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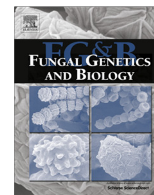

# The heat shock protein (Hsp) 70 of *Cryptococcus neoformans* is associated with the fungal cell surface and influences the interaction between yeast and host cells

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ABSTRACT

The pathogenic yeast *Cryptococcus neoformans* secretes numerous proteins, such as heat shock proteins, by unconventional mechanisms during its interaction with host cells. Hsp70 is a conserved chaperone that plays important roles in various cellular processes, including the interaction of fungi with host immune cells. Here, we report that sera from individuals with cryptococcosis infection recognize a recombinant *C. neoformans* Hsp70 (Cn_rHsp70). Moreover, immunofluorescence assays using antibodies against Cn_rHsp70 revealed the localization of this protein at the cell surface mainly in association with the capsular network. We found that the addition of Cn_rHsp70 positively modulated the interaction of *C. neoformans* with human alveolar epithelial cells and decreased fungal killing by mouse macrophages, without affecting phagocytosis rates. Immunofluorescence analysis showed that there was a competitive association among the receptor, GXM and Cn_rHsp70, indicating that the Hsp70-binding sites in host cells appear to be shared by glucuronoxylomannan (GXM), the major capsular antigen in *C. neoformans*. Our observations suggest additional mechanisms by which Hsp70 influences the interaction of *C. neoformans* with host cells.

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## 1. Introduction

*Cryptococcus neoformans* is an encapsulated yeast pathogen that causes opportunistic disease in humans. During host infection, *C. neoformans* is inhaled and then deposited into the lungs. In immunocompromised individuals, the fungus may disseminate to the brain causing meningoencephalitis (Mitchell and Perfect, 1995).

Macrophages are the first line of defense against *C. neoformans* (Mansour and Levitz, 2002). Moreover, macrophages play multiple roles during cryptococcosis that may include fungal killing, polysaccharide sequestration, cytokine production and antigen presentation (García-Rodas and Zaragoza, 2012). However, macrophages may also function as replicative niches for*C. neoformans* (Tucker and Casadevall, 2002; Alvarez and Casadevall, 2006; Johnston and May, 2012). One of the main virulence attributes of *C. neoformans* is the polysaccharide capsule, which is mainly composed of glucuronoxylomannan (GXM). This polysaccharide interacts with a number of surface components, including other polysaccharides, lipids and glycoproteins (Zaragoza et al., 2009; De Jesus et al., 2009; Jesus et al., 2010; Ramos et al., 2012).

Heat shock proteins (Hsps) are well-conserved proteins that participate in a wide range of biological processes. Hsps are involved in protein folding, the stabilization of biological substrates, the assembly of macromolecules and the degradation of polypeptides as well as the regulation of transcription mechanisms, splicing and translation (Bukau et al., 2006). Hsp70 is a cytoplasmic protein that is detected on the surface of fungal cells after heat stimulation (Guimarães et al., 2011). During the cellular response of eukaryotes to heat shock, Hsp70 is inserted into the plasma membrane prior to its release to the extracellular environment (Multhoff, 2007). In its membrane-bound form, Hsp70 can activate macrophages (Asea et al., 2000) and may be involved in cell adhesion mechanisms, molecular trafficking, receptor expression and macromolecule internalization (Vega et al., 2008).

*Abbreviations:* Hsp70, heat shock protein 70 kDa; GXM, glucuronoxylomannan; Cn_rHsp70, *Cryptococcus neoformans* recombinant heat shock protein 70 kDa.

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Hsps have been identified as dominant antigens in a variety of systemic infection models, including candidiasis (Eroles et al., 1997), aspergillosis (Burnie and Matthews, 1991; Gomez et al., 1992) and histoplasmosis (Deepe and Gibbons, 2002). Hsps have been characterized as key antigens inducing humoral responses in *C. neoformans* (Kakeya et al., 1997, 1999). Furthermore, Hsps are localized at the cell surface of a number of fungal pathogens (reviewed in Nimrichter et al., 2005; López-Ribot et al., 1996) and Hsp70 contributes to *Candida*–host interactions (Sun et al., 2010) acting as an invasin. In *C. neoformans*, Hsps have been described as extracellular components that are exported by vesicular mechanisms (Rodrigues et al., 2008), implying the existence of trans-cell wall secretory mechanisms for protein release and at least a transitory surface distribution of Hsps. However, the role of *C. neoformans* Hsps in host cell interactions and the infection process has not been previously reported.

In the present study, we identified Hsp70 as a surface protein of *C. neoformans* colocalized with GXM and associated with the capsular network. Based on the well-known ability of surface fungal proteins to impact fungi-host cell interactions (Bohse and Woods, 2005; Coleman et al., 2009; Desai et al., 2011), we evaluated the role of Hsp70 in the adhesion of *C. neoformans* to different cell lines and investigated its ability to stimulate phagocytosis. Our results indicate that Hsp70 may play important roles in the pathogenesis of *C. neoformans*.

## 2. Materials and methods

### 2.1. Fungal strain and culture media

The standard serotype A *C. neoformans* H99 strain (ATCC 208821) was maintained on YPD medium (1% yeast extract, 2% peptone, 2% dextrose and 1.5% agar). Cells were grown under constant agitation at 30 °C for 48 h in 50 ml of yeast nitrogen base (YNB), harvested by centrifugation at 5000g for 5 min, washed in phosphate-buffered saline (PBS) and counted in a Neubauer chamber.

### 2.2. Cloning, expression and purification of His-tagged recombinant protein

The *HSP70* gene sequence was obtained from the Broad Institute (www.broadinstitute.org). The amplified cDNA sequence was cloned into the pUC18 vector at the SmaI site and subcloned into the expression plasmid pET-23d(+) between the XhoI and NcoI sites (Invitrogen Corp., Carlsbad, California, USA). The sequence of the cloned ORF was verified by DNA sequencing. For the expression of the recombinant protein, the BL21(DE3) pLysS *Escherichia coli* strain was transformed with the plasmid, and protein expression was induced with 1 mM IPTG for 3 h. The recombinant protein was purified by nickel affinity chromatography using a Hi-Trap column (GE Healthcare, *formerly Amersham Biosciences*, Uppsala, Sweden). The purified recombinant protein, termed Cn_rHsp70, was dialyzed against water and treated with Triton X-114 to minimize any activation as a result of contaminating lipopolysaccharides (Yasuda et al., 2004).

### 2.3. Anti-Cn_rHsp70. sera

Female 6–8-week-old BALB/c mice were intraperitoneally immunized with 20 μg of Cn_rHsp70 mixed with complete Freund's Adjuvants (CFA, Sigma–Aldrich, St. Louis, Missouri, USA) and boosted 2 and 4 weeks post-immunization with incomplete Freund's adjuvant (Sigma–Aldrich). Polyclonal anti-Cn_rHsp70 antibody was obtained 6 weeks after the first immunization. All animal procedures were approved by the Ethics Committee for Animal Experimentation of the Universidade Federal do Rio Grande do Sul, Brazil.

Enzyme-linked immunosorbent assays (ELISAs) were used to examine the specificity of polyclonal anti-Cn_rHsp70 sera for recombinant protein Hsp70 from *C. neoformans*. Briefly, Cn_rHsp70 was incubated in 96-well polystyrene plates (Costar 9018; Corning Inc., New York, USA) overnight at 4 °C in PBS. The wells were blocked with 1% BSA (Sigma–Aldrich) in PBS for 1 h at 37 °C. The wells were then washed three times with 0.1% Tween-20 in PBS and coated with the anti-Cn_rHsp70 polyclonal sera. After 1 h at 37 °C, the plates were washed, coated with peroxidase-labeled anti-mouse IgG (Sigma–Aldrich) and incubated for 1 h at 37 °C. Serologic reactions were measured by the addition of TMB (Invitrogen) and spectrophotometrically determined at 450 nm. Pre-immune serum was used as a negative control. All experiments were performed in duplicate and statistically analyzed using Student's *t*-tests.

### 2.4. Analysis of mice and human sera

To determine the specificity of the polyclonal anti-Cn_rHsp70, a Western blot of *C. neoformans* protein extract was performed. Total protein was isolated as described by Crestani et al. (2012). Cell extract was separated by SDS–PAGE, transferred to a polyvinylidene fluoride (PVDF, GE Healthcare) membrane and probed with anti-Cn_rHsp70 at the dilutions of 1:5000 and 1:10,000. Detection was performed using an ECL-plus system (GE Healthcare) according to the manufacturer's instructions.

Due to the well-described ability of cryptococcal Hsp70 to induce humoral responses in human patients (Kakeya et al., 1999), we evaluated the serological properties of Cn_rHsp70 by Western blotting assays using sera from individuals diagnosed with cryptococcosis. Sera were kindly donated by Dr. Liline Martins (Universidade Federal do Piauí, Brazil). Cn_rHsp70 was separated by SDS–PAGE, transferred to a polyvinylidene fluoride membrane and probed with pooled sera from 5 patients with cryptococcosis at a dilution of 1:10. Detection was performed using an ECL-plus system (GE Healthcare) according to the manufacturer's instructions. Prestained Molecular Weight Marker (Sigma–Aldrich) was used as a protein ladder.

### 2.5. Surface distribution of Hsp70

ELISA with intact yeast cells was used to examine whether Hsp70 associated with the surface components of *C. neoformans*. The assay was performed based on the protocols described by Lopes et al. (2010). Briefly, *C. neoformans* ($5 \times 10^5$ cells/well) were incubated in 96-well polystyrene plates (Costar 9018; Corning Inc., New York, USA) for 2 h at 37 °C in PBS. Unattached yeast cells were removed by washing with PBS, and attached cells were blocked for 1 h at 37 °C using 2% BSA (Sigma–Aldrich) in PBS supplemented with 0.05% Tween-20. The plates were washed three times with 0.1% Tween-20 in PBS and coated with anti-Cn_rHsp70 polyclonal serum that was serially diluted 1:2 in the blocking solution. After 1 h at 37 °C, the plates were washed, coated with peroxidase-labeled anti-mouse IgG (Sigma–Aldrich) and incubated for 1 h at 37 °C. Serologic reactions were measured by the addition of TMB (Invitrogen) and determined by OD measurements at 450 nm. Polyclonal antiserum against β-tubulin (Sigma–Aldrich) produced in mice was used as a negative control.

### 2.6. Immunolocalization of Hsp70

Fungal cells were grown in YNB at 30 °C for 48 h, washed with PBS and fixed in 4% paraformaldehyde (Sigma–Aldrich). Cells were

blocked with 1% BSA (Sigma–Aldrich) and incubated with anti-Cn_rHsp70 polyclonal serum (1:50, v/v) for 1 h at 37 °C. Alternatively, the cells were incubated under the same conditions with a mouse monoclonal antibody to GXM (mAb 18B7, 10 μg/ml), a kind gift from Arturo Casadevall (Albert Einstein College of Medicine of Yeshiva University, New York). After washing with PBS, the cells were incubated with Alexafluor 488-conjugated anti-mouse IgG (Invitrogen) for 1 h at 37 °C. The cells were washed again and mounted on glass slides using a 50% glycerol/50% PBS/0.1 M *N*-propyl gallate solution. Finally, stained cells were analyzed using an Olympus FluoView™ 1000 fluorescence microscope (Olympus Optical Co., Melville, New York, USA).

The surface association of Hsp70 and GXM was also analyzed by double immunostaining using the polyclonal serum raised against Cn_rHsp70 and mAb 2D10, a kind gift from Arturo Casadevall (Albert Einstein College of Medicine of Yeshiva University, New York). After washing, cells were fixed with 4% paraformaldehyde and blocked for 1 h at 37 °C in PBS-1% BSA. Cells were washed three times with PBS and incubated with anti-Cn_rHsp70 polyclonal serum, for 1 h at 37 °C followed by incubation with Alexafluor 488-labeled anti-mouse IgG (Invitrogen). After washing, a second round of immunofluorescence staining was performed. Cells were incubated with mAb 2D10 and then incubated with 568-conjugated IgM anti-mouse (Invitrogen). Yeast cells were analyzed using an Olympus FluoView 1000 microscope (Olympus Optical Co., Melville, New York, USA). The acapsular mutant CAP67 of *C. neoformans* was analyzed by immunofluorescence as described above to verify the putative association of Hsp70 with surface, non-capsular components.

### *2.7. Binding of Cn_rHsp70 to mammalian cells*

The human type II alveolar epithelial cell line A549 and the macrophage-like cell lineage J774.1 were maintained and grown to confluence in culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a 7.5% $CO_2$ atmosphere. To evaluate whether Cn_rHsp70 affects cell viability, the ability of mammalian cells to reduce XTT ((sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H tetrazolium inner salt) was assayed. Monolayers ($10^6$ cells/well) were incubated for 18 h with varying concentrations of Cn_rHsp70 (0–250 μg/ml). After this period, 158 μl of fresh medium supplemented with 1 mg/ml XTT and 1 mM menadione was added, followed by 3 h of incubation at 37 °C. The absorbance (490 nm) was measured by spectrophotometry (Jin et al., 2004). Statistical analysis was performed using an ANOVA test (Graphpad Prism 5).

A549 and J774 cells ($10^6$ cells/well) were incubated with purified Cn-rHSP70 (10 μg/ml in DMEM) at 37 °C for 60 min. After washing to remove unbound proteins, the cells were fixed with 4% paraformaldehyde and blocked for 1 h at 37 °C in PBS supplemented with 1% BSA. The cells were washed three times with PBS and incubated with anti-Cn_rHsp70 polyclonal serum (1:50, v/v) for 1 h at 37 °C, followed by incubation with Alexafluor 488-labeled anti-mouse IgG (Invitrogen). Confocal microscopy was performed using an Olympus FluoView 1000 microscope (Olympus Optical Co., Melville, New York, USA). A549 and J774 cells incubated without the recombinant protein and prepared for microscopy as above were used as controls.

### *2.8. Influence of Cn_rHsp70 on the interaction of C. neoformans with host cells*

J774.1 macrophages were stimulated using 500 ng/ml *E. coli* LPS (Sigma–Aldrich) and 100 U/ml IFN-γ (Sigma–Aldrich) and pre-incubated with different concentrations of Cn_rHsp70 to evaluate whether the recombinant protein interfered with rates of phagocytosis, killing of *C. neoformans* or nitric oxide (NO) production. The macrophages were cultivated in 96-well plates (TPP, Trasadingen, Switzerland) at a density of $10^5$ cells/well at 37 °C with 7.5% $CO_2$. Cn_rHSP70 (0–25 μg/ml) was added to cell monolayers for 2 h. The medium was then replaced with fresh medium containing *C. neoformans* at a ratio of 10 fungal cells per phagocyte. Mammalian cells were incubated in the presence of *C. neoformans* for 18 h at 37 °C with 7.5% $CO_2$. Unattached yeast cells were removed by washing with PBS. Fungal survival was evaluated after macrophage lysis with sterile ice-cold Milli-Q water and subsequent plating on YPD for CFU determination. Phagocytosis indices were evaluated by measuring the fluorescence of infected macrophages in systems where fungal cells were labeled with FITC (Sigma–Aldrich). The assay was performed as described above with the modification that after macrophage lysis, labeled cells were transferred to a 96-well plate (Black Opaque 96-well Microplate, Perking Elmer, Massachusetts, USA), and the absorbance was read at 490–525 nm in a VICTOR™ *X*3 Multilabel Plate Reader (Perking Elmer, Massachusetts, USA). The NO production was measured using the Griess Reagent (Sigma–Aldrich) (Rivera et al., 2002). Fungal survival, phagocytosis indices and NO production were determined in duplicate and statistically analyzed using Student's *t*-test.

To evaluate the NO production by epithelial cells, A549 cells were stimulated using 100 U/ml IFN-γ (Sigma–Aldrich) and cultivated in 96-well plates (TPP, Trasadingen, Switzerland) at a density of $10^5$ cells/well at 37 °C with 7.5% $CO_2$. Cn-rHSP70 (0–25 μg/ml) was added to cell monolayers and the systems were incubated for 2 or 18 h. The medium was then replaced with fresh medium containing *C. neoformans* at a ratio of 10 fungal cells per host cell. Mammalian cells were then incubated in the presence of *C. neoformans* for 18 h at 37 °C with 7.5% $CO_2$. NO production was measured as described above.

To evaluate the attachment of *C. neoformans* to the A549 cells, $10^4$ epithelial cells/well were distributed in 96-well plates and incubated with 10 μg/ml of Cn_rHsp70 for 1 h at 37 °C. *C. neoformans* cells ($10^5$ cells/well) were incubated with 10 μg/ml of purified Cn_rHsp70 polyclonal serum for 1 h at 37 °C. The opsonized *C. neoformans* cells were incubated with epithelial cells at 37 °C in a 7.5% $CO_2$ atmosphere for 18 h. Unattached yeast cells were washed with PBS, A549 cells were lysed with sterile cold water and yeast suspensions were plated on YPD agar. Control systems were treated without antibody or with polyclonal antiserum against β-tubulin produced in mouse at the same concentration used for anti-Cn_rHsp70. After 48 h, the number of CFUs was determined. All experiments were performed in duplicate and statistically analyzed using Student's *t*-test.

### *2.9. Binding of Cn_rHsp70 and GXM in phagocytes*

To verify the distribution of Cn_rHsp70 and GXM in mammalian cells, J774.1 cells were grown and maintained as described in Section 2.7. J774.1 cells ($10^6$ cells/well – CellView Greiner Bio One) were double-immunostained for Cn_rHsp70 (10 μg/ml) and GXM (10 μg/ml). Three systems were analyzed as follows: (A) Cn_rHsp70 was incubated for 60 min, GXM was added and the incubation proceeded for an additional 60 min (Cn_rHsp70_GXM); (B) GXM was incubated for 60 min, Cn_rHsp70 was added and the incubation proceeded for 60 min (GXM_Cn_rHsp70); (C) Cn_rHsp70 and GXM were co-incubated for 60 min (Cn_rHsp70 + GXM). After washing, cells were fixed with 4% paraformaldehyde and blocked for 1 h at 37 °C in PBS-1% BSA. Cells were washed three times with PBS and incubated with anti-Cn_rHsp70 polyclonal serum or 2D10 mAb, a kind gift from Arturo Casadevall (Albert Einstein College of Medicine of Yeshiva University, New York), for 1 h at 37 °C followed by incubation with

Alexafluor 488-labeled anti-mouse IgG (Invitrogen) or 568-conjugated anti-mouse IgM. After washing, a second round of immunofluorescence staining was performed. Cells were incubated with 2D10 mAb or anti-Cn_rHsp70 polyclonal serum and then incubated with 568-conjugated IgM anti-mouse or Alexafluor 488-labeled anti-mouse IgG (Invitrogen), respectively. Finally, stained cells were analyzed using an Olympus FluoView 1000 microscope (Olympus Optical Co., Melville, New York, USA).

The binding of GXM and Cn_rHsp70 was also analyzed using ImageJ software to quantify the fluorescence intensity. To perform the quantitative colocalization analysis, the Pearson's coefficient (Parmryd et al., 2003) was calculated using Olympus FluoView software.

The expression of macrophage GXM receptors after exposure to Cn_rHsp70 was assessed by a molecular approach. Macrophages were incubated with 10 μg/ml of Cn_rHsp70 or 10 μg/ml of GXM for 1 h. Total mRNA from was isolated by Trizol (Invitrogen) to evaluate the expression of TLR2 and TLR4. RNA was treated with DNAse (Promega) and was reverse-transcribed using MMLV reverse transcriptase (Promega) using oligo-dT. qRTPCR was performed on a Real-time PCR StepOne Real-Time PCR System (Applied Biosystems). PCR thermal cycling was 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s, 55° C for 15 s and 60 °C for 60 s. Platinum SYBR green qPCR Supermix (Invitrogen) was used as a reaction mix, supplemented with 5 pmol of each primer and 1 μl of the cDNA template in a final volume of 20 μl. Melting curve analysis was performed to confirm a single PCR product. The data were normalized with the transcript for ß-actin amplified in each set of qRT-PCR experiments. A non-template control was included. The relative expression level of genes was determined by the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001). Statistical analyses were conducted with the ANOVA test.

## 3. Results

### 3.1. Cn_rHsp70. is recognized by serum antibodies

Cn_rHSP70 was successfully cloned into the pET23d plasmid vector. *E. coli* BL21(DE3)pLysS cells were transformed with the resulting plasmid and induced with 1 mM IPTG for 3 h. After purification by affinity chromatography, the recombinant protein was resolved by SDS–PAGE for further evaluation of the serologic reactivity. As shown in Fig. 1A, the polyclonal anti-Cn_rHsp70 serum

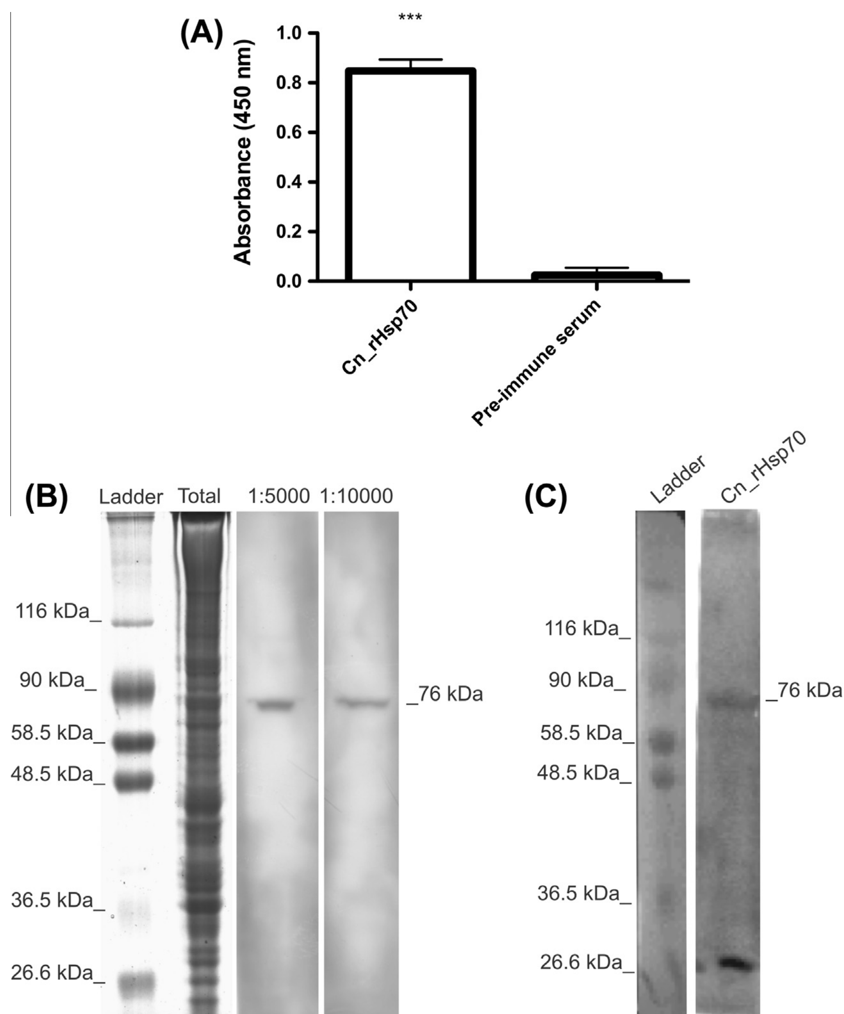


**Fig. 1.** Cn_rHsp70 is recognized by sera from immunized mice and patients with cryptococcosis. (A) A polyclonal serum raised against *C. neoformans* Hsp70 interacts with Cn_rHsp70. The recombinant protein was used to coat 96-well polystyrene plates for ELISA. Positive reactions were observed when the antigen was probed with the polyclonal serum. The reactivity of the protein pre-immune serum was similar to the background level. Asterisks denote $p < 0.05$. (B) Western blot analysis of *C. neoformans* cell extracts after separation by SDS–PAGE confirmed the specificity of a polyclonal serum raised against *C. neoformans* Hsp70. (C) Serologic reactivity of Cn_rHsp70 with a pool of sera obtained from cryptococcosis patients. Western blot analysis after separation of Cn_rHsp70 by SDS–PAGE revealed a band with a migration rate corresponding to Hsp70. Molecular mass markers are shown in the right column.

recognized soluble Cn_rHsp70. This reactivity was abolished when the pre-immune serum was incubated with soluble Cn_rHsp70. *C. neoformans* cell extracts were probed with mice sera raised against Cn_rHsp70 demonstrating a high specificity of the polyclonal antibody (Fig. 1B). Cn_rHsp70 was also probed with sera from patients with cryptococcosis. A band with a migration rate and molecular mass corresponding to Cn_rHsp70 (76 kDa) was recognized by the patients' sera (Fig. 1C).

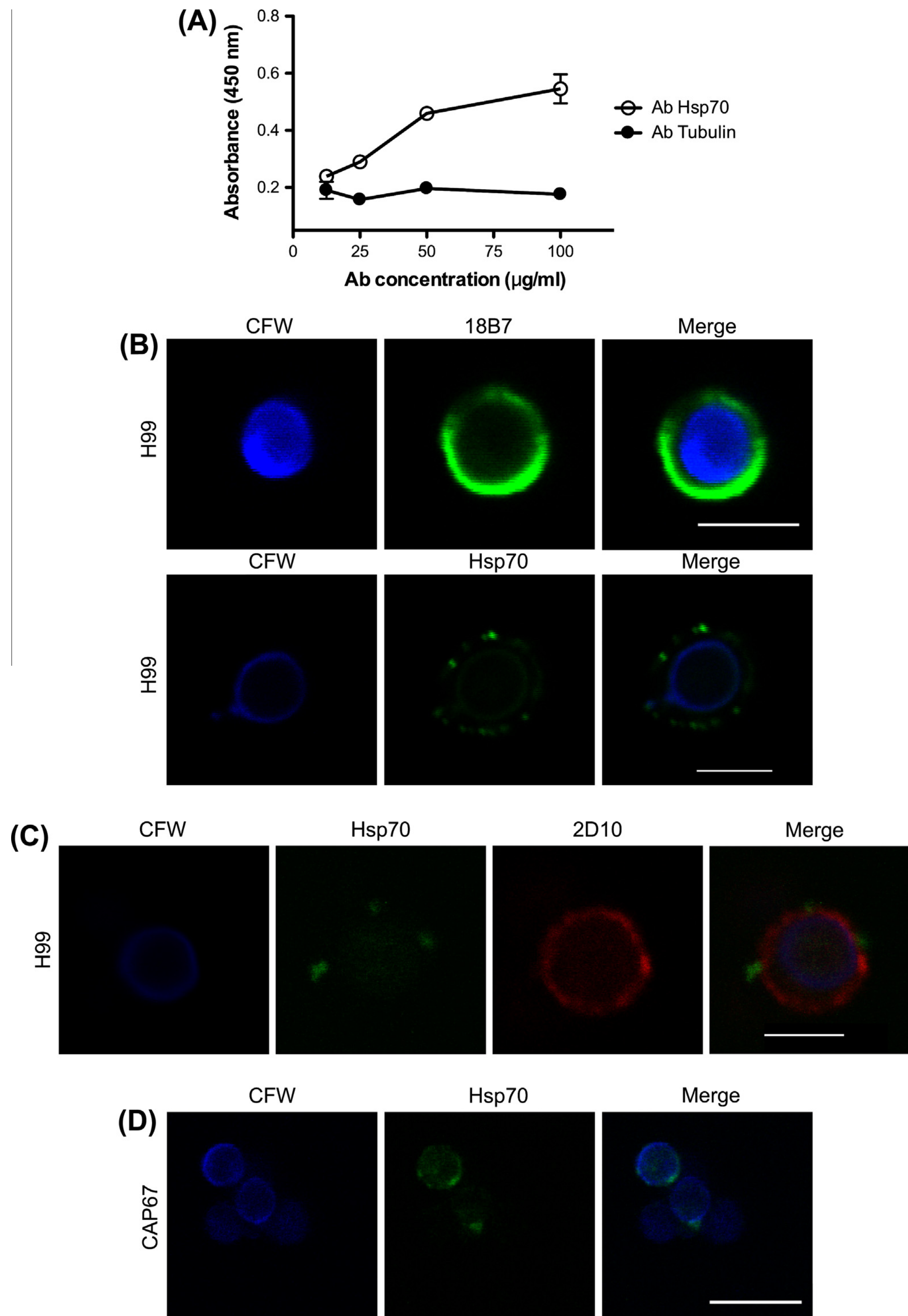


**Fig. 2.** Hsp70 is associated with the capsule. (A) The anti-Hsp70 polyclonal serum binds to the cell surface of *C. neoformans.* ELISA using whole cells revealed efficient serum binding at concentrations greater than 25 µg/ml. An irrelevant antibody (β-tubulin) used as a control showed no binding to *C. neoformans* cells. Data represent mean ± SD from three experimental replicates. The experiment was repeated twice with similar results. (B) Immunofluorescence analysis of *C. neoformans* yeast cells revealed preferential Hsp70 detection at the cryptococcal capsule. Capsular structures are stained with mAb 18B7 (green fluorescence – upper panel); Hsp70 appears in green (lower panel). The cell wall was stained with calcofluor white (blue fluorescence). (C) *C. neoformans* H99 yeast cells were double-immunostained for Cn_rHsp70 (anti-Cn_rHsp70, green fluorescence) and GXM (mAb 2D10, red fluorescence). The cell wall was stained with calcofluor white (blue fluorescence). (D) Staining of the acapsular mutant CAP67 confirmed that Hsp70 is also located on the cell wall; Hsp70 appears in green. The cell wall was stained with calcofluor white (blue fluorescence). The scale bar corresponds to 5 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### 3.2. Hsp70 is capsule associated

ELISA using intact *C. neoformans* cells demonstrated that the anti-Cn_rHsp70 serum reacted with the fungus (Fig. 2A). Binding was not observed in control fungal cells using polyclonal antiserum against β-tubulin produced in mice.

The cellular distribution of Hsp70 was analyzed by confocal microscopy using the polyclonal serum raised against Cn_rHsp70.

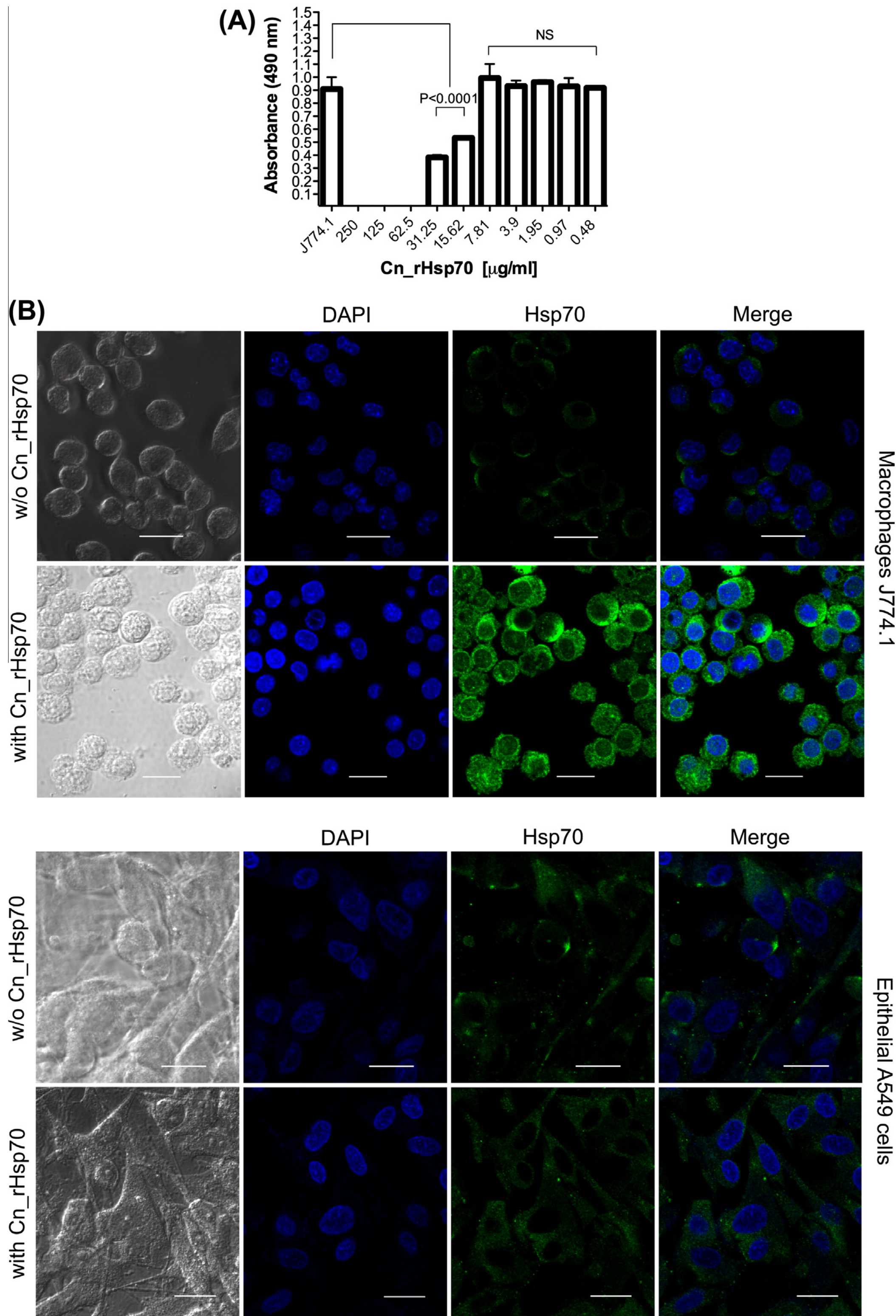


**Fig. 3.** Cn_rHsp70 binds to host cells. (A) J774.1 cells were incubated with different concentrations of Cn_rHsp70 overnight followed by tetrazolium (XTT) reduction assays. Cell viability was affected by Cn_rHsp70 at concentrations greater than 15.62 μg/ml. Asterisks denote $p < 0.05$. Data represent mean ± SD from three experimental replicates. (B) J774.1 and A549 cells were incubated with Cn_rHsp70 (10 μg/ml) for immunofluorescence analysis (with Cn_rHsp70). Negative controls consisted of cells that were not exposed to the recombinant protein (w/o Cn_rHsp70). J774.1 cells were incubated with Cn_rHsp70 for 60 min. Similarly, alveolar A549 cells were treated with the protein for 60 min. The nucleus was stained with DAPI (blue fluorescence). Alexafluor 488-conjugated anti-mouse IgG produced the green fluorescence. The scale bar corresponds to 7.3 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Capsular structures were stained with mAb 18B7, which reacts with GXM (Mukherjee et al., 1993). Hsp70 was clearly associated with the capsular network (Fig. 2B). Alternatively, the surface association of Hsp70 and GXM was analyzed by double immunostaining using the polyclonal serum raised against Cn_rHsp70 and mAb 2D10, respectively. Hsp70 was clearly associated with the capsular network (Fig. 2C). Hsp70 ofC. *neoformans* also seems to be located on the cell wall, as observed in the acapsular mutant CAP67 (Fig. 2D).

### *3.3. Cn_rHsp70 is recognized by phagocytes and epithelial cells*

The surface exposure of Hsp70 led us to hypothesize that host cells may recognize this molecule. Before addressing this hypothesis, we evaluated the potential toxic effects of Cn_rHsp70 towards mammalian cells. J774.1 macrophages were exposed to varying concentrations of Cn_rHsp70 (0–250 μg/ml) to evaluate the cell viability by XTT reduction. The protein was toxic to host cells at relatively high concentrations (Fig. 3A) and, thus, we selected the nontoxic concentration of 10 μg/ml for further experiments.

To evaluate the presence of Hsp70-binding sites at the cell surface of two different cell types, A549 alveolar epithelial cells and J774.1 macrophages were incubated with Cn_rHsp70 followed by further incubation with the anti-Cn_rHsp70 polyclonal serum. Binding of Cn_rHsp70 was demonstrated for both cell lineages (Fig. 3B). The macrophage-like J774.1 cells appeared to have a higher affinity for Cn_rHsp70 than did alveolar A549 cells. For this reason, we selected J774.1 cells as prototypes for further immunofluorescence assays.

### *3.4. Cn_rHsp70 affects the interaction of C. neoformans with host cells*

Because surface proteins are the first points of contact of pathogens with host cells, the role of Cn_rHsp70 was evaluated during this interaction. The addition of Cn_rHsp70 had effects on the killing of *C. neoformans* by macrophages, but not in the phagocytosis process (Fig. 4A and B). The pretreatment of macrophages with Cn_rHsp70 increased fungal survival and caused a decrease in NO production (Fig. 4C). Furthermore, macrophages pretreated with Cn_rHsp70 showed a decrease in NO production in the absence of *C. neoformans* cells (Fig. 4D).

Due to the hypothesis that epithelial cells might play a role in the lungs producing inflammatory mediators such as NO, A549 cells were treated with different concentrations of Cn_rHsp70 to evaluate whether the recombinant protein interfered with NO production. A549 cells were treated with Cn_rHsp70 for 2 or 18hs in the presence or absence of *C. neoformans*. In the absence of *C. neoformans,* Cn_rHsp70 (0.31–25 μg/ml) did not stimulate NO production (data not shown). In the presence of *C. neoformans* a slight increase in NO levels ($p < 0.05$) was observed (Fig. 5A and B).

The treatment of alveolar cells with Cn_rHsp70 in combination with *C. neoformans* coated with anti-Cn_rHsp70 serum decreased the association between the yeast and host cells (Fig. 5C). When A549 cells were incubated with 1 μg/ml of Cn_rHsp70 and *C. neoformans* cells coated with 1 μg/ml of anti-Cn_rHsp70, there was a decrease in the fungal cell recovery. The same effect was observed when A549 cells were incubated with 3 μg/ml of Cn_rHsp70 and *C. neoformans* cells coated with 1 μg/ml or 10 μg/ml of anti-Cn_rHsp70. However, fungal attachment was not observed without antibody coating, even when A549 cells were incubated with 1 or 3 μg/ml of Cn_rHsp70. The same result was observed in controls where the interaction was performed with *C. neoformans* cells coated with anti-Cn_rHsp70 or with anti-tubulin.

### *3.5. Cn_rHsp70 and GXM are colocalized on the surface of host cells*

It has been reported that human A549 cells express receptors for GXM (Barbosa et al., 2007). The activation of A549 cell surface

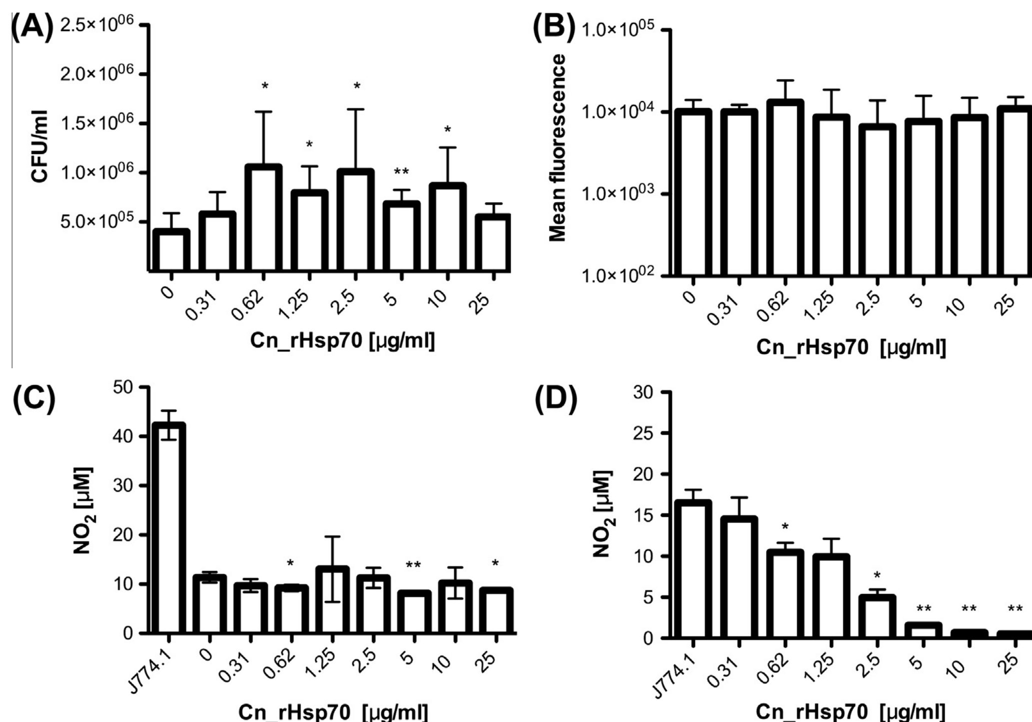


**Fig. 4.** Cn_rHsp70 influences fungal survival and NO production by macrophages (J774.1). Fungicidal activity (A), phagocytosis index (B) and NO production (C) are shown. (D) Macrophages were pretreated with Cn_rHsp70 in the absence of *C. neoformans* cells and NO production was measured. The results are representative of two independent experiments. Asterisks denote $p < 0.05$ and are relative to J774.1 cells incubated with *C. neoformans* cells without pretreatment with Cn_rHsp70 (A and C) or J774.1 cells alone (D).

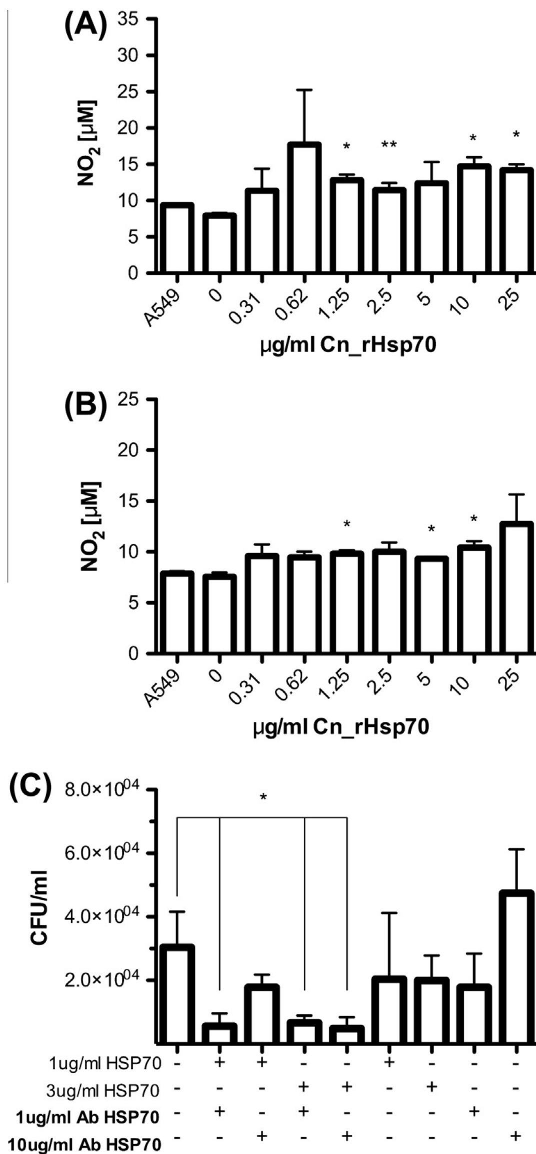


**Fig. 5.** Hsp70 modulates the adhesion of *C. neoformans* and increases the NO production by A549 cells. Epithelial cells were pretreated with Cn_rHsp70 for 2 h (A) or 18 h (B), and then incubated with *C. neoformans* cells for 18 h to evaluate NO production. Asterisks denote $p < 0.05$ and are relative to A549 cells incubated with *C. neoformans* cells without pretreatment with Cn_rHsp70. (C) The treatment of fungal cells with anti-Cn_rHsp70 and of human cells with soluble Cn_rHsp70 caused a significant inhibition ($p < 0.05$) of the association between fungal and host cells. The inhibitory effect was not observed when an antibody against α-tubulin was used under the same conditions. Control systems consisted of A549 or *C. neoformans* cells that were not exposed to the antibody or to the recombinant protein, respectively.

receptors by GXM induces the release of IL-8, a response that is also observed when these cells are stimulated with microbial Hsp (Yamaguchi et al., 1999). We then hypothesized that GXM and Hsp70 may share the same cellular binding sites in host cells. As macrophages are the first line of defense in pulmonary fungal infection, we evaluated this hypothesis using J774.1 macrophage-like cells. When both Cn_rHsp70 and GXM antigens were simultaneously incubated with the macrophages, the molecules were detected at the plasma membrane, although colocalization did not occur (Fig. 6A, lower panel). Previous exposure of the J774.1 cells to GXM followed by Cn_rHsp70 incubation resulted in a similar pattern of antigen binding to these cells (Fig. 6A, upper panel). However, when the cells were treated with Cn_rHsp70 followed by GXM, both antigens clearly colocalized (Fig. 6A, middle panel). The calculated Pearson correlation coefficient of 0.84 confirmed this high degree of colocalization. Independent staining experiments employing only one antigen (GXM or Cn_rHsp70) were performed as a control (Fig. 6B). Furthermore, the fluorescence levels were quantified using ImageJ software (Fig. 6C and D) to compare the three systems of incubation. When the cells were incubated with GXM followed by Cn_rHsp70, the intensity of green (Fig. 6C) and red (Fig. 6D) fluorescence increased in comparison with the respective controls ($p < 0.05$). However, when the cells were treated with Cn_rHsp70 followed by GXM, a significant increase in the red fluorescent intensity was observed (Fig. 6D) with ($p < 0.0001$). Collectively, these results suggest that Cn_rHsp70 may modulate the association of GXM with macrophage cells.

The expression of macrophage TLR2 and TLR4 receptors after exposure to Hsp70 was evaluated to confirm the involvement of these receptors in the Hsp70–GXM interaction. Our results indicated that TLR4 was upregulated in the macrophages after exposure to the fungal components (Fig. 7B).

## 4. Discussion

Previous studies of the functions of Hsps in *C. neoformans* suggest that the deletion of *SSA1*, a gene that is homologous to the *HSP70* gene, results in increased capsular dimensions (Zhang et al., 2006). In the current study, we provide evidence that *C. neoformans* Hsp70 is both surface-associated and distributed within the fungal cytoplasm. Our immunofluorescence observations demonstrate that Hsp70 and the capsule are at least transiently associated, leading us to hypothesize that Hsp70 is associated with *C. neoformans* surface components during secretion. The mechanism by which Hsp70 becomes localized at the cell surface is unclear, although it was recently demonstrated that hydrogen bonds might promote interactions between the surface components of *C. neoformans* (Ramos et al., 2012). The *C. neoformans HSP70* gene lacks secretory signal sequences, suggesting that the protein is not surface localized. However, it is now clear that fungal cells are extremely efficient in exporting cytoplasmic proteins by unconventional mechanisms (Albuquerque et al., 2008; Rodrigues et al., 2008; Vallejo et al., 2012; Kubitschek-Barreira et al., 2013; Oliveira et al., 2010) that may provide an explanation for the detection of Hsp70 at the cell surface of *C. neoformans*. For example, Hsps 60 and 70-kDa from *Histoplasma capsulatum* have also been detected on the cell surface (Long et al., 2003).

The interaction between macrophages and cryptococci is complex and may have different outcomes. Fungal killing by the phagocytes has been previously demonstrated, although intracellular replication of the pathogen is now recognized as an important consequence of the phagocytic process (reviewed in García-Rodas and Zaragoza, 2012). There was an increase in fungal survival when macrophages were pre-incubated with Cn_rHsp70 prior to incubation with fungal cells, followed by a reduction in NO production. We speculated that Cn_rHsp70 could inhibit the macrophage activation avoiding fungal killing by the oxidative mechanism. However, Cn_rHsp70 was actually less efficient than an NO inhibitor, as inferred from a comparison between the current data and the study by Chiapello et al. (2008). Kawakami et al. (1997) demonstrated that *C. neoformans* inhibits NO production in peritoneal macrophages stimulated with LPS and IFN-γ and that this inhibition is independent of the capsule. Our findings are in agreement with these previous studies, suggesting that surface molecules of the fungus may be involved in the suppression of NO production in macrophages.

**Fig. 6.** Cn_rHsp70 and GXM colocalized in J774.1 cells. (A) GXM (2D10, red fluorescence) was incubated for 60 min prior to the addition of Cn_rHSP70 (anti-Cn_rHsp70, green fluorescence) to the system (GXM_ Cn_rHsp70; upper panel). Cn_rHsp70 (anti-Cn_rHsp70, green fluorescence) was incubated for 60 min prior to the addition of GXM (2D10, red fluorescence) to the system (Cn_rHsp70 _GXM; middle panel). Cn_rHsp70 and GXM were incubated simultaneously for 60 min with J774.1 cells (Cn_rHsp70 + GXM; lower panel). (B) GXM (2D10, red fluorescence) was incubated for 60 min with J774.1 cells (GXM). Cn_rHsp70 (anti-Cn_rHsp70, green fluorescence) was incubated for 60 min with J774.1 cells (Cn_rHsp70). Binding sites were determined after the addition of antibodies raised against Hsp70 (anti-Cn_rHsp70) or GXM (2D10), followed by the addition of secondary antibodies. The merged image (right panel) demonstrates the colocalization of Cn_rHsp70 and GXM at the surface of J774.1 cells. ImageJ software was used to measure the green (C) and red (D) fluorescence intensity (average of five cells) per area in each layer. The scale bar corresponds to 10.070 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

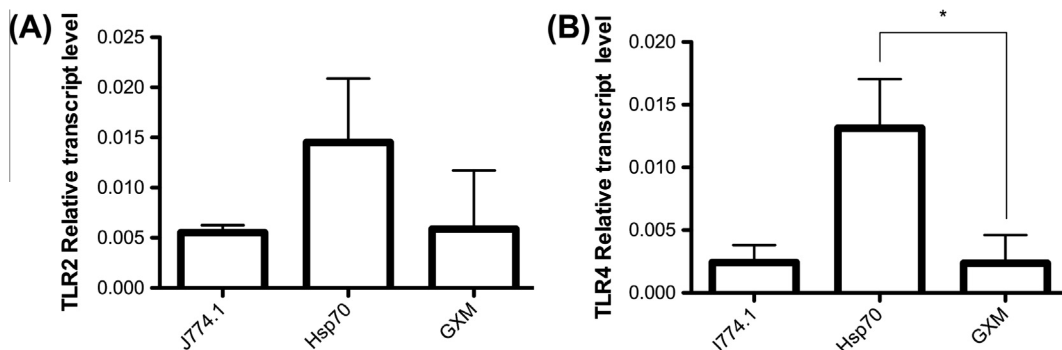


**Fig. 7.** TLR2 and TLR4 expression in macrophages stimulated with Cn_rHsp70 or GXM. Macrophages were treated with 10 μg/ml of Cn_rHsp70 or 10 μg/ml of GXM. The levels of TLR2 (A) and TLR4 (B) mRNAs were determined by qRT-PCR. The relative transcript levels were normalized to actin. Data shown are representative of three independent experiments.

Human lung epithelial cells might play a role in innate immunity in response to bacterial diseases by producing NO (Roy et al., 2004). Moreover, lung epithelial cells interact with *C. neoformans* GXM (Barbosa et al., 2006) with consequent production of IL-8 (Barbosa et al., 2007). In our model, we observed that, in the presence of *C. neoformans,* A549 epithelial cells treated with Cn_rHsp70 released NO. Our findings led us to speculate that Hsp70 could have a dual role during infection. Cn_rHsp70 could act as an effector molecule inducing NO production by epithelial cells (Roy et al., 2004) or as an immunosuppressive molecule reducing NO production by macrophages (Rossi, 1999).

GXM binds to cellular receptors, including CD14, TLR2, and TLR4, *in vitro* (Levitz, 2002; Yauch et al., 2004; Monari et al., 2005). Some of these receptors, such as TLR2 and TLR4, also interact with Hsp70 (Thèriault et al., 2005). For example, it has been demonstrated that Hsp activates innate immunity through interaction with the TLR4/CD14/MD2 complex (Triantafilou et al., 2008). Therefore, we speculate that *C. neoformans* Hsp70 may bind to host cells by mechanisms similar to those used by capsular GXM. In other models, Hsp70 has been correlated with processes of fungal adhesion to epithelial cells (Ganendren et al., 2006; Coleman et al., 2009; Bailão et al., 2012). In our study, Cn_rHsp70 promoted the adhesion of *C. neoformans* to A549 cells. One must consider that Hsp70 is regularly secreted by *C. neoformans* (Rodrigues et al., 2008) and the extracellular form will not be affected by antibody treatment. If this form of the protein associates with host receptors, it might promote increased adhesion of *C. neoformans* to host cells, since this protein also interacts with capsular components. Previous studies demonstrated that *C. neoformans* binds to alveolar epithelial cells in processes that involve the capsule and other surface structures, culminating in fungal internalization (Merkel and Scofield, 1997; Barbosa et al., 2006). The adhesion of microbes to host cells is a multifactorial process that involves multiple host receptors and microbial ligands (Mendes-Giannini et al., 2005). In this context, it is reasonable to suppose that *C. neoformans* uses several different molecules to interact with epithelial cells.

A549 cells express receptors for GXM (Barbosa et al., 2007) and also for microbial Hsp (Yamaguchi et al., 1999). In our model, potentially similar sites of adhesion were observed for both molecules during macrophage interaction. We speculate that the exposure of macrophage cells to Cn_rHsp70 may facilitate further binding of GXM. This sequential event may be explained by the direct interaction between host cell-bound Cn_rHsp70 and GXM or, alternatively, by a change in the expression of GXM receptors in the host cells after exposure to the Hsp. Further studies on the interaction of Hsp70 with GXM will contribute to the understanding of this step of the interaction of *C. neoformans* with phagocytes cells.

The capsule plays important roles in the interaction of *C. neoformans* with macrophages, which mainly involve protection against phagocytosis (reviewed in Zaragoza et al., 2009). In *H. capsulatum*, however, Hsp60 mediates the recognition of yeast cells by macrophages through binding to the CD18 receptor (Long et al., 2003). Because Hsp70 was found to be associated with the capsular components, we hypothesized that similar processes may occur in *C. neoformans*. Based on the immunofluorescence analysis, we suggest that Cn_rHsp70 binds to host cells through an unidentified receptor. Other studies have suggested that cryptococci interact with host cells by mechanisms that likely involve the CD14 receptor (Barbosa et al., 2007; Levitz, 2002; Yauch et al., 2005). However, Hsp70 binds to TLR2 and TLR4 with minimal affinity (Thèriault et al., 2005) and activates innate immune receptors, including the TLR4/CD14/MD2 complex (Triantafilou et al., 2008). Our results demonstrated that TLR4 was upregulated in the macrophages stimulated with Cn_rHsp70. Therefore, we speculate that *C. neoformans* Hsp70 may bind to host cells by mechanisms similar to those used by capsular GXM.

The characterization of unconventional secretion in *C. neoformans* is ongoing (Kmetzsch et al., 2011.), and its role in the modulation of host cell responses has been described (Oliveira et al., 2010). Moreover, non-classically secreted proteins were found in the cell surface of several fungi and shown to modulate the interaction with host cells (Long et al., 2003; Nogueira et al., 2010). Our results provide evidence that *C. neoformans* Hsp70 may be involved in the association of cryptococcal cells with alveolar epithelial or macrophage cells. Further studies will elucidate the complexassociation of Hsp70 with molecules involved in the interaction between host cells and *C. neoformans* as well as the impact of such an association on the infection process.

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