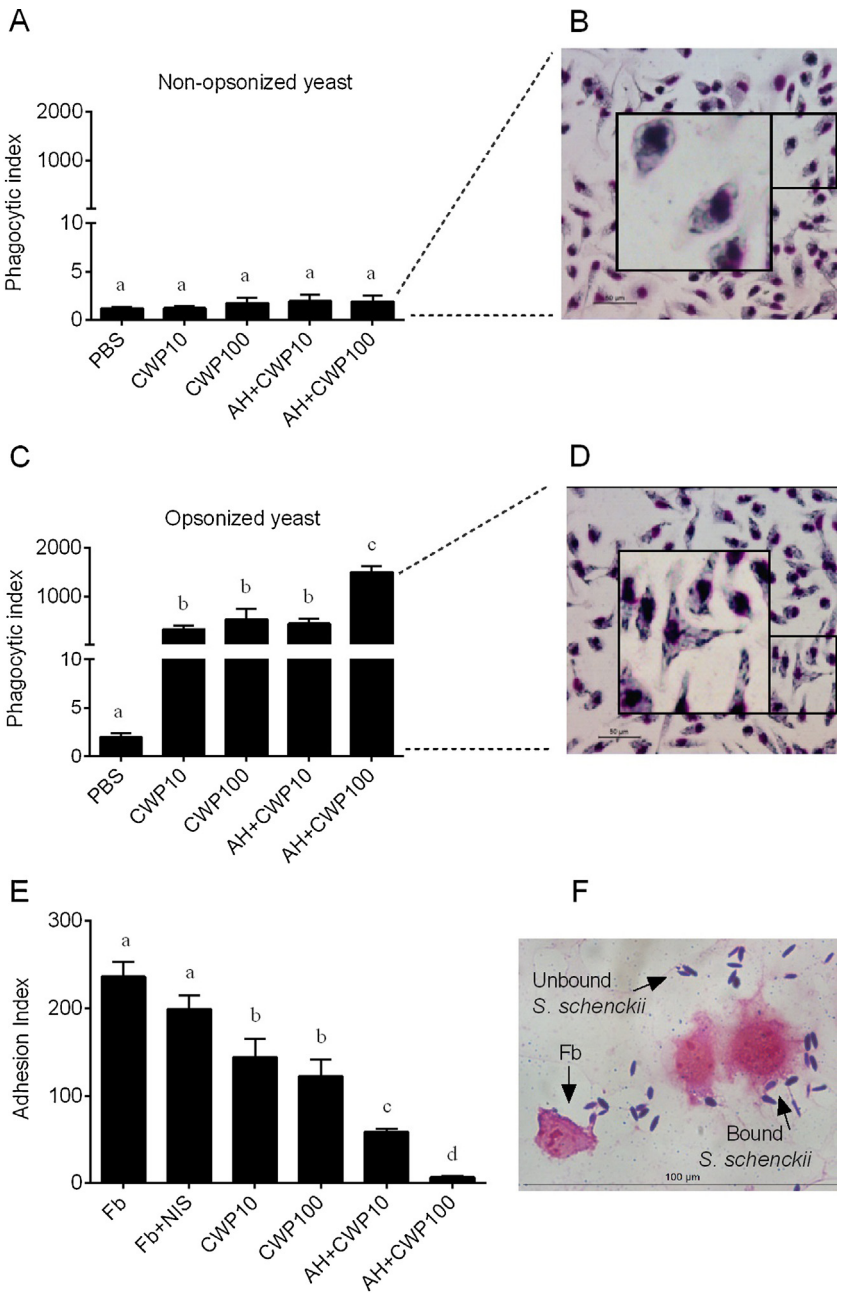

Fig. 5. Phagocytosis-enhancing and adhesion-inhibiting properties of the anti-ssCWP sera. (A–D) Thioglycollate-elicited peritoneal macrophages collected from mice immunized with the indicated formulations were incubated with opsonized or PBS-treated *S. schenckii* yeasts at a 1:4 macrophage to yeast ratio. (A and C) Phagocytic index for opsonized or non-opsonized yeasts, as indicated. (B and D) Representative photos of the AH+CWP100 treatment group in each experiment. (E and F) Opsonized or PBS-treated *S. schenckii* yeasts were transferred to fibroblast (Fb)-coated plates and incubated for 3 h. (E) Adhesion index for each indicated condition. (F) Representative photo of the PBS-treated control (PBS). Results are presented as the mean ± SD from three independent experiments. Statistical significance was determined by one-way ANOVA using Tukey's multiple comparisons test and a 95% confidence interval. Different letters represent significant differences between each pair of means ($p < 0.05$). NIS: serum from non-immunized mice.

immunization with AH+CWP100 discussed later. Lastly, recognition of two protein bands by serum from AH+CWP10-, but not from CWP10-immunized mice, further evidences AH's immune-enhancing effect.

For many years, the protective role of antibody-mediated immunity in fungal infections was controversial, whereas cell-mediated immunity was considered the fundamental mechanism of host defense. However, in the last two decades, a role for antibodies in host defense against different pathogenic fungi (Casadevall and Pirofski, 2012b), including *S. schenckii* (Almeida, 2012), has been shown in various forms, including opsonization and inhibition of fungus adherence onto host cells, complement activation, and antibody-dependent cellular cytotoxicity (ADCC) (Casadevall and Pirofski, 2012a). It is thus expected that an effective antifungal vaccine would be able to induce antibodies against the fungus' cell wall components in order to trigger some of the above-mentioned mechanisms. Accordingly, sera from both AH-CWP10- and AH+CWP100-immunized mice strongly inhibited adhesion of *S. schenckii* yeasts to fibroblasts, as well as significantly enhanced the fungus' uptake by macrophages *in vitro*.

As expected, passive transference of the AH+CWP100 serum was able to afford *in vivo* protection, matching the results obtained in

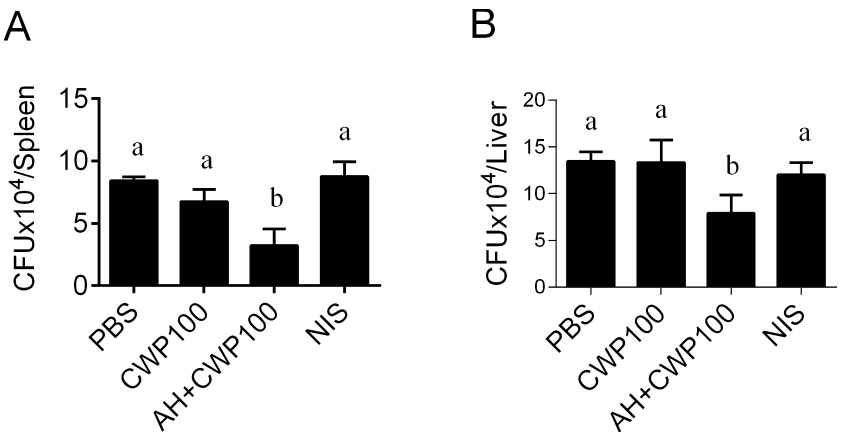

Fig. 6. Assessment of protection after passive transference of the anti-ssCWP sera. Mice were inoculated with the indicated formulations, or with NIS (serum from non-immunized mice) or PBS alone as negative controls two hours prior to infection with 10^6 *S. schenckii* yeasts. Protection was assessed by determining the number of CFUs recovered from mice's spleen (A) and liver (B) on day 5 post-infection. Results are presented as the mean ± SD of 7 mice from one of three separate experiments. Statistical significance was determined by one-way ANOVA using Tukey's multiple comparisons test and a 95% confidence interval. Different letters represent significant differences between each pair of means ($p < 0.05$).

the *in vitro* assays. Interestingly, although the CWP100 serum was effective in enhancing phagocytosis and inhibiting adhesion of *S. schenckii* yeasts to fibroblasts, its passive transference was not able to afford protection in a subsequent challenge with *S. schenckii*. Since both ssCWP-specific IgG1 and IgG2a antibodies were efficiently induced by immunization with AH+CWP100, whereas immunization with CWP100 induced production of IgG1 antibodies only, we thus suggest that IgG2a induction by AH+CWP100 may have been a key factor determining this formulation's protection-affording capability; in mice, IgG2a antibodies participate in complement fixation and binding to Fcγ receptors in order to stimulate phagocytosis and ADCC (Perez et al., 2013). Furthermore, our findings resemble those reported by Almeida's group showing a significant reduction in the number of CFUs recovered from the spleen and liver of anti-gp70-treated mice when the mAb was injected before, during, or after the *S. schenckii* infection (de Almeida et al., 2015; Nascimento et al., 2008); they also found that opsonization with an anti-gp70 mAb led to increased phagocytosis of *S. schenckii* yeasts by macrophages (Franco Dde et al., 2012). Since we could not detect gp70 in our protein extract, it seems that targeting of other antigens may be sufficient for enhancing phagocytosis and controlling the *S. schenckii* infection.

Interestingly, immunization with AH+CWP100 led to an increased *ex vivo* release of IL-12, IFN-γ, IL-4, and IL-17, suggesting a balance between Th1, Th2, and Th17 responses. Such profile is consistent with that reported by Hung et al. (2011). These authors showed that induction of this same Th1/Th2/Th17 balance on C57BL/6 mice that were immunized with an attenuated strain of *Coccidioides posadassi* was responsible for affording protection in a subsequent challenge. Also, a study performed by our group using a murine model of systemic infection showed a balance between Th1 and Th2 responses induced in an antigen-specific manner against the *S. schenckii* exoantigen (Maia et al., 2006). More recently, we showed the development of a mixed Th1 and Th17 response *in vivo*, and that the latter was required for optimal fungal clearance (Ferreira et al., 2015). Further studies are needed to clarify to what extent the cytokine profile found *in vitro* could contribute to afford *in vivo* protection in a future vaccination therapy.

In conclusion, this study showed that the AH+CWP100 formulation is immunogenic and able to promote phagocytosis-enhancing and adhesion-inhibiting, as well as *in vivo* protection-affording antibodies. Directly challenging vaccinated animals should promote a much better protection than just the passive transference of immune sera, as numerous vaccination-triggered cell-mediated effectors, plus additional humoral immune response elements (*e.g.*, complement proteins, which are absent from the inactivated sera), would aid in the clearance of the *S. schenckii* infection. Nevertheless, our present results suggest that the AH+CWP100 formulation is a viable vaccine candidate for further testing. Futures studies will allow us to better evaluate our proposed vaccine candidate protective properties in an actual immunization protocol and to determine its effectiveness as a tool for sporotrichosis' immunoprophylaxis in high risk areas.

Conflicts of interest

The authors declare no commercial or financial conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2015.10.005.

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