UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Análise do papel da Hsp70 e catalase 2 na fisiologia e patogênese do fungo *Cryptococcus neoformans*

Tese de Doutorado

Carolina Pereira Silveira

Porto Alegre, junho 2013

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do Grau de Doutor em Ciências.

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Porto Alegre, junho 2013

Este trabalho foi desenvolvido no Laboratório de Biologia de Fungos de Importância Médica e Biotecnológica, situado no Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul.

AGRADECIMENTOS

A minha orientadora, professora Dra. Marilene Henning Vainstein pela oportunidade de desenvolver meu conhecimento técnico; total confiança no meu trabalho; incentivo às novas idéias que surgiram ao longo deste trabalho e pela orientação durante todo meu doutoramento.

Ao meu co-orientador, professor Dr. Augusto Schrank, pelo incentivo na busca de conhecimento.

À minha comissão de acompanhamento, professor Dr. Arnaldo Zaha e professora Dra. Irene Schrank, pelas discussões dos resultados.

Ao professor Dr. Charley Staats pela contribuição na geração de dados e principalmente pela amizade.

À Dra. Lívia pela colaboração e ensinamentos que muito contribuíram para o desenvolvimento deste trabalho, mas principalmente a amiga Lívia, pela constante força, carinho e amizade que demonstrou durante todo o tempo.

À Juli e Melissa, por todo o companheirismo e amizade.

À pequena Alicia por toda a disposição e contribuição para este trabalho.

Aos colegas do laboratório 217, 220 e 222 pela convivência e companheirismo.

Ao Laboratório de Sequenciamento da Rede Sul de Análise de Genomas PIGS – Cbiot UFRGS pelo sequenciamento de todos os plasmídeos utilizados neste trabalho.

À Unidade de Química de proteínas e Espectrometria de Massas – UNIPROTE-MS pela identificação dos peptídeos das proteínas recombinantes.

Ao Centro de Microscopia Eletrônica da UFRGS – CME por todas as imagens obtidas neste trabalho.

Aos funcionários Milton e Terezinha, por facilitarem o trabalho de todos com a limpeza e esterelização dos materiais.

Aos secretários do PPGBCM, Luciano e Silvia, pelo constante auxílio e atenção oferecidos aos alunos.

À FINEP e a Ludwig Biotec pelo apoio financeiro.

Ao meu amor, Thiago, pelo total incentivo, apoio e compreensão.

Aos meus irmãos, Roberto e Gustavo, pelo amor incondicional.

Aos meus pais, Mercedes e Alexandre, agradeço pela minha formação e educação, mas mais ainda por serem meus grandes incentivadores e estarem do meu lado em qualquer decisão.

À todos que contribuíram de forma direta e indireta para a realização desta tese.

Sumário

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

°C	graus Celsius
%	porcento
anti-GXM	anticorpos anti glucoronoxilomanana
APC	células apresentadoras de antígenos
Arg1	enzima arginase que hidrolisa arginina
ATP	adenosina trifosfato
BSA	albumina de soro bovino
C3b	elementos resultante da clivagem do componente 3 do complemento
cDNA	DNA complementar
$CD4^+$	linfócitos T auxiliares positivos para o marcador CD4
$CD8^+$	linfócitos T auxiliares positivos para o marcador CD8
CD14	receptor CD14
CFU	unidade formadora de colônia, do inglês *Colony Forming Units*
CLSI	do inglês *Clinical and Laboratory Standards Institute*
Cn_rCAT	*Cryptococcus neoformans* recombinant catalase protein
Cn_rHsp70	*Cryptococcus neoformans* recombinant heat shock protein
CpG	sequências de DNA ricas em citosina e guanina
CO_2	dióxido de carbono
CR1	receptor de complemento 1
CR2	receptor de complemento 2
CR3	receptor de complemento 3
DMSO	dimetilsulfóxido
DNA	ácido desoxirribonucléico
DNAse	enzima que degrada DNA
DTT	dietiltritol
EDTA	ácido etileno diamino tetra-acético
FITC	isotiocianato de fluoresceína
g	força gravitacional

gp43	glicoproteína de 43kDa
GXM	glucoronoxilomanana
h	horas
H_2O	água
H_2O_2	peróxido de hidrogênio
HIV	do inglês *human immunodeficiency virus*
Hsp	proteína de choque térmico
Hsp60	proteína de choque térmico de 60 Kda
Hsp70	proteína de choque térmico de 70 Kda
IFN-γ	interferon gama
IgG	imunoglobulina G
IL-4	interleucina 4
IL-8	interleucina 8
IL-10	interleucina 10
IL-12	interleucina 12
IL-13	interleucina 13
IL-17	interleucina 17
IgM	imunoglobulina M
iNOs	óxido nítrico sintase isoforma induzível
IPTG	Isopropyl β-D-Thiogalactoside
kb	kilobases
kDa	kilodalton
LPS	lipopolissacarídeo
M	molar
mAb	anticorpo monoclonal
MALDI-TOF	dessorção / ionização a laser assistida por matriz - tempo de vôo
MAPK	proteínas kinases activadas por mitógenos
$MgSO_4$	sulfato de magnésio
MHC I	complexo principal de histocompatibilidade da classe I
MHC II	complexo principal de histocompatibilidade da classe II
min	minutos
ml	mililitros

mM	milimolar
mm	milímetro
mmol	milimol
M-MLV-RT	Transcriptase reversa do vírus da leucemia murina Moloney
mRNA	RNA mensageiro
MyD88	do inglês *Myeloid differentiation primary response gene (88)*
NaCl	cloreto de sódio
ng	nanogramas
NF-kB	fator nuclear kappa beta
nm	nanômetro
NO	óxido nítrico
O_2^-	íon óxido
O_2	dióxido
OD	densidade ótica, do inglês *optical density*
ORF	do inglês *open reading frame*
PBS	solução salina tamponada com fosfato
PCR	reação em cadeia da polimerase
pH	potencial hidrogeniônico
PIPES	do inglês *piperazine-N,N'-bis(2-ethanesulfonic acid)*
pmol	picomol
PVDF	do inglês *Hydrofobic polyvinylidene difluoride*
qPCR	PCR quantitativo
RNA	ácido ribonucleico
RNAse	enzima que degrada RNA
ROS	espécies reativas de oxigênio, do inglês *reactive oxygen species*
RT-PCR	transcrição reversa seguido da reação em cadeia da polimerase
s	segundos
SDS	dodecil sulfato de sódio, do inglês *sodium dodecyl sulfate*
SDS-PAGE	eletroforese desnaturante em gel de poliacrilamida-SDS

SSa1	gene SSa1 da família das Hsp70
TE	Tris-EDTA
Th1	células T *helper* do tipo 1
Th2	células T *helper* do tipo 2
Th17	células T *helper*
TLR2	receptor tipo toll 2
TLR4	receptor tipo toll 4
TLR9	receptor tipo toll 9
TMB	tetrametil benzidina
TNF-α	fator de necrose tumoral alfa
U	unidades
YPD	do inglês *yeast extract*, *peptone*, *dextrose*
YNB	do inglês *yeast nitrogen base*
µg	micrograma
µl	microlitros

LISTA DE FIGURAS

RESUMO

A criptococose acomete pacientes imunocomprometidos, principalmente pacientes com a resposta imune mediada por células T debilitada, sendo portanto, suscetíveis a patógenos oportunistas. *C. neoformans* possui um repertório de fatores de virulência que permitem o estabelecimento da infecção e sua disseminação para o sistema nervoso central, causando meningoencefalite. O tratamento da criptococose é baseado apenas na utilização prolongada de antifúngicos e provoca muitos efeitos colaterais nos pacientes. Por estes motivos, novas terapias estão sendo abordadas, com o intuito de promover a ativação do sistema imune do hospedeiro para debelar a infecção. Para que tais terapias possam ser desenvolvidas se faz necessário identificar moléculas que estimulem a resposta imune celular. Proteínas de choque térmico de patógenos têm sido utilizadas como alvo no tratamento de muitas doenças infecciosas em seres humanos. Estas proteínas estimulam a resposta imune mediada por células T e apresentam resultados promissores em tratamentos contra infecções fúngicas. Da mesma forma, proteínas que fazem parte do sistema de defesa antioxidante, como as catalases, por exemplo, desempenham um papel importante no combate a radicais livres produzidos pelo hospedeiro e parecem ter outras funções no processo da infecção. O entendimento da biologia destas proteínas bem como a sua participação na interação com células do sistema imune do hospedeiro é fundamental para a validação da sua aplicabilidade no tratamento da criptococose. Nesse contexto, apresentamos aqui um estudo das funções biológicas de uma das proteínas de choque térmico (Hsp70) e de uma das enzimas envolvidas na detoxificação de espécies reativas de oxigênio (catalase 2) do fungo *C. neoformans*. Também foram avaliados os efeitos terapêuticos destas proteínas no tratamento da criptococose. Demonstramos que a proteína Hsp70 de *C. neoformans* está localizada na cápsula polissacarídica, sendo importante no processo de adesão da levedura a células epiteliais A549. Demonstramos também que Cn_rHsp70 co-localiza com o principal componente da cápsula polissacarídica a glucuronoxilomanana (GXM) na superfície de macrófagos. A Cn_rHsp70 quando em contato com células do fungo, diminui sua viabilidade celular e não altera o tamanho da cápsula, mas interfere na secreção de GXM. Em relação à catalase 2, foi demonstrado que a proteína é localizada na parede celular do fungo e possui atividade de degradação de peróxido de hidrogênio quando associada à superfície celular. Em ensaios de infecção experimental em animais imunizados com construções de DNA contendo as sequências de Hsp70 e catalase 2, nenhuma das proteínas conferiu proteção, mas ocorreu estímulo da resposta

mediada por células Th1 e Th2. O mecanismo pelo qual estas proteínas estimulam o sistema imune ainda precisa ser elucidado.

ABSTRACT

Cryptococcus neoformans affects immunocompromised patients, especially patients with impaired T cell-mediated immune response, and therefore are susceptible to opportunistic pathogens. *C. neoformans* has a repertoire of virulence factors that allows the establishment and dissemination of the infection in the central nervous system, causing meningoencephalitis. The virulence factors of *Cryptococcus* has been subject of many studies that have showed the involvement of gene products in the infection process. However, the treatment of cryptococcal meningoencephalitis consists of antifungal therapy and displays many side effects. For these reasons, new therapies are being addressed, in order to promote the activation of the host immune system to overcome the infection. To develop these therapies, it is necessary to identify molecules that stimulate cell-mediated immune response. Heat shock proteins from many pathogens have been used as a target for the treatment of many humans infectious diseases. These proteins stimulate the T cell-mediated immune response and show promising results in treatment against fungal infections. Similarly, proteins from antioxidant system such as catalase for example, play an important role against free radicals produced by the host and appear to have other functions in the infection process. The understanding of these proteins biology and their involvement with host immune system cells is essential for the validation of its applicability in the treatment of cryptococcosis. In this context, we present here a study of the biological function of a heat shock protein (Hsp70) and one of the enzymes involved in detoxification of reactive oxygen species (catalase 2) of the fungus *C. neoformans*. We evaluated the therapeutic effects of these proteins in the treatment of cryptococcosis. We have demonstrated that the protein Hsp70 from *C. neoformans* is located in the polysaccharide capsule, and it is important in the adhesion process of the yeast to epithelial cells A549. Also demonstrated that Cn_rHsp70 co-localizes with the main component of the polysaccharide capsule, glucuronoxilomanana (GXM), on the surface of macrophages. When Cn_rHsp70 are in contact with fungal cells, the cellular viability decreased and does not change the capsule size however, interferes with the GXM secretion. Catalase 2 is deposited on the fungal cell wall and showed activity by hydrogen peroxide degradation. In vivo experiments using animals infected and treated with Hsp70 DNA vaccines and catalase 2, no protection were observed, but there are immune response mediated by Th1 and Th2 cells. The mechanism by which these proteins stimulate the immune system has yet to be elucidated.

1. JUSTIFICATIVA

A levedura *Cryptococcus neoformans* é um dos agentes etiológicos da criptococose, doença que acomete principalmente pacientes imunocomprometidos. Com o aumento do número de indivíduos imunossuprimidos, causado pelo vírus da imunodeficiência humana (HIV) e por tratamento com agentes imunossupressores, a casuística da criptococose também tem aumentado (Friedman *et al*., 2005; Chayakulkeeree & Perfect., 2006; Leal *et al*., 2008). A terapia convencional utilizada no tratamento desta micose sistêmica inclui a administração intravenosa de antifúngicos como a anfotericina B acompanhada por administração oral de fluconazol (Negroni, 2012). Entretanto, o tratamento gera alta taxa de resistência e reincidência da doença ao longo do tempo (Espinel-Ingroff A., *et al*., 2012). Além disso, o tratamento provoca efeitos colaterais (Laniado-Laborìn & Cabrales-Vargas, 2009). Novas abordagens para o tratamento desta infecção têm sido avaliados e envolvem características fundamentais da biologia da levedura (Kinjo *et al.,* 2007; Rodrigues *et al*., 2007; Rachini *et al*., 2007; Bryan *et al.*, 2010; Rosas *et al*., 2001). A vacinação com um epítopo da cápsula polissacarídica, um dos principais fatores de virulência do fungo, confere proteção em modelos murinos (Datta *et al*., 2008). A defesa do hospedeiro contra infecções causadas por *C. neoformans* ocorre com a participação da imunidade mediada por células (Jain *et al*., 2009). Primariamente, a imunidade inata atua pelo reconhecimento das células de *C. neoformans* por receptores de antígenos presentes na superfície do patógeno e posterior apresentação destes antígenos pelo sistema MHCI ou MHCII. Estas células podem então, ativar os linfócitos T a produzir citocinas pró-inflamatórias e

promover a resposta imune adaptativa. Uma vez ativada, as células T atuam contra a proliferação e a disseminação do patógeno (Zhou & Murphy, 2006). Se, apesar destas defesas o patógeno consegue se estabelecer, ele atinge a corrente sanguínea e se dissemina para o sistema nervoso central causando a forma mais grave da doença, a meningoencefalite. Se não for tratada, a criptococose pode levar à morte (Prado *et al*., 2009). Como os pacientes imunocomprometidos apresentam deficiências na imunidade mediada por células, a identificação de um antígeno do microrganismo que induza a resposta imune celular é de extrema importância para ativar o sistema imune destes individuos e, assim, propiciar o controle da infecção.

Proteínas de choque térmico, bem como as proteínas que compõem o mecanismo de defesa contra estresse oxidativo, possuem um papel fundamental na biologia do fungo e podem ter participação no desenvolvimento da infecção (revisado por Hartl & Hayer-Hartl, 2002; Cox *et al*., 2003). Proteínas de diversas vias metabólicas, assim como proteínas que influenciam na virulência, podem ser protegidas pela atividade de chaperonas, como a Hsp70. De forma semelhante, durante a infecção do hospedeiro, o fungo encontra muitos ambientes desfavoráveis para seu crescimento e proliferação. Nesse contexto, as catalases, enzimas que fazem parte do mecanismo de defesa antioxidativo, são importantes na defesa do fungo, evitando assim, algumas das defesas do hospedeiro.

Neste contexto, o presente trabalho visa a entender a participação da Hsp70 e da catalase 2 no processo de infecção. Além disso, essas sequências foram utilizadas para elicitar a resposta imune mediada por células em experimentos com animais. Até o momento, poucos estudos

envolvendo estas duas proteínas foram realizados.

2. REVISÃO DA LITERATURA

2.1 *Cryptococcus neoformans* e criptococose

As principais espécies do gênero *Cryptococcus* causadoras de doenças em seres humanos são *Cryptococcus neoformans* e *C. gattii*. *C. neoformans* acomete pacientes imunocomprometidos, sendo portanto, considerado um patógeno oportunista. Em contrapartida, *C. gattii* acomete pacientes imunocompetentes, sendo considerado um patógeno primário (Lin & Heitman, 2006).

Apesar de ambas as espécies causarem a mesma doença, a criptococose, estudos em modelos animais sugerem que *C. gattii* acomete principalmente o pulmão, ao passo que *C. neformans* inicia a infecção pelos pulmões mas podem atingir o sistema nervoso central (Ngamskulrungroj *et al*., 2012). Esta diferença no estabelecimento inicial da infecção também é refletida no padrão de citocinas produzidas em ambas as espécies (Schoffelen *et al*., 2013).

C. neoformans é uma levedura basideomicética patogênica que é capaz de infectar todos os órgãos humanos, podendo atingir o cérebro, levando ao quadro mais grave da doença, a meningoencefalite (Barnett, 2010). Este fungo possui distribuição mundial e é encontrado em diversos habitats sendo frequentemente isolado do solo e de excretas de pombos (Nielsen *et al*., 2007).

A criptococose está se tornando um problema de saúde pública pois acomete tanto pacientes imunocomprometidos quanto imunocompetentes.

Além disso, o número de pacientes imunocomprometidos vêm aumentando, visto que ocorre um aumento de número de pessoas infectadas pelo HIV, transplantadas ou que recebem imunoterapia. Dados epidemiológicos mostram que, anualmente, em torno de um milhão de pessoas infectadas pelo HIV apresentam quadros de criptococose e que destes, aproximadamente 600.000 vão à óbito (Park *et al*., 2009).

A infecção causada por espécies de *Cryptococcus* ocorre através da inalacão dos esporos da levedura (resultado da reprodução sexual) (Giles *et al.*, 2009) assim como de leveduras dissecadas na natureza. Estas partículas inaladas depositam-se nos alvéolos pulmonares colonizando o trato respiratório e em muitas vezes, não causam sintomas, gerando uma forma latente da infecção. Entretanto, em quadros de comprometimento da imunidade, a forma dormente pode se tornar ativa e disseminar por via hematogênica, causando a infecção sistêmica (Liu *et al*., 2012). Outras formas de infeção já foram documentadas, como, por exemplo, infecção gastrointestinal ou cutânea, porém a forma mais conhecida é através da inalação (Del Poeta & Casadevall, 2012).

Para atingir o sistema nervoso central e causar meningite, a célula fúngica precisa atravessar a barreira hematoencefálica. Existem 3 mecanismos pelo qual a célula de *Cryptococcus sp.* consegue atravessar essa barreira e invadir o cérebro (Figura 1): (a) **Modelo transcelular,** neste mecanismo as células da levedura aderem-se e atravessam as células endotelias microvasculares do cérebro (HBMEC) sem causar danos à morfologia e integridade destas células (Chang *et al*., 2004); (b) **Modelo paracelular,** este modelo pode ser utilizado pelo patógeno, embora poucas

evidências demonstrarem o uso dele pela levedura *Cryptoccocus sp*. Neste modelo, as células da levedura degradam a proteína transmembrana das junções entre as células epiteliais e atravessam a barreira hematoencefálica causando mudança morfológicas nas células dos hospedeiro (Chen *et al*., 2003)**; (c) Modelo cavalo de Tróia**, neste mecanismo a levedura consegue atingir o cérebro através de fagócitos mononucleares que fagocitam a levedura, transportando o fungo através a barreira hematoencefálica, não expondo as células de *Cryptococcus sp.* ao sistema imune (Charlier *et al*., 2009).

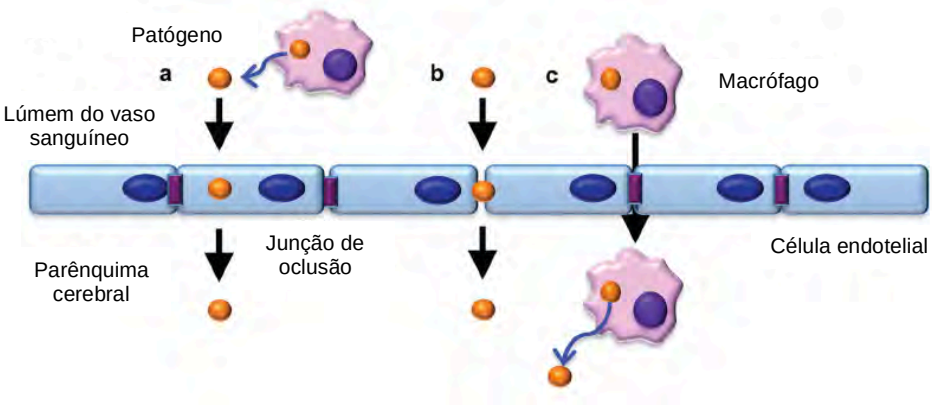

Figura 1. Possíveis mecanismos utilizados por *Cryptococcus* para atravessar a barreira hematoencefálica: Mecanismo transcelular **(a)**, onde o patógeno atravessa a barreira hematoencefálica por endocitose sem causar dano à célula hospedeira; mecanismo paracelular **(b)** onde o patógeno penetra entre as células hospedeiras, podendo gerar dano e o mecanismo cavalo de Tróia **(c)** onde o patógeno transmigra dentro de fagócitos infectados. Figura adaptada de Liu *et al*. 2012.

2.2 Fatores de virulência

Um grande número de fatores de virulência de *C. neoformans*, que contribuem para o estabelecimento da infecção, já foram caracterizados. Entretanto, na literatura, são descritos 3 fatores clássicos que possibilitam à levedura infectar seres humanos, dentre eles, a capacidade de crescimento 37°C, a produção de uma cápsula polissacarídica e a produção de melanina (revisado por Karkowska-Kuleta *et al*., 2009). Muitos produtos gênicos envolvidos nas mais diversas vias de sinalização regulam a expressão destes fatores de virulência.

2.2.1 Crescimento à 37°C

A capacidade de desenvolvimento do fungo em altas temperaturas é um fator necessário, porém não suficiente para torná-lo patogênico. A tolerância da levedura à temperatura corpórea dos mamíferos, de 37°C, é uma pré-condição para o estabelecimento da doença. Existem muitos genes cujos produtos estão relacionados ao desenvolvimento a 37°C e, na maioria dos casos, estes produtos gênicos estão associados à virulência (Perfect, 2006). Um dos principais fatores associados ao crescimento a 37°C é a calmodulina, produto do gene CNAC1, que possui atividade de fosfatase dependente de Ca^{2+}. Esta proteína é ativada na presença de estresse térmico, defosforilando um grupo de proteínas necessárias para as células de *C. neoformans* crescerem a 37°C (revisado por Olszewski *et al*., 2010).

2.2.2 Cápsula

A levedura *C. neoformans* possui em sua superfície uma cápsula de natureza majoritariamente polissacarídica, cujos componentes são produzidos dentro da célula, exportados pela parede celular através de

vesículas e depositados na superfície celular. Os principais constituintes da cápsula são os polissacarídeos glucuronoxilomanana (GXM) e galactoxilomanana (GalXM), que se organizam de forma complexa formando longos polímeros. Apesar de muitos estudos elucidarem a composição e a estrutura desta cápsula, pouco se sabe sobre o processo de montagem desta estrutura (Rodrigues *et al*., 2009). Uma das principais características da cápsula é a habilidade de alterar o seu tamanho e sua complexidade. Estas duas características são reguladas pelas condições ambientais onde se encontra a levedura, como, por exemplo, a presença de CO_2, fosfolipídeos e condições limitantes de ferro (O'Meara & Alspaugh, 2012).

A cápsula polissacarídica possui função antifagocítica, uma vez que serve de barreira física impedindo o reconhecimento pelos receptores das células do hospedeiro. Além disso, a cápsula é capaz de modular a atividade de macrófagos, já que é reconhecida por alguns receptores presentes na superfície dos fagócitos, como CD14, TLR2 e TLR4, culminando na translocação de NF-kB para o núcleo, e consequente inibição da produção de TNF-α e não ativação de macrófagos (revisado por García-Rodas & Zaragoza, 2011).

2.2.3 Produção do pigmento melanina pela enzima lacase

Além da cápsula que envolve a célula, a presença do pigmento melanina ligado à parede celular também é relatada como importante para a proteção do fungo contra oxidação, fagocitose e para o estímulo de resposta imunológica. A melanina tem alta afinidade por metais, sendo importante na proteção da célula contra radicais livres, principalmente à 37°C, onde a atividade da superóxido-dismutase está aumentada (revisado por Nosanchuk

& Casadevall, 2003). A enzima responsável pela produção da melanina, a lacase, possui outras funções, como, por exemplo, a atividade de ferro-oxidase, que protege a levedura contra macrófagos alveolares. Dentro do fagolisossomo, a levedura competete pelo ferro livre e evita que o macrófago produza radicais hidroxila, evitando assim sua morte por estresse oxidativo (Liu *et al*., 1999). A atividade da enzima é regulada por diversos fatores ambientais, sendo induzida em presença de cálcio, ferro e cobre e inibida por glicose, nitrogênio e altas temperaturas (Zhu *et al*., 2004).

Apesar de existirem muitos estudos acerca da produção da melanina em relação à expressão de genes potencialmente envolvidos na virulência de *Cryptococcus sp.*, pouco se sabe sobre o processo da melanização. Eisenman e colaboradores (2009) sugeriram um modelo para tentar explicar como ocorre a melanização na célula da levedura. Neste trabalho o autor propõe que a síntese do pigmento ocorre nas vesículas presentes na parede da célula. Esta teoria explica muitas questões ainda não respondidas à respeito deste processo, porém ainda precisa ser comprovada.

Sabe-se que *C. neoformans* possui dois genes de lacase, CNLAC1 e CNLAC2 com alto grau identidade (Zhu *et al*., 2004). Pouco se sabe à respeito do gene CNLAC2. Porém, CNLAC1 já foi bem caracterizado e verificou-se que está envolvido na disseminação extrapulmonar mas não na persistência pulmonar do organismo (Noverr *et al*., 2004).

2.3 Parasitismo intracelular

Os macrófagos alveolares desempenham um papel importante na defesa do hospedeiro contra a criptococose. Após a inalação, o fungo pode residir no ambiente extracelular dos alvéolos ou ser fagocitado e permanecer

dentro dos macrófagos. Uma vez dentro dos macrófagos, o fungo pode escapar dos mecanismos de defesa dos fagócitos, permanecer em estado latente e estabelecer infecção ou, ainda, pode ser morto e eliminado pelo organismo (revisado por García-Rodas & Zaragoza, 2012).

Células de *Cryptococcus sp.* conseguem evitar a fagocitose pela presença da cápsula ou de proteínas antifagocíticas (Stano *et al*., 2009). Entretanto, caso seja fagocitada, as células da levedura ficam contidas em organelas chamadas fagolisossomos, que contém pH ácido, inumeras enzimas hidrolíticas e radicais livres capazes de matar a levedura. Neste contexto, *Cryptococcus sp.* é capaz de sobreviver à este ambiente inóspito e se replicar. Neste processo, a cápsula desempenha um papel importante, uma vez que evita a morte da célula e interfere na função dos macrófagos (revisado por García-Rodas & Zaragoza, 2012). Uma vez dentro de macrófagos, as células de *Cryptococcus sp.* são capazes de sobreviver e serem liberadas dos macrófagos através de um processo de exocitose não lítica, onde as células dos macrófagos permanecem viáveis, em um processo chamado de vomocitose (Nicola *et al*., 2011; Johnston & May, 2013). Este processo é altamente regulado, controlando a fusão do fagossomo à membrana plasmática e liberação das células da levedura (revisado por Johnston & May, 2013). O processo de sobrevivência da levedura é crítico para a progressão e disseminação da infecção.

Células de *Cryptococcus sp.* presentes no pulmão podem apresentar alterações morfológicas, produzindo células gigantes. Estas células gigantes conferem proteção às células menores durante o estágio inicial de infecção pulmonar e não são fagocitadas devido ao seu tamano celular (Okagaki *et al*.,

2012). Além disso, estas células são resistentes à condições de estresses, sendo de extrema importância na sobrevivência do fungo durante um período prolongado, sendo, portanto, relacionado com o estado de latência de *Cryptococcus sp..*

2.4 Sistema imune

O mecanismo de defesa do organismo para combater *Cryptococcus sp.* conta com a participação do sistema imune inato e adaptativo (Figura 2). Portanto, a resposta imune mediada por células T é o principal mecanismo de defesa imune. Células $CD4^+$ tem um importante papel no recrutamento de macrófagos e são as principais populações de células não funcionais em pacientes HIV positivo, o que os torna suscetíveis à criptococose. As células $CD8^+$ também contribuem para imunidade protetora mediada por células contra infecções de *C. neoformans.* Essas células são importantes para o recrutamento de células efetoras e secreção de IFN-γ (Zhou & Murphy, 2006).

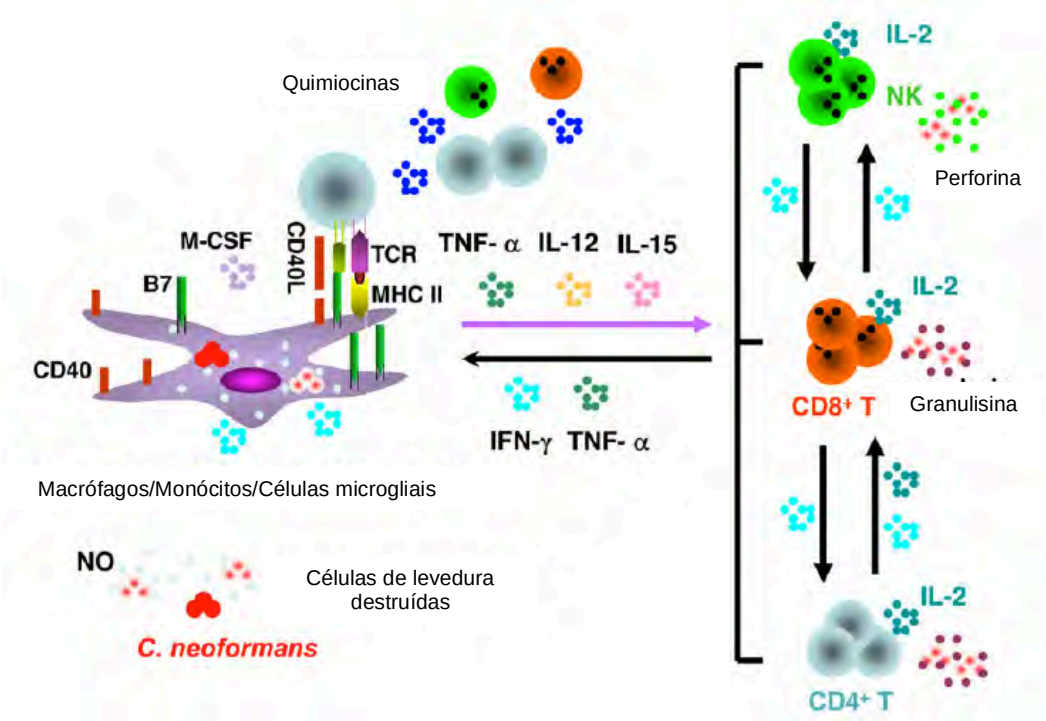

Figura 2: Mecanismo de defesa do sistema imune em infecções causadas por *C. neoformans*. Uma vez inalada, a célula da levedura pode permanecer no ambiente extracelular dos pulmões ou ser fagocitada pelos macrófagos. Estas células fagocíticas podem servir de célula apresentadora de antígeno e ativar os linfócitos T $CD4^+$ e $CD8^+$ a secretar citocinas pro-inflamatórias (TNF-α e IFN-γ) e promover a produção de óxido nítrico pelos macrófagos ativados. Figura adaptada de Zhou & Murphy, 2006.

Quando a levedura é inalada e deposita-se no pulmão, entra em contato com macrófagos, que secretam uma série de citocinas, modulando o desenvolvimento e a expressão de células efetoras do tipo Th1, Th2 e Th17. Uma vez ativada, as células efetoras produzem mais citocinas, que por sua vez aumentam a função dos macrófagos. O balanço das citocinas Th1 e Th2 são importantes na imunidade mediada por células. As principais citocinas da imunidade mediada por células Th1 responsáveis pelo combate a patógenos

intracelulares são IL-12 e IFN-γ. Essas citocinas causam ativação clássica dos macrófagos com a produção de óxido nítrico (NO) que atua como molécula fungicida (Chawla, 2010). Outras citocinas estão envolvidas no mecanismo de imunidade celular mediada por células Th1. TNF-α, por exemplo, é importante para estimular a produção de IL-12 e IFN-γ (Herring *et al*., 2002). Em contraste, a imunidade mediada por células Th2, as citocinas IL-4, IL-10 e IL-13 contribuem para o progresso da infecção, inibindo a proliferação de células T. Estas citocinas ativam os macrófagos de forma alternativa, aumentando a fagocitose e crescimento intracelular da levedura (Olszewski *et al*., 2011).

Portanto, a eliminação do patógeno ou o estabelecimento da infecção depende do tipo de ativação dos macrófagos. Estas células podem funcionar a favor do hospedeiro, uma vez que agem eliminando o patógeno do organismo, ou podem servir como reservatório para o fungo se replicar e disseminar, aumentando assim, a infecção. Ainda não se conhece que fatores dos macrófagos desencadeiam o papel de proteção do hospedeiro ou proteção do fungo contra a ação de outras células imunes (McQuiston & Williamson, 2012) (Figura 3).

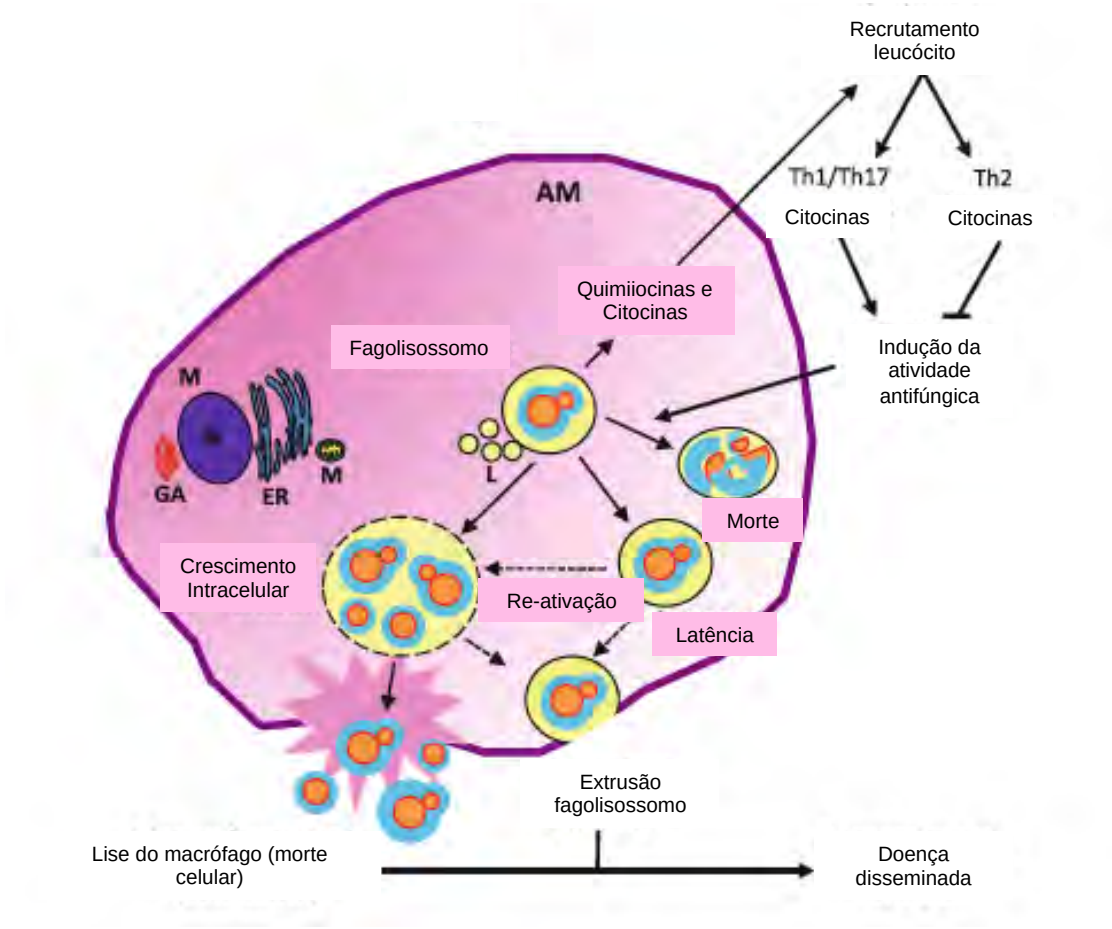

Figura 3: Estratégia da levedura *C. neoformans* para sobreviver dentro do macrófago. Uma vez fagocitada, a levedura permanece dentro de estruturas chamadas fagolisossomo, podendo seguir 3 destinos dentro da célula: ser morta pela atividade antifúngica das citocinas secretadas pelas células recrutadas; permanecer em estado de latência para que seja reativada quando houver baixa no sistema imunológico do hospedeiro; ou a levedura pode replicar-se levando a lise do macrófago e disseminação da doença. Figura adaptada por McQuiston & Williamson, 2012.

A resposta imune mediada por células Th17 é pouco caracterizada, porém, foi demonstrado que ela consegue prevenir a criptococose, uma vez

que ativa macrófagos e evita a proliferação intracelular da levedura (Antachopoulos & Walsh, 2011).

A resposta imune mediada por células B também contribui para a proteção do organismo contra infecções causadas por *Cryptococcus sp..* O anticorpo anti-GXM, principal componente capsular, foi capaz de gerar proteção em modelos murinos (Feldmesser *et al*., 2002; Datta *et al*., 2008). Tem sido relatado que o anticorpo anti-GXM consegue promover o reconhecimento e a ingestão das células de levedura pelas células apresentadores de antígenos (Kelly *et al*., 2005).. Além disso, o anticorpo consegue aumentar a fagocitose através da opsonização

A imunidade inata também contribui na defesa contra infecção causada por *C. neoformans*, uma vez que ela é essencial para o início da resposta adaptativa (Kawakami, 2004). As principais células efetoras deste mecanismo de defesa incluem as células dendríticas e natural *killer*, assim como macrófagos.

As células da levedura se ligam a receptores das células fagocíticas e disparam a resposta imune inata. Receptores do tipo toll têm sido bem caracterizados no reconhecimento de microrganismos. Estes receptores possuem um domínio extracelular transmembrana e um domínio intracelular que interage com proteínas adaptadoras, como, por exemplo, MyD88. Após a interação de MyD88 com o receptor, ocorre a ativação de NF- kB e MAPK, que, por sua vez, levam à secreção de citocinas e a expressão de moléculas co-estimulatórias e MHC na superfície das células (revisado por Takeda & Akira, 2005).

Anticorpos e proteínas do sistema complemento agem como opsoninas e induzem a fagocitose por *C. neoformans.* Na ausência de opsoninas, os macrófagos não se ligam eficientemente às células da levedura por causa da cápsula polissacarídica (Saylor *et al*., 2010). Porém, quando as células são opsonizadas com anticorpo anti-capsular, a levedura pode ser fagocitada via receptores de imunoglobulinas Fc. A cápsula polissacarídica induz a ativação de complemento, resultando na deposição de C3b na cápsula, a qual induz a fagocitose via receptor CR1, CR3, CR4 (Zaragoza *et al*., 2003).

Os receptores CD14, TLR2 e a proteína adaptadora MyD88 reconhecem o polissacarídeo capsular presente na célula de *Cryptococcus sp.* (Yauch *et al*., 2004; Biondo *et al*., 2005). Além disso, a via de sinalização TLR9 contribui para o desenvolvimento da imunidade adaptativa contra infecções causadas por *C. neoformans,* após a ingestão e exposição de sequência CpG de DNA (Zhang *et al*., 2010; Qiu *et al*., 2012)

2.5 Proteínas de choque térmico (Hsps)

As proteínas de choque térmico (Hsps) são proteínas altamente conservadas, sendo encontradas em todos os grupos de seres vivos. Estas proteínas estão envolvidas em muitos processos biológicos importantes para a fisiologia e manutenção das células (Bukau *et al*., 2006). Hsps funcionam como chaperonas que reconhecem e se ligam a peptídeos nascentes para promover o enovelamento, prevenindo a agregação e o correto dobramento das proteínas (revisado por Hartl & Hayer-Hartl, 2002).

As Hsps são expressas constitutivamente e geralmente têm o aumento da sua expressão sob condições de estresse, estando localizadas

principalmente no citosol (Basu *et al*., 2000). As Hsps citosólicas não possuem peptídeo-sinal que permita o transporte para a membrana. Porém, a presença de Hsp no ambiente extracelular também ocorre. O mecanismo de secreção das Hsps parede ser complexo e ainda não foi bem elucidado. Um dos mecanismos que tenta explicar a localização extracelular destas proteínas é através do transporte vesicular, onde a proteína se funde a exossomos e é liberada na membrana celular (Lancaster & Febbraio, 2005; Mambula *et al*., 2007; Rodrigues *et al*., 2008).

Estas proteínas podem exercer outras funções nas células, como, por exemplo, serem apresentadoras de antígenos (APC) recrutando linfócitos T $CD8^+$ e linfócitos T CD4+ (revisado por Murshid *et al.,* 2012; Tsan *et al*., 2009). Além da função de APC, as Hsps localizadas na superfície celular podem ativar o sistema imune induzindo a produção de citocinas pro-inflamatórias (Tsan & Gao, 2004). Porém, Hsps purificadas parecem não ativar a resposta imune *in vitro* (Tsan *et al*., 2009). Warger e colaboradores (2006) demonstraram que a proteína Hsp aumenta a ativação de células dendríticas por produtos bacterianos e induz à resposta imune adaptativa.

Muitos receptores expressos nos monócitos e nas células dendríticas têm sido descritos como capazes de promover a ligação das Hsps, os quais precedem a apresentação de antígenos via MHC I e MHC II (Murshid *et al*., 2012). As Hsps podem se ligar em receptores *scavenger* Lox-1 e CD91 e a receptores do tipo Toll (TLR2 e TLR4), apesar da ligação a estes receptores parecer controversa (Borges *et al*., 2012).

Os fungos patogênicos que causam infecções em seres humanos precisam se adaptar à temperatura corpórea e manter a homeostase para

estabelecer a infecção. Portanto, as proteínas de choque térmico são fundamentais para manutenção celular e apresentam-se como imunodominantes em infecções causadas por estes fungos patogênicos (Burnie *et al*., 2006). Dado o efeito estimulatório das proteínas de choque térmico em infecções causadas por fungos patogênicos, alguns trabalhos estão sendo realizados no intuito de utilizar estas proteínas como antígenos no tratamento destas infecções, sendo, portanto, potenciais antígenos no desenvolvimento de vacinas (Raska *et al*., 2005; Ribeiro *et al*., 2009; Ribeiro *et al*., 2010).

O anticorpo monoclonal anti- Hsp90 de *C. albicans* tem sido utilizado no tratamento de candidíase invasiva (Hodgetts *et al*., 2008). No fungo *H. capsulatum*, foi identificado que a proteína Hsp60 está localizada na superfície do fungo, promove a ativação de células T e a proteção contra animais infectados com este fungo (Gomez *et al.,* 1995). Outra Hsp60 foi avaliada e conferiu proteção em modelos animais contra infecções causadas pelo fungo *Paracoccidioides brasiliensis* (de Bastos *et al*., 2008).

No genoma de *C. neoformans* foram identificadas onze proteínas de choque térmico, sendo que em infecções causadas por esta levedura, somente uma proteína da família das Hsp70 foi identificada como antígeno imunodominante, tanto em infecções em camundongos como em infecções humanas (Kakeya *et al*., 1997; Kakeya *et al*., 1999).

2.5.1 Hsp70

Hsp70 são proteínas pertencentes à família das proteínas de choque-térmico. São proteínas de 70 kDa, compostas de dois domínios funcionais, um domínio de ligação ao ATP e outro de ligação ao subtrato. Estas proteínas

estão envolvidas na translocação de proteínas através da membrana e na regulação da resposta ao estresse térmico (Burnie *et al*., 2006).

Apesar do mecanismo pró-inflamatório mediado por Hsp70 já ter sido descrito, Borges e colaboradores (2012) propuseram um mecanismo anti-inflamatório para Hsp70 extracelular (Figura 4). Neste modelo, as Hsp70 extracelulares ativam a resposta imune inata através da ligação à receptores na superfície da célula, induzindo a sinalização por TLR2 à produção de IL-10. Os autores acreditam que, dependedo de como a Hsp70 se encontra (livre, associada à peptídeos ou a nucleotídeos) no ambiente extracelular, ela pode se ligar a diferentes receptores.

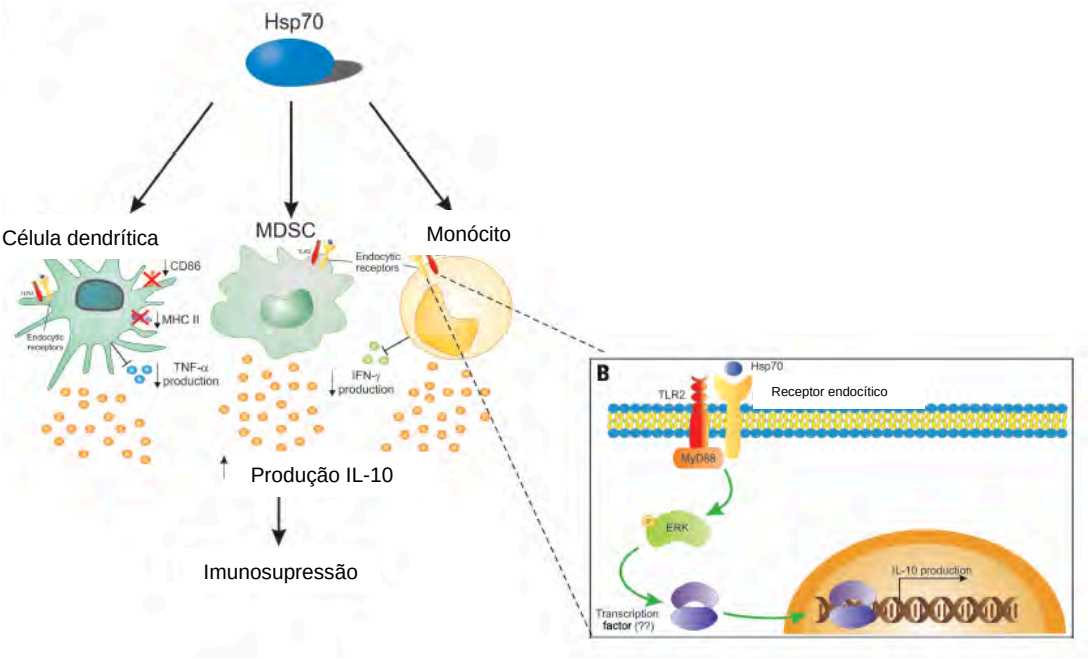

Figura 4: A atividade imunosupressora de Hsp70. Segundo este modelo, Hsp70 pode ligar-se à um receptor endocítico, sinalizar receptores de sinalização para que ocorra a ativação de Myd88 e produção de IL-10, levando à quadros de imunosupressão. Figura adaptada de Borges *et al*., 2012.

Além das funções citadas acima, a proteína Ssa1, da família das Hsp70, possui atividade de co-ativador transcricional do gene da lacase em *Cryptococcus sp..* A deleção deste gene reduziu a produção da atividade da lacase, diminuiu a virulência em modelo animal e aumentou o tamanho da cápsula polissacarídica (Zhang *et al*., 2006). Estes dados indicam que, em *C. neoformans*, a modulação da expressão de fatores de virulência pode ocorrer via proteínas de choque térmico.

Portanto, a função das proteínas Hsp70 no processo de infecção e seu envolvimento na proteção do fungo bem como na modulação dos fatores clássicos de virulência em *C. neoformans* precisam ser melhor elucidados.

2.6 Catalases

Para combater os patógenos intracelulares, as células fagocíticas produzem espécies reativas de oxigênio (ROS), sendo um dos componentes mais efetivos do sistema imune inato (Yang *et al*., 2013). Portanto, a capacidade de *Cryptococcus sp.* sobreviver dentro destas células exige que a levedura consiga detoxificar essas ROS e reparar os danos causados por estas moléculas.

Muitos genes que codificam enzimas envolvidas na proteção contra estresse oxidativo e nitrosativo têm sido avaliados quanto à influência na virulência (Cox *et al*., 2003; Missall *et al*., 2004). Estes genes estão envolvidos em mecanismos redundantes na defesa antioxidante. Peroxiredoxina (tsa1), tioredoxina redutase (Trx1 e Trx2), glutationa peroxidase (Gpx1 e Gpx2), superóxido dismutase (Sod1), citocromo c peroxidase (Ccp1) e oxidase alternativa (Aox1) são enzimas envolvidas na

resistência à estresse oxidativo em *C. neoformans* (Missall *et al*., 2005a; Missall *et al*., 2005b; Cox *et al.*, 2003; Giles *et al*., 2005; Akhter *et al*., 2003). Upadhya e colaboradores (2013) fizeram uma análise global dos genes expressos durante a exposição a peróxido de hidrogênio e demonstraram que a adaptação metabólica de *C. neoformans* frente a esta molécula é diferente da adaptação que ocorre em outros fungos (Vivancos *et al*., 2006; Cuéllar-Cruz *et al*., 2008). Além disso, este grupo identificou genes relacionados com a resistência a antifúngicos, o que está em consonância com relatos anteriores, que descreveram uma relação entre a atividade de catalase e a resistência à anfotericina B e agentes antineoplásicos em *C. albicans* (Linares *et al*., 2006; Blum *et al*., 2008).

Catalases são enzimas envolvidas na degradação de péroxido de hidrogênio em água e oxigênio. Estas enzimas conferem resistência a mecanismos oxidativos produzidos pela célula, conferindo resistência à morte celular causada por danos oxidativos às células (Hansberg *et al*., 2012). As catalases localizam-se dentro dos peroxissomos ou no citoplasma, mas elas também podem ser secretadas e associadas à parede celular (Guimarães *et al*., 2008; Momany *et al*., 2004).

Estas enzimas podem participam da virulência em muitos microrganismos uma vez que conferem proteção à célula do fungo e atenuam a virulência em modelos animais (Wysong *et al*., 1998, Holbrook *et al*., 2013). Além da função no combate às espécies reativas de oxigênio, a catalase apresenta um papel importante na ligação ao plasminogênio, podendo contribuir para a invasão do hospedeiro (Crowe *et al*., 2003).

Existem 3 famílias de genes da catalase: o grupo das catalases que

possuem manganês no sítio ativo (Mn-catalases), encontrada nos procariotos; as catalases bifuncionais, peroxissomos catalases, encontradas tanto em procariotos quanto eucariotos inferiores; e as catalases monofuncionais, encontradas em procariotos e eucariotos (Zámocký *et al.,* 2012).

Em *C. neoformans*, 4 genes de catalases foram identificados: dois genes que codificam catalases específicas de esporos (CAT1 e CAT3), uma catalase de peroxissomo (CAT2) e uma catalase citosólica (CAT4). As catalases CAT1 e CAT3 pertencem a um grupo de catalases relacionadas a produção de esporo ou germinação. A deleção da família inteira de catalase em *C. neoformans* demonstrou que estes genes não são relacionados com a virulência em modelo animal (Giles *et al*., 2006). Além disso, *C. neoformans* não possui atividade de catalase no sobrenadante de cultivo, consistente com a ausência da família das catalases secretadas (Upadhya *et al*., 2013).

A função das catalases na defesa antioxidante ainda precisa ser melhor elucidada. Acredita-se que estas enzimas possuam uma função interligada com outros constituintes do mecanismo de defesa contra estresse oxidativo. Esta hipótese é baseada no fato de que a perda da atividade de catalase não está relacionada com estresse oxidativo ou defeitos no desenvolvimento em muitos fungos (Giles *et al*., 2006). *H. capsulatum* possui três genes que codificam catalases, porém, somente duas catalases são funcionais. CATB é uma catalase extracelular, conhecida como antígeno M, localizada na parede celular e imunodominante em infecções causadas por este fungo. Já a CATP, se localiza no citoplasma, e tem uma função mais crítica na defesa contra ROS. Porém, a perda de CATP ou CATB

individualmente não altera a sobrevivência e crescimento nos pulmões demonstrando uma função redundante na defesa de *H. capsulatum* contra estresse oxidativo (Holbrook *et al*., 2013).

Zaragoza e colaboradores (2008) demonstraram que o aumento da cápsula polissacarídica durante a fagocitose confere resistência à estresse oxidativo. Porém, este mecanismo não é dependente da atividade de catalase. Assim, outros mecanismos devem existir para o controle destes radicais causadores de estresse oxidativo em espécies de fungos patogênicos.

2.7 Tratamento da criptococose

O tratamento utilizado no combate às infecções fúngicas inclui o uso de 4 principais agentes antifúngicos. Polienos, azóis e equinocandinas agem na membrana, na síntese da parede celular e no DNA das células fúngicas, respectivamente (Chen *et al*., 2010) Porém, o tratamento utilizando esses medicamentos possui muitos aspectos negativos, que incluem a toxicidade e o prolongado tempo de tratamento, que gera a resistência dos patógenos (Perfect *et al*., 2012). Por isso, novas estratégias para o combate dessas infecções são de extrema importância.

Novas abordagens, como por exemplo, o desenvolvimento de vacinas para o tratamento de infecções fúngicas vem sendo extensivamente estudados. Tem sido discutido que as vacinas são ferramentas importantes na prevenção e/ou tratamento de criptococose, uma vez elas estimulam o sistema imune mediado por células (Zhou & Murphy, 2006).

Estudos da imunomodulação da criptococose focam, principalmente, na função das citocinas e anticorpos monoclonais. Anticorpos monoclonais contra GXM, melanina, glicosilceramidas e ß- glicanas têm demonstrado efeito protetor em modelos animais (Rodrigues *et al*., 2007; Rachini *et al*., 2007; Antachopoulos & Walsh, 2012). Além disso, a imunoterapia utilizando citocinas, principalmente IFN-γ e IL-12, em associação com antifúngicos, também demonstrou resultados promissores (Joly *et al*., 1994; Clemons *et al*., 1994). Em 2010, a Sociedade Americana de Doenças Infecciosas recomendou o uso de IFN-γ recombinante no tratamento da criptococose (Antachopoulos & Walsh, 2012).

2.8 Vacina de DNA

A criptococose é uma doença de difícil tratamento, uma vez que a taxa de reincidência é alta e a terapia antifúngica possui muitos efeitos colaterais. Muitos estudos estão sendo realizados no intuito de desenvolver uma estratégia terapêutica mais eficaz. Sequências de DNA não metiladas, glucosilceramidas, ß-glicanas, radioterapia e imunização passiva são efetivas na prevenção da doença (Kinjo *et al.,* 2007; Rodrigues *et al*., 2007; Rachini *et al*., 2007; Bryan *et al.*, 2010; Rosas *et al*., 2001). Outras doenças fúngicas têm sido tratadas utilizando vacinas de DNA e apresentam resultados promissores (Pinto *et al*., 2000; Ribeiro *et al*., 2010).

As vacinas de DNA oferecem muitas vantagens em relação às vacinas clássicas. O custo mais baixo da produção, os fáceis controle de qualidade e armazenamento são características que contribuem para o sucesso desta terapia (Shedlock & Weiner, 2000).

As vacinas de DNA são seguras e induzem forte resposta imune celular no hospedeiro (Shedlock & Weiner, 2000). Acredita-se que a baixa imunogenicidade seja devida ao mecanismo de entrega do DNA na célula do hospedeiro. Porém, muitas estratégias têm sido avaliadas para aumentar a eficiência da transfecção. Os mecanismos mais comuns de entrega da vacina de DNA é por injeção intramuscular, intradérmica, bombardeamento e eletroporação (Ferraro *et al*., 2011).

Outras ferramentas têm sido avaliadas para aumentar a imunogenicidade das vacinas de DNA. Formulações da vacina com micropartículas ou lipossomos parecem aumentar a eficiência da transfecção. Adjuvantes à vacina de DNA também têm sido testados e demonstram um aumento no estímulo do sistema imune (Abdulhaqq *et al*., 2008). Contudo, a ferramenta vacina de DNA precisa ser avaliada em infecções causadas por *C. neoformans.*

3. OBJETIVOS

3.1 OBJETIVO GERAL

Este trabalho tem como objetivo estudar a função biológica de uma Hsp70 e da catalase 2 do fungo *Cryptococcus neoformans*, bem como avaliar o efeito terapêutico destas proteínas no tratamento da criptococose.

3.2 OBJETIVOS ESPECíFICOS

- Estudar a localização da Hsp70 e catalase 2 na célula da levedura *C. neoformans.*

- Avaliar a interação das proteínas Hsp70 e catalase 2 com células do hospedeiro.

- Avaliar o efeito terapêutico destas proteínas no tratamento de camundongos infectados com *C. neoformans.*

- Utilizar vacina de DNA contendo as sequências da Hsp70 ou catalase 2 no tratamento da criptococose.

- Caracterizar o perfil de citocinas produzidas pelo hospedeiro tratado com as vacinas de DNA contendo as sequência de Hsp70 e catalase 2.

Capítulo I

Artigo formatado para ser submetido ao período *Eukaryotic Cell.*

A putative peroxisomal catalase from *Cryptococcus neoformans* is anchored on the cell wall, exhibited activity and is recognized by macrophage cells

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ABSTRACT

Cryptococcus neoformans is an opportunist fungal pathogen that resists to oxidative stress inside the macrophages during host infection. *C. neoformans* developed the ability to grow and survive in hostile environment, such as that presented by the phagosome. The plethora of enzymes involved in this mechanism are considered one of the most important virulence traits. In the present study, we report that a cell wall-associated putative peroxisomal catalase 2 protects yeast cells against oxidative stress. Furthermore, the recombinant catalase 2 protein is recognized by sera from patients diagnosed with criptococcosis. The recombinant catalase 2 was found to be capable to bind and associate to phagocytes, promoting the adhesion of yeast cells. The binding site of the recombinant catalase 2 is different from that of the major capsular polyssacharide, glucoronoxylomann - GXM, binding site, as a distinct pattern of anti-catalase 2 or anti-GXM antibodies recognition in host cell surface was observed in macrophages treated with the conjunction of GXM and recombinant catalase 2. Our data suggest additional functions to catalase 2 that can influence the interaction of *C. neoformans* with host cells.

1. INTRODUCTION

Cryptococcus neoformans is a basidiomycete considered an opportunist yeast pathogen that causes disease in immune compromised patient (1). *C. neoformans* has a repertory of well-characterized mechanisms that contribute to fungal pathogenesis. Once the infective propagules are inhalated by the host, fungal cells come into contact with host immune response and use mechanisms that allow fungal survival (2). Alveolar macrophages phagocyte *C. neoformans* cells during initial stages of infection. and represent a hostile environment producing both oxidative and nitrosative agents that kill phagocytized yeast. However, *C. neoformans* cells survive and replicate within phagocytes (3,4). In response to the oxidative and nitrosative environment, the antioxidant system from *C. neoformans* cells produce enzymes such as peroxidases, superoxide dismutase and catalases (5). A major virulence factor, the polysaccharide capsule, confers resistance against oxidative stress and inhibits macrophage activation (6,7). Other important

virulence factor that plays protective role in stress resistance is laccase, an enzyme that produces melanin (8).

Among the many reactive species produced by the phagocytes, the superoxide íon is the most active (9). To hamper the toxic effects of superoxide, *C. neoformans* expresses superoxide dismutase, a metalloenzyme that belongs to the antioxidant system and catalyzes the dismutation of O_2^-, yelding H_2O_2 and O_2. This enzyme uses copper and zinc as cofactors and contributes to the yeast virulence by resisting oxidative damage within macrophages (10, 11). Besides, peroxidases, that remove peroxides, are also an important virulence factor to fungal pathogen (12).

Catalases are involved in decomposition of H_2O_2 to H_2O, also conferring resistance to oxidative radicals, mainly in the peroxisome and cytoplasm (9). Moreover, catalases are found associated with spores and in extracellular environment in *Paracoccidioides brasiliensis* (13). Despite the enzymes that protect fungi against reactive oxygen species are related to virulence, all catalases from *C. neoformans* do not confer resistance to oxidative stress or or influenced virulence (14). Catalases have been identified in the cell surface of other fungal pathogens such as *Aspergillus fumigatus* and *Histoplasma capsulatum* (15,16). The catalase from *H. capsulatum* was found to be located on the yeast cell surface and presented enzymatic activity in such environment (16). In another yeast pathogen, *Candida albicans*, peroxisomal catalase was identified as plasminogen-binding protein that can enable tissue invasion and play a role in the disease process (17).

The goal of this study is to evaluate the biological functions of catalase 2 (annotated as peroxisomal catalase) from the *C. neoformans.* We demonstrated that catalase 2 can be located in the cell wall, accessible to effector cells of the immune system and can be recognized by antibodies from cryptococcosis patients. Surface catalase 2 can contribute to antioxidant defense system. In addition, we show that catalase 2 can bind to the macrophages cell at different sites that GXM, suggesting that catalase 2 binding to host cells is a mediator that facilitates the infection process.

MATERIALS AND METHODS

Fungal strain and growth condition

C. neoformans var. grubii H99 strain (ATCC 208821) was maintained in 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 YNB (Yeast Nitrogen base), collected by centrifugation at 5000 *g* for 5 minutes, washed in phosphate-buffered saline (PBS) and counted in Neubauer chamber.

Cloning, expression, and purification of His-tagged recombinant protein

Catalase 2 gene sequence (CNAG_05256.2) was obtained from the Broad Institute (www.broadinstitute.org). The cDNA sequence was amplified using primers CATF (GGATCCATGACTGAGAAAACACCCA) and CATR (CTCGAGTTACTTTCCCTTGTTGAGCT) and was cloned into the SmaI site of pUC18, followed by subcloning into the *XhoI* and *NcoI* sites of the expression plasmid pET-23d(+) (Invitrogen Corp., Carlsbad, California, USA). The sequence of the cloned ORF was verified by DNA sequencing (Brazilian Genome Network at the Center of Biotechnology, CBiot-UFRGS-RS). BL21(DE3) pLysS *E. coli* was transformed with the plasmid and protein expression was induced with 1 mM IPTG for 3h. The recombinant protein was purified using nickel affinity chromatography Hi-Trap (GE Healthcare, *formerly Amersham Biosciences,* Uppsala, Sweden). The purified protein, named Cn_rCAT2, was eluted by 4 M urea buffer, dialyzed against water and treated with Triton X-114 to minimize contamination with lipopolysaccharide (18).

To confirm identity of recombinant catalase 2, the Cn_rCAT2 protein was separated by SDS-PAGE, excised from gel, alkylated and digested with trypsin, followed by extraction of peptides (19). Peptides were identified by MALDI-TOF at the Unidade de Química de Proteínas e Espectrometria de Massas (Uniprote-MS), Cbiot, UFRGS.

Anti_rCAT2 sera

Female 6-8-week-old BALB/c mice were immunized intraperitoneally with 20, 50 and 100 µg of Cn_rCAT2 mixed with complete Freund's Adjuvants (CFA, Sigma, St. Louis, Missouri, USA) and boosted 2 and 4 weeks post

immunization with incomplete Freund's adjuvant (Sigma-Aldrich). Polyclonal anti-Cn_rCAT2 antibody was then collected 6 weeks after the first immunization. The Federal University of Rio Grande do Sul Ethics Committee approved animal manipulation.

Enzyme-linked immunosorbent assays (ELISA) were used to examine the specificity of polyclonal anti-Cn_rCAT2 antibody to recombinant protein CAT from *C. neoformans*. Briefly, Cn_rCAT2 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 1h at 37°C. The plates were washed three times with 0.1% Tween-20 in PBS and coated with the anti-Cn_rCAT2 polyclonal sera. After 1h at 37°C, the plates were washed, coated with peroxidase-labeled antibodies raised to mouse IgG (Sigma-Aldrich) and incubated for 1h at 37°C. Serologic reactions were measured by addition of TMB (Invitrogen Corp., Carlsbad, California, USA) and measurement of OD at 450 nm. Pre- immune serum was used as negative control. All experiments were performed in twice sets and statistically analyzed by Student's *t* test.

Analysis of human sera

Due to the property of cryptococcal catalase 2 to induce humoral responses in human patients (M. Polese, 2009, unpublished data), we evaluated the serological properties of Cn_rCAT2 in Western blotting assays using sera from individuals diagnosed for cryptococcosis. Sera were kindly donated by Dr. Liline Martins (Universidade Federal do Piauí, Brazil). Cn_rCAT2 was separated by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF, GE Healthcare) membrane and probed with pooled sera from 5 patients with cryptococcosis at the dilution of 1:10. Detection was performed using an ECL-plus system (GE Healthcare) according to the manufacturer's instructions.

Cytoplasmatic distribution of catalase

The catalase cytoplasmic localization was performed as described by Zámocky *et al*. 2013 (9). Briefly, *C. neoformans* H99 cells were grown in YNB

at 30°C for 48h, washed with PBS and fixed in 4% paraformaldehyde. Cells were washed 5 times with PEM buffer (50 mM PIPES, 25 mM EDTA, 5 mM $MgSO_4$) and cell wall was digested with digestion buffer [50 mg/ml Lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich), 5mM EDTA, pH 7] at 37°C for 5min. Cells were washed with PEM buffer, immersed in cold methanol for 10min and washed twice with PEM buffer. Then, cells were blocked with 1% BSA (Sigma-Aldrich) and incubated with the anti-Cn_rCAT2 polyclonal serum (1:50, v/v) for 1h at 37°C. After washing with PBS, the cells were incubated with Alexafluor 488-conjugated IgG (Invitrogen Corp.) for 1 hour at 37°C. The cells were again washed and mounted onto glass slides using a 50% glycerol/50% PBS/0.1 M *N*-propyl gallate solution. Cells were analyzed using Axioskop 40 (Zeiss) immunofluorescence microscope. Nucleus was stained with 0,25 µg/ml of 4',6-diamidino-2-phenylindole (DAPI).

Cell Surface catalase detection

ELISA using intact yeast cells was used to examine whether catalase would be associated to surface components of *C. neoformans*, based on the protocols described by Lopes *et al*. 2010 (20). Briefly, *C. neoformans* yeast cells ($5x10^5$ per well) were incubated in 96-well polystyrene plates (Costar 9018) for 2h at 37°C in PBS. The wells were blocked with 2% BSA (Sigma-Aldrich) in PBS supplemented with 0.05% Tween-20 for 1h at 37°C. The plates were washed three times with 0.1% Tween-20 in PBS and coated with the anti-Cn_rCAT2 polyclonal sera, serially diluted 1:2 in the blocking solution. After 1h at 37°C, the plates were washed, coated with peroxidase-labeled antibodies raised to mouse IgG (Sigma-Aldrich) and incubated for 1h at 37°C. Serologic reactions were measured by addition of TMB (Invitrogen Corp.) and measurement of OD at 450 nm. Polyclonal antiserum against β-tubulin (Sigma-Aldrich) produced in mouse was used as negative control. Whole cell ELISA was performed in duplicate and statistically analyzed using Student's *t* tests.

The wild type and acapsular mutant of C. *neoformans* was analyzed by immunofluorescence assay. Fungal cells from *C. neoformans* H99 and CAP67 were grown in YNB at 30°C for 48h, washed with PBS and fixed in 4%

paraformaldehyde. Cells were blocked with 1% BSA (Sigma-Aldrich) and incubated with the anti-Cn_rCAT2 polyclonal serum (1:50, v/v) for 1h at 37°C. After washing with PBS, the cells were incubated with Alexafluor 488-conjugated IgG (Invitrogen Corp.) for 1h at 37°C. The cells were again washed and mounted onto glass slides using a 50% glycerol/50% PBS/0.1 M *N*-propyl gallate solution. Stained cells were finally analyzed with an Olympus FluoView™ 1000 fluorescence microscope (Olympus Optical Co., Melville, New York, USA).

To confirm the surface localization of catalase, we evaluated different cell fractions from *C.neoformans* using Western blotting assays. Cell wall was extracted following as described by Stie *et al*. 2009 (21). Briefly, *C. neoformans* was grown for 48h at 30°C in 50 ml of YPD. Capsule was extracted using DMSO for 30 min at room temperature, washed 2 times with Milli-Q water and frozen for 24h at -80°C. Cells were suspended in cold buffer (10mM Tris, pH7.4; proteases inhibitor) and lysed with glass beads for 12 cycles for 2min, with ice-intervals. Cells were sonicated and centrifuged at 3000 *g* for 10min. Then, cells were incubated with decreasing concentrations of NaCl. Finally, cell wall proteins were extracted using buffer containing SDS (50mM Tris, pH 8; 0.1M EDTA; 2%SDS; 10mM DTT), centrifuged at 3000 *g* for 10min and lyophilized. Intracellular proteins were used as control. Total protein was isolated as described by Crestani *et al*. 2012 (22). Supernatant was collected after centrifugation, lyophilized and stored at -20°C. Cell fractions were separated by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF, GE Healthcare) membrane and probed with anti-Cn_rCAT2 at the dilution of 1:800. To ensure that cell wall fraction does not have intracellular proteins, polyclonal antiserum against β-tubulin was used as control at the dilution 1:2000.

Determination of catalase activity on the cell surface

To verify if catalase located on the cell surface has activity in *C. neoformans,* catalase activity was measured as described in the Worthington enzyme manual (Worthington Biochemical Corp., Freehold, N.J., 1972). First, cells were grown in 50 ml of YPD for 96 h and aliquots from culture were

collected at 24 h intervals. Decomposition of hydrogen peroxide was measured spectrophotometrically at 240 nm. Measurements were made at 20 s intervals for the first 3 min after the cells were mixed with the substrate. One unit of catalase was defined as the amount required to catalyze the decomposition of 1 mmol of hydrogen peroxide per min in 0.05 M hydrogen peroxide at 25 °C.

Synergistic effect of catalase 2 with fluconazole and amphotericin B

To test the synergistic effect of Cn_rCAT2 with antifungals, a disk diffusion methodology was performed following the CLSI M44-A disk diffusion method guidelines CLSI (23). Briefly, the entire surface of Mueller-Hinton agar supplemented with 2% dextrose agar plates (150-mm plates) were inoculated using a swab dipped in the yeast inoculum suspensions at OD530 with the range between 0.11 and 0.14. The inoculated agar was allowed to dry for 15 to 30min, and the disks (fluconazole: 25 µg/disk, amphotericin B: 100 µg/disk; Cn_rCAT2: 10 µg/disk) were applied to the inoculated agar. The plates were incubated at 30°C for 48h and the inhibition zone diameter was determined. All experiments were performed in triplicate and statistically analyzed using Student's *t* tests.

To verify whether Cn_rCAT2 inhibits melanin production, cells were spotted on niger seed agar plates supplemented with 10 µg/ml of Cn_rCAT2. The plates were incubated at 30°C and evaluated for 2 days.

The ability of Cn_rCAT2 to reduce lung and brain CFU burden was evaluated. 20 µg of Cn_rCAT2 was administrated intraperitoneally in BALB/c mice (female, 4–6 weeks old) at day 1. Mice were infected intranasally with $2x10^5$ *C. neoformans* cells and immunized with 20 ug of Cn_rCAT2 at day 8. Twenty four hours post-infection, mice received 20 ug of Cn_rCAT2 and 1,5 mg/Kg amphotericin B by intraperitoneal administration. Mice received amphotericin B for 7 days. Two weeks after infection, mice were sacrificed and CFU burden determined. Data are representative of one experiment with 5 mice per group.

Proliferation cell assay and activation of macrophages cells by catalase 2

The macrophage-like cell lineage J774.1 was 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_rCAT2 would affect cellular 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) in presence of diferent Cn_rCAT2 concentrations was assayed. Monolayers (10^6 cells/well) were incubated for 18h with 7 varying concentrations of Cn_rCAT2 (0 to 100). After this period, 158 µl of fresh medium supplemented with 1 mg/ml XTT and 1mM menadione was added, followed by 3 hours of incubation at 37°C. The absorbance (490 nm) was measured spectrophotometrically (24). Statistical analysis was performed using the Student's *t* tests.

The ability of Cn_rcCAT2 to stimulate NO production by macrophages was measured using the Griess Reagent (Sigma-Aldrich) (25). This assay was performed in duplicate and statistically analyzed by Student's *t* test.

Influence of Cn_rCAT2 in the interaction of *C. neoformans* with host cells

To evaluated the *C. neoformans* attachment to the A549 cells, 10^4 cells/well of epithelial cells were distributed in 96-well plates and incubated with 10 µg/ml of Cn_rCAT2 for 1h at 37°C. *C. neoformans* (10^4 cells per well) were incubated with 10 µg/ml of purified Cn_rCAT polyclonal antibody for 1h at 37°C, washed with PBS and then incubated with epithelial cells at 37°C in a 7.5% CO_2 atmosphere for 18 hours. Unattached fungi were washed with PBS and A549 cells were lysed with sterile cold water and fungi suspensions were plated on YPD agar. Control systems were treated with no antibody or polyclonal antiserum against β-tubulin produced in mouse at same concentration used for anti-Cn_rCAT2. After 48h, the CFU was determined

and statistically analyzed by Student's *t* test. Experiments were performed only once.

Binding of Cn_rCAT 2 and GXM to phagocytes

To verify the association of Cn_rCAT2 and GXM in mammalian cells, cells were maintained as described above. J774.1 cells (10^6 cells/well-CellView Greiner Bio One) were double immunofluorescence stained with Cn_rCAT2 (10 µg/ml) and GXM (10 µg/ml). Three systems were analyzed: A) Cn_rCAT2 was incubated for 60min, then GXM was added for 60 minutes (Cn_rCAT_GXM); B) GXM was incubated for 60 minutes, then Cn_rCAT was added for 60 minutes (GXM_Cn-rCAT); C) Cn_rCAT2 and GXM were co-incubated for 60 min (Cn_rCAT2+GXM).

After washing, cells were fixed with 4% paraformaldehyde and blocked for 1 hour (37°C) in PBS-1%BSA. Cells were washed 3 times with PBS and incubated with anti-Cn_rCAT polyclonal serum or 2D10 mAb (gently provided by Arturo Casadevall, Albert Einstein College of Medicine of Yeshiva University, New York) for 1h at 37°C following by incubation with Alexafluor 488-labeled anti-mouse IgG (Invitrogen) or Alexafluor 568-labelled anti-mouse IgM. After washing, the second immunofluorescence was performed. Cells were incubated with 2D10 mAb or anti-Cn_rCAT2 polyclonal serum, and then incubated with 568 -conjugated IgM anti-mouse or Alexafluor 488- labeled anti-mouse IgG (Invitrogen Corp.) respectively. Stained cells were finally analyzed with an Olympus FluoView™ 1000 fluorescence microscope (Olympus Optical Co., Melville, New York, USA).

Results

Cn_rCAT2 is recognized by serum antibodies

Expression vector (pET23d) containing catalase sequence (pET23CAT) was confirmed by restrict enzyme digestion (Fig.1A). BL21(DE3) pLysS *E. coli* was transformed with resulting plasmid and induced with 1 mM IPTG for 3 hours (Fig. 1B). Starting 1h after induction, recombinant protein was expressed. A recombinant catalase 2 from *C. neoformans* was produced in *E. coli* and purified using affinity chromatography (Fig. 1C). Polyclonal

antibody anti-Cn_rCAT2 was produced in mice immunized with 20, 50 or 100 µg of recombinant protein and assayed against soluble Cn_rCAT (Fig. 1D). The recombinant catalase protein from *C. neoformans* (Cn_rCAT2) was resolved in SDS-PAGE for further evaluation of serologic reactivity. Cn_rCAT2 was probed with sera from cryptococcosis patients. A recognition signal with predicted molecular mass corresponding to Cn_rCAT2 (56 kDa) and a truncated catalase (30kDa) could be detected in Western-blot experiments employing sera from cryptococcosis diagnosed patients (Fig. 1E). The identity of the Cn_rCAT2 peptides was confirmed by MALDI-Tof spectrometry (Fig. 1F).

Catalase 2 is located on the surface

Catalases have been reported to locate at the cell surface in fungi (16). In order to verify the localization of the catalase 2 in *C. neoformans* we performed ELISA using intact yeast cells and demonstrated that the anti-Cn_rCAT2 serum reacted with the fungus cell surface (Figure 2A). Polyclonal antiserum against β-tubulin produced in mouse was used as negative control and showed no binding to the fungal cell.

An important characteristic of *C. neoformans* is the presence of a polysaccharide capsule associated with the cell wall (26). The cellular distribution of catalase 2 from wild type and acapsular mutant CAP67 were analyzed by confocal microscopy using the Cn_rCAT2 serum (Fig.2B). Catalase 2 is located on the cell surface, mainly on the cell wall. In addition, catalase 2 is a putative peroxisome enzyme, therefore, the cytoplasmic localization was performed using permeabilized protoplasts (Figure 2C). As expected, catalase 2 was observed in the cytoplasm.

The cell wall fraction from *C. neoformans* was evaluated to confirm that catalase 2 is located on the cell surface. After cell treatment with DMSO to remove capsule, total protein (E), cell wall proteins (CW) and supernatant (S) were isolated and resolved by SDS-PAGE (Fig. 2D). As showed in Fig. 2E, catalase was present in total protein and cell wall fractions. No catalase 2 was detected in supernatant, indicating that catalase 2 is not secreted to extracellular media. A polyclonal antiserum against β-tubulin was used as

control to rule out contamination with intracellular proteins in cell wall fraction.

Taken together our results, allow to speculate that catalase 2 from *C. neoformans* is secreted by non-conventional mechanisms, since bioinformatic analysis employing Signal P revealed that this protein does not have classical secretion motives. Moreover, the immunological detection in cytoplasmic and cell wall fraction contributes to this assumption.

Cell surface catalase has activity

To investigate whether catalase 2 at the surface of *C. neoformans* is active we quantified the catalase activity of the whole cell by an indirect method. This method relies on the descrease in OD_{240} caused by degradation of hydrogen peroxyde (16). Time course analysis revealed that catalase activity increased with the incubation time 48, 72 and 96 h (Fig. 3A). We normalized the catalase activity by the number of cells in the aliquots (OD_{600}). We also performed a CFU determination, at each incubation time, to verify whether the increase of catalase activity observed was not a consequence of fungal cell death and release of intracellular catalase activity. As demonstrated in Fig. 3B, the increase of incubation time led to an increase of CFU counts of cryptococcal cells, indicating that catalase activity detected in whole cells is not due to the release of intracellular catalase. These results indicates that catalase located on the cell wall has activity.

Catalase 2 does not enhance antifungal effect in vitro and in vivo or interfere in melanin production

To evaluate a possible synergistic effect of catalase 2 with antifungal usually used in cryptococcosis treatment, a disk diffusion test was performed. Inhibition zone does not have a significative difference between disk diffusion agar containing fluconazole (25 µg/ml), amphotericin B (100 µg/ml) or co-incubated with 10 µg/ml of recombinant protein (Fig. 4A), demonstrating that Cn_rCAT does not have antifungal activity, since when incubated alone, no inhibition zone was observed. The same results were obtained to *C. albicans* and C. *parapsilosis.* To evaluate whether catalase 2 interferes in cell melanization, recombinant protein (10 µg/ml) was incubated with fungal cell and plated on agar niger. The recombinant protein did not interfere in (Fig.

4B) *C. neoformans and C. gattii* melanization. *C. albicans* was used as a control since does not produce melanin pigment.

Since Cn_rCAT have not shown an antifungal activity, we speculated that could be effective in stimulate immune response in mice infection. BALB/c mice were immunized with 20 µg/ml of Cn_rCAT and infected by intranasal inhalation with *C. neoformans* cells. Then, mice received 2 doses of protein and were treated with 1,5 mg/kg of amphotericin B every day. Mice were sacrificed and CFU burden in the lungs (Fig. 4C) and brain (Fig. 4D) were determined. As demonstrated in Fig. 4C, mice that received Cn_rCAT treatment had no significatively decreased the fungal burden compared to the group non- treated (control – H99). However, mice treated with amphotericin B in association with Cn_rCAT, had an increased in fungal burden compared to the group that were treated with amphotericin B alone (p <0.05). Relative to the brain burden, when mice were treated with amphotericin B, no fungal cells could be detected. No difference was observed when mice were treated with Cn_rCAT compared to the group non- treated.

We conclude that Cn_rCAT does not have antifungal activity and does not have a synergistic effect with fluconazole and amphotericin B; does not interferes in melanization and does not protect mice against *C. neoformans.*

Catalase had a toxic effect on macrophages cells and did not increased NO production

J774.1 macrophages were exposed to 7 concentrations of Cn_rCAT2 (0 to 100 µg/ml) to evaluate the cell viability after recombinant protein incubation by XTT reduction. The protein was toxic to host cells at relatively low concentrations (Fig. 5A). The pre-incubation of J774 cells with 1.56, 3.125 and 6.25 µg/ml of Cn_rCAT were not sufficient to induce NO production by macrophages ($p > 0.05$) (Fig. 5B).

Influence of Cn_rCAT on the interaction of *C. neoformans* with host cells

The treatment of alveolar cells with Cn_rCAT2 in combination with treatment of *C. neoformans* with the anti-Cn_rCAT2 serum was evaluated using 10^4 cells. Fig. 6 shows that when 10^4 yeast cells were coated with 1

μg/ml or 3 μg/ml of anti-Cn_rCAT and A549 cells were incubated with 1 μg/ml or 10 μg/ml of Cn_rCAT (1 fungal cell to 1 epithelial cell) less adherent cells were observed. A polyclonal antibody β-tubulin was used as a control at the same concentrations.

These results indicate that catalase could influence the adhesion process of cryptococcal cells to lung epithelial cells.

Cn_rCAT2 and GXM have different binding sites in host cells

Based on the cell surface localization of catalase 2, we hypothesized that GXM and catalase 2 could share the same cellular binding sites in host cells. Both catalase 2 and GXM were detected at the plasma membrane level of host cells (J774.1 cells, Fig 7A). To show that the binding of catalase 2 and GXM to host cells is specific and mediated by different receptors, J774.1 cells were incubated with both Cn_rCAT2 and GXM. Independently of the order of adition Cn_rCAT2 did not prevent GXM binding and *vice-versa* (Fig 7B). Hence, catalase and GXM bind to different sites at host cells.

Discussion

Antioxidant defenses are a powerful mechanism to allow fungal survival in adverse environments. When in contact with host cells yeast has to overcame different kinds of stressors during fungal infection. Once yeast escapes from killing by macrophages it can invades other organs and can disseminate to the central nervous system. Due to the importance of antioxidant system, the activity of several molecules can overlap and compensate protective activity from enzymes responsible to combat oxidative species, since the lost of entire family of catalases does not alter the virulence in *C. neoformans* (13). Surprisingly, catalase mutant did not inhibit an increase in sensibility to exogenous oxidative stress (13) and lead us to speculate that catalase may have alternative function than protective defense. Recombinant catalase protein from *C. neoformans* was recognized by patients sera and the polyclonal antibody produced was assayed against entire yeast cell. As previously described for *H. capsulatum* (16), catalase 2 from *C. neoformans* was also located on the cell surface, mainly on the cell

wall, as observed by an acapsular mutant CAP67 that still has catalase 2 at cell surface. Despite supposed that *C. neoformans*, apparently, does not have secreted catalase (13), we demonstrated that putative peroxidase catalase is secreted and is associated with cell wall.

C. neoformans has four catalases, where CAT1 and CAT3 are putatively spore-specific; CAT4 is putatively cytosolic and CAT2 is putatively peroxisomal (13). We analyzed the putative peroxisomal catalase 2, which does not contain canonic sequence signals to be exported to the membrane or even secreted. As some virulence factors are secreted by unconventional mechanisms through vesicles, including catalase A from *C. neoformans* (CNAG_00575.1) (27), it would be possible that peroxisome catalase 2 can be secreted by this mechanism. The catalase 2 located on the cell surface showed activity to degraded exogenous hydrogen peroxide. The presence of non-classically secreted proteins in the cell wall of several fungi has been reported and metabolic enzymes at the cell suface can modulate the interaction with host cells as described in *Paracoccidioides brasiliensis* and *H. capsulatum* (28, 29).

It has been reported that fluconazole and amphotericin B cause oxidative damage to plasma membrane (30,31). It was also reported that antineoplasic drugs contribute to the fungal resistance by catalase activity (32). Therefore, we evaluated the possible synergistic effect of catalase with fluconazole and amphotericin B using disk diffusion test. However, we observed that recombinant catalase 2 did not increase the antifungal effect. In addition, a monoclonal antibody against catalase conferred protection to BALB/c mice infected with *Nocardia brasiliensis* (33). Differently we observed that the recombinant catalase 2 protein did not confer protection to mice experimentaly infected with *C. neoformans.*

It was reported that oxidative and nitrosative stress are related, since a mutant of thiol peroxidase gene is hypersensitive to NO (11) and that catalase inhibited NO production by activated macrophages (34). Then, we evaluated if macrophages incubated with Cn_rCAT2 display alterations in NO production. As observed Cn_rCAT2 did not induce NO production compared to macrophages incubated with LPS. Further studies are necessary to verify if Cn_rCAT2 inhibits NO production by activated macrophages and incubated

with *C. neoformans* cells.

Despite the participation of catalase 2 in the adhesion process has not been reported yet, previous studyes demonstrated that *C. neoformans* binds to alveolar epithelial cells through the capsule and other surface structures (35). In our study, yeast cells interfered on the adhesion assay, since 10^4 incubated with Cn_rCAT promoted the adhesion of *C. neoformans* to A549 cells.

Cellular surface components interact with the host cell receptor. In fact, GXM binds to the epithelial cells allowing the yeast cell to be internalized (36). Therefore, we evaluated the potential binding sites of catalase and GXM. Immunofluorescence analysis demonstrated that catalase 2 binds to the macrophages. Moreover, Cn_rCAT binds to macrophages in different sites as capsular GXM does. So, we speculate that, because of the cell wall localization not associated to the capsular network, Cn_rCAT2 did not interfere in the association of GXM and host cell.

In the present study, we have showed that a predicted peroxisomal catalase, catalase 2, from *C. neoformans* anchored on the cell wall and exhibited activity. Additionally, we demonstrated that catalase 2 binds to the host cell, in different sites that GXM does and may mediate yeast/cell adhesion. Herein, the antioxidant system show a complex way to protect yeast cells against oxidative stress indicating that enzymes involved in this process can perform more than one function in different sites. Further studies are necessary to address the role of secreted catalases other than oxidative defense, to help understand the early stages of *C. neoformans* infection.

Acknowledgments

This work was supported by grants from FINEP (Financiadora de Estudos e Projetos, Brazil), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Brazil), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Brazil) and Ludwig Biotecnologia. The authors thank to Dr. Arturo Casadevall for providing the monoclonal antibody anti-GXM (2D10). Automated DNA sequencing was performed at the facilities of the Brazilian Genome Network at the Center of Biotechnology, CBiot-UFRGS-

RS. We also thank the Electron Microscopy Center of the Federal University of Rio Grande do Sul (CME, UFRGS) for the confocal microscopy analysis and Henrique Biehl for technical assistance.

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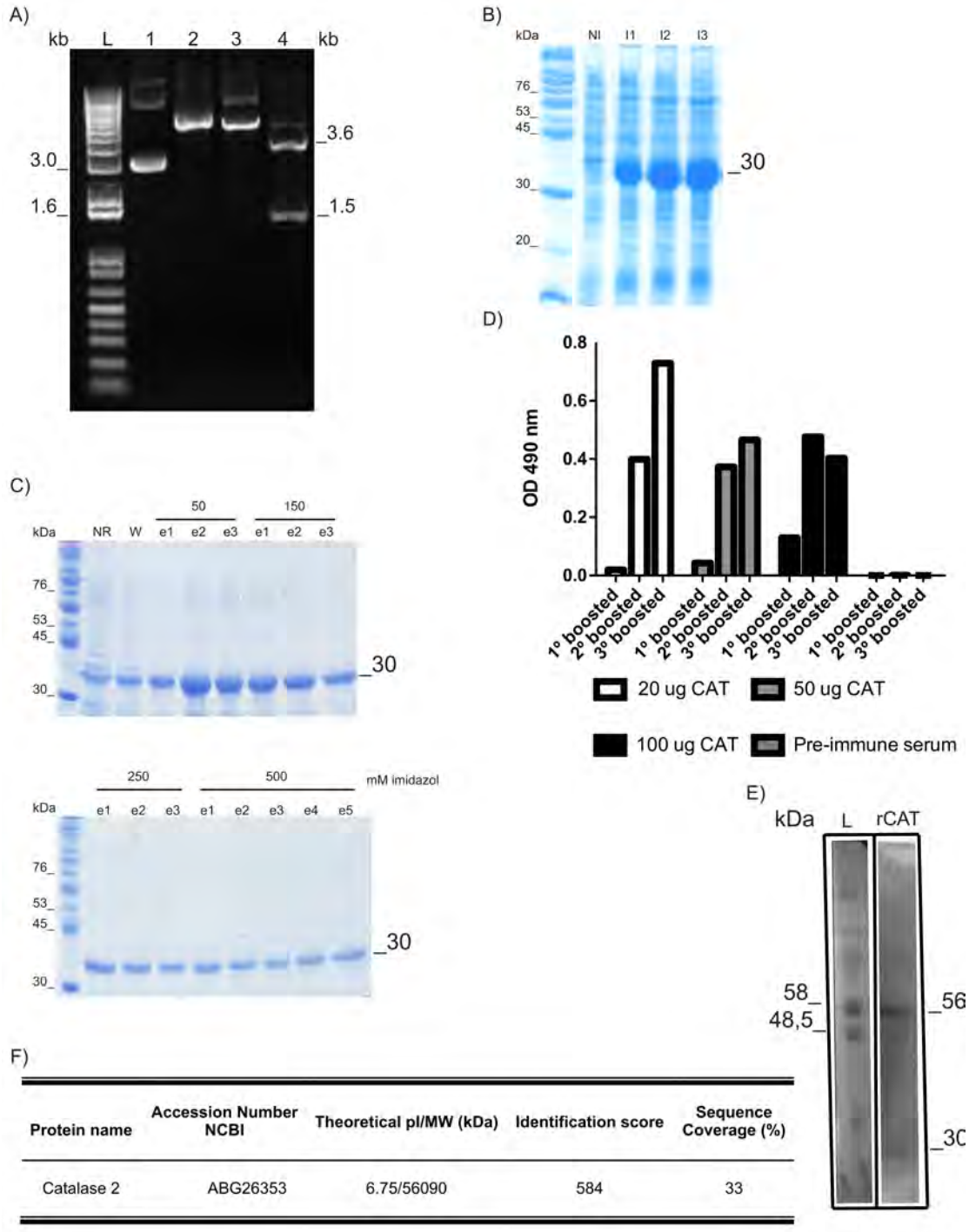
FIGURES AND LEGENDS



F)

Protein name	Accession Number NCBI	Theoretical pI/MW (kDa)	Identification score	Sequence Coverage (%)
Catalase 2	ABG26353	6.75/56090	584	33

Fig 1 Cn_rCAT is recognized by a polyclonal serum raised against *C. neoformans*. A) Catalase sequence was cloned into pET23d vector and the restriction enzymes were used to confirm plasmid construct. L: 1kb ladder; 1: pET23dCAT; 2: pET23dCAT linearized with NcoI; 3: pET23dCAT linearized

with XhoI; 4: double digestion of pET23dCAT with NcoI and XhoI releasing a catalase sequence (1.5kb) and pET23d (3.6 kb). B) *E.coli* BL21(DE3)pLysS was transformed with pET23dCAT, non-induced (NI) and induced with 1 mM IPTG for 1 h(I1), 2 hrs (I2) and 3 hrs (I3). A 30kDa band corresponding to catalase protein appeared starting 1 hour of induction. C) Recombinant protein was purified using increasing concentration of imidazole. NR: not retained; W: wash; e1, e2, e3 50: eluted 1, 2 and 3 with 50 mM imidazole respectively. e1, e2, e3 150: eluted 1, 2 and 3 with 150 mM imidazole respectively. e1, e2, e3 250: eluted 1, 2 and 3 with 50 mM imidazole respectively. e1, e2, e3, e4 and e5 500: eluted 1, 2 and 3 with 500 mM imidazole respectively. A 30kDa band corresponding to the probable truncated catalase 2 protein was eluted in all imidazole concentrations. D) A quantity of 20 µg, 50 µg and 100 µg of recombinant protein was used to coat 96 wells polystyrene plates for ELISA. Positive reactions were observed when the antigen was probed with the polyclonal serum. Asterisks denote $p < 0.05$. E) Serologic reactivity of Cn_rCAT2 with a pool of sera (1:10) obtained from cryptococcosis patients. Western blot analysis after separation of Cn_rCAT2 by SDS-PAGE revealed a band with migration rate corresponding to monomer of catalase (56 kDa) and truncated protein (30 kDa). Molecular mass markers are shown on the right column. F) Catalase peptides were identified by MALDI-TOF and confirmed the identity of recombinant protein from *C. neoformans.*

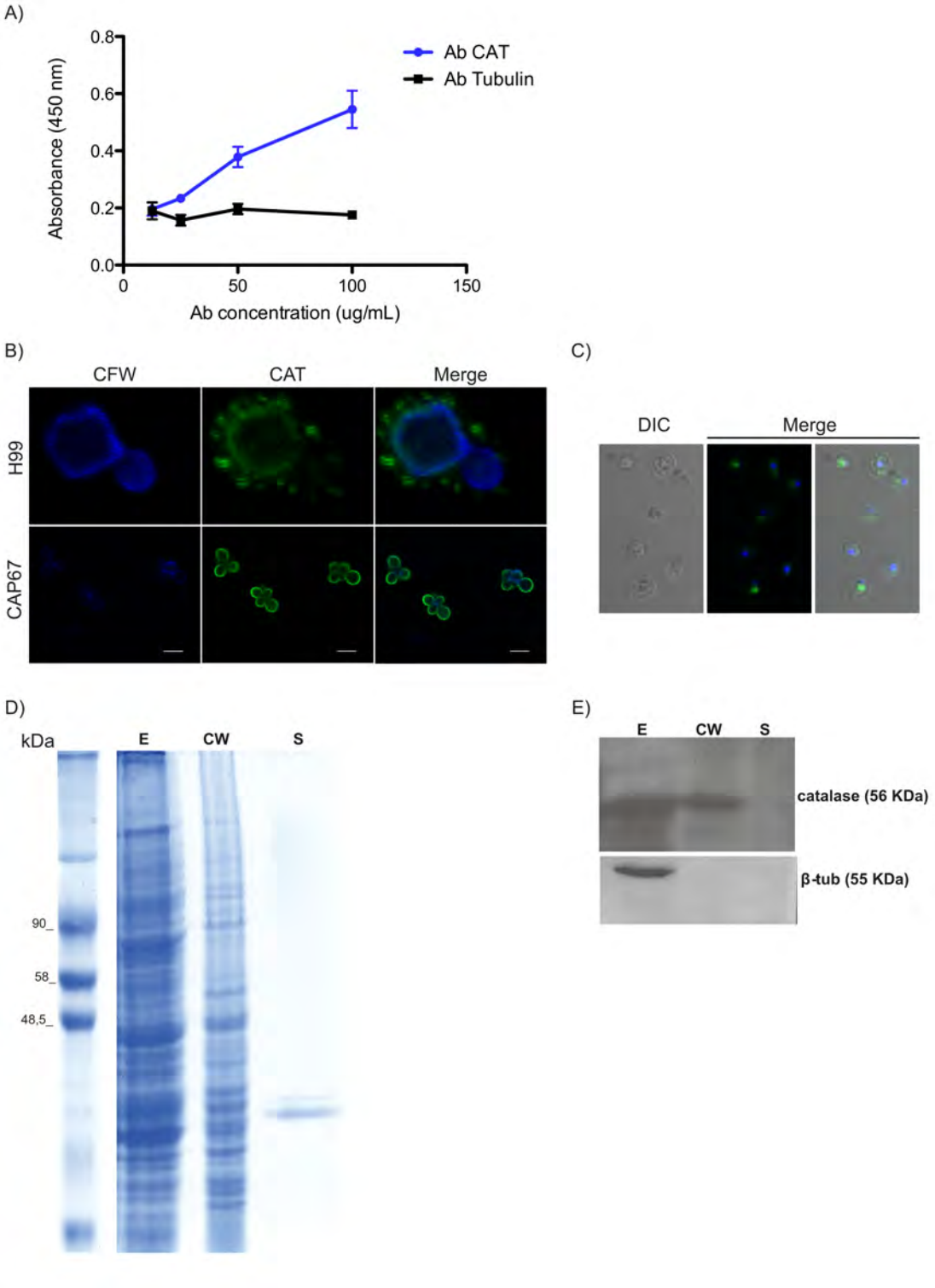


Fig 2 The anti-CAT2 polyclonal serum binds to the cell surface of *C. neoformans***.** A) ELISA with whole cells revealed efficient serum binding at concentration ranges higher than 25 µg/ml. As control, antibody anti-β-tubulin was used and showed no binding to *C. neoformans* cells. The experiment was repeated twice with similar results. B) Catalase 2 localizes to the fungal cell surface and cell

wall in wild type (H99). Acapsular mutant CAP67 confirmed that catalase 2 is also located on the cell wall (CAP67); Catalase 2 appears in green. The cell wall was stained with calcofluor white (blue fluorescence). The scale bar corresponds to 5 μm. C) A cytoplasmic localization of catalase 2. The nucleus was stained with DAPI (blue fluorescence) and cytoplasmatic catalase was stained with Alexa 488 (green fluorescence). D) Total extract protein, including intracellular proteins (E), cell wall proteins (CW) and supernatant (S) were resolved by SDS-PAGE and immune reacted with polyclonal antibody anti-Cn_rCAT2 confirming the cell wall localization of catalase 2 **(E, upper panel)**. As control of contamination of cell wall fraction with intracellular proteins, antibody anti-β-tubulin was reacted against cellular fractions **(E, lower panel)**.

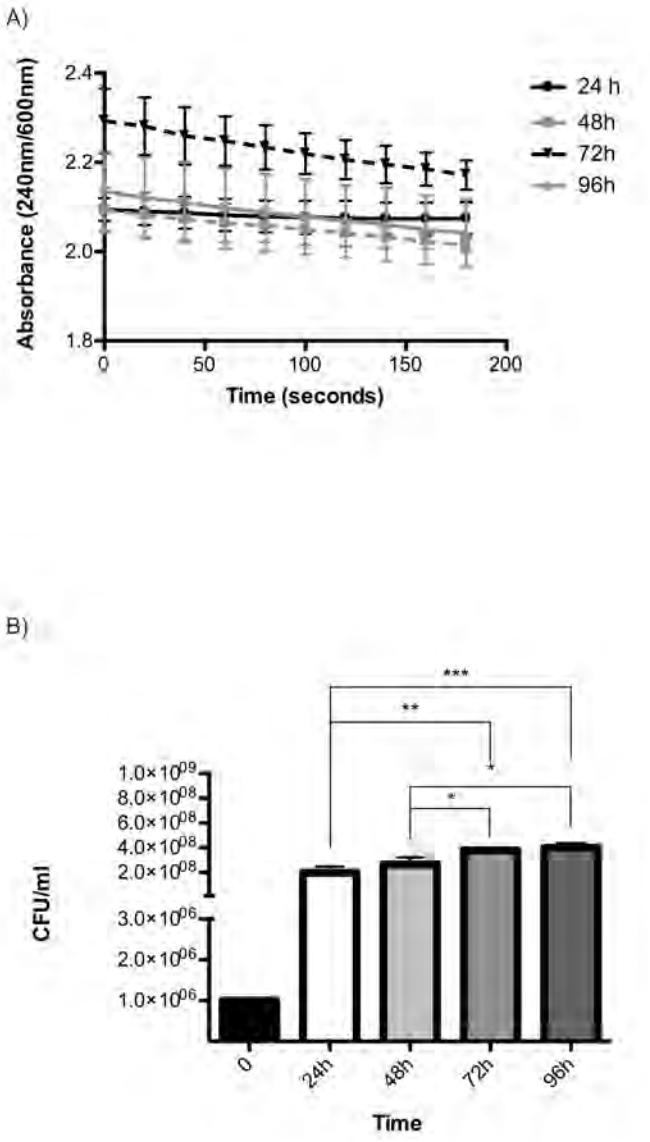


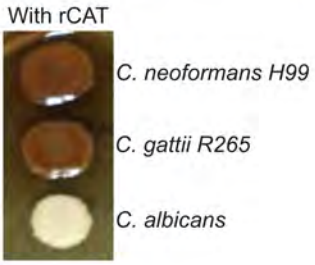
Fig 3 Catalase cell wall has activity to degrade hydrogen peroxide. The catalase activity of whole cell was evaluated by degradation of hydrogen peroxide spectrophotometrically at 240 nm every 20 seconds for 3 min. (A) The activity was evaluated in cells cultured for 24, 48, 72 and 96 h. Experiments were repeated three times. (B) Growth curve was evaluated to rule out the release of catalases from dead cells.

A)

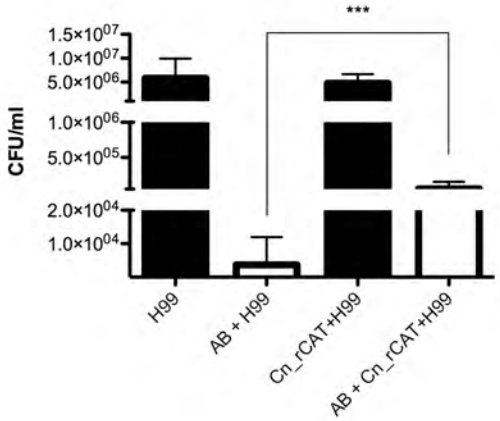
Microrganisms	Zone diameter (mm)		
	Candida albicans	*Candida parapsilosis*	*Cryptococcus neoformans*
Saline	0	0	0
CAT	0	0	0
AB + CAT	18,4 ± 0,54	19,83 ± 1,47	24,2 ± 1,09
AB	18,6 ± 2,19	19 ± 3,57	22,8 ± 4,54
Fluco + CAT	0	36,5 ± 1,87	28,6 ± 4,03
Fluco	0	31,5 ± 9,71	28,8 ± 6,38

AB: Amphotericin B (100 µg/disk); Fluco: fluconazol (25 µg/disk); HSP70 (10 µg/disk)

B)



C)



D)

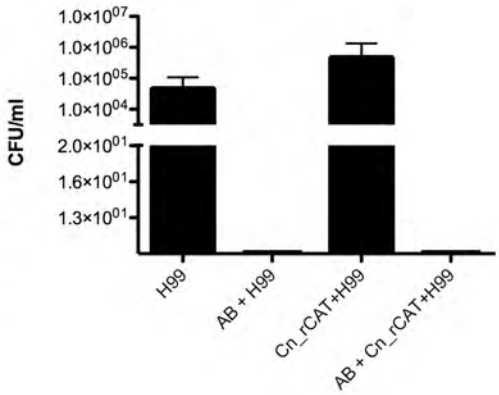


Fig 4 Catalase does not have influence with fluconazole and amphotericin B activity in vitro and in vivo and does not interferes in melanin production. A) Inhibition zone of fluconazole (Fluco) and amphotericin B (AB) disks in association with 10 µg/ml of Cn_rCAT2 (CAT) was measured to *C. albicans*, C. *parapsilosis* and *C. neoformans.* B) *C. neoformans* and *C. gattii* incubated

with Cn_rCAT2 on niger agar produced melanin. *C. albicans* was used as control of a yeast that produce pigment. C) Fungal burden in the mice lungs were evaluated after infection, treatment with Cn_rCAT2 and amphotericin B. Asterisks denotes P <0.05. D) CFU burden in the mice brain were evaluated after infection, treatment with Cn_rCAT and amphotericin B. No detectable cells were observed in the group that received amphotericin B. Asterisks denotes P <0.05. Bars are representative of 6 mice infected per group.

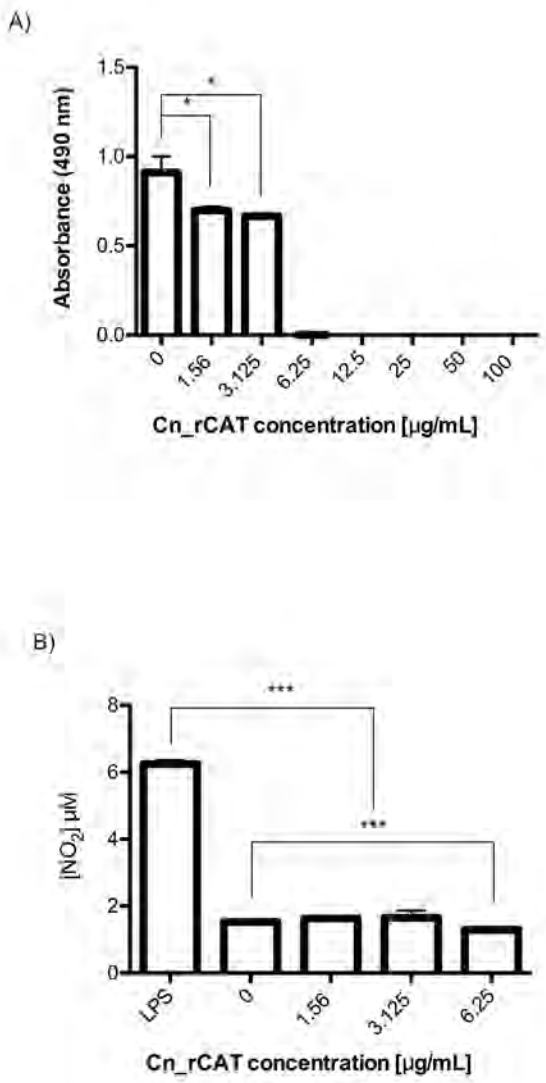


Fig 5 Effects on macrophage viability and NO production. A) J774.1 cells were incubated with 7 different concentrations of Cn_rCAT2 overnight, followed by tetrazolium (XTT) reduction assays. Cell viability was affected by all concentrations of Cn-rCAT2 used. Asterisks denotes P<0.05. B) J774.1 cells were incubated with 1.56; 3.125 and 6.25 µg/ml of Cn_rHCAT2. Negative controls consisted of cells that were not exposed to the recombinant protein. Positive control consisted of cells that were exposed to 500 ng/ml *E. coli* LPS. NO production in the supernatant was evaluated using Griess reagent. Experiment was performed only once. Asterisks denote p <0.05.

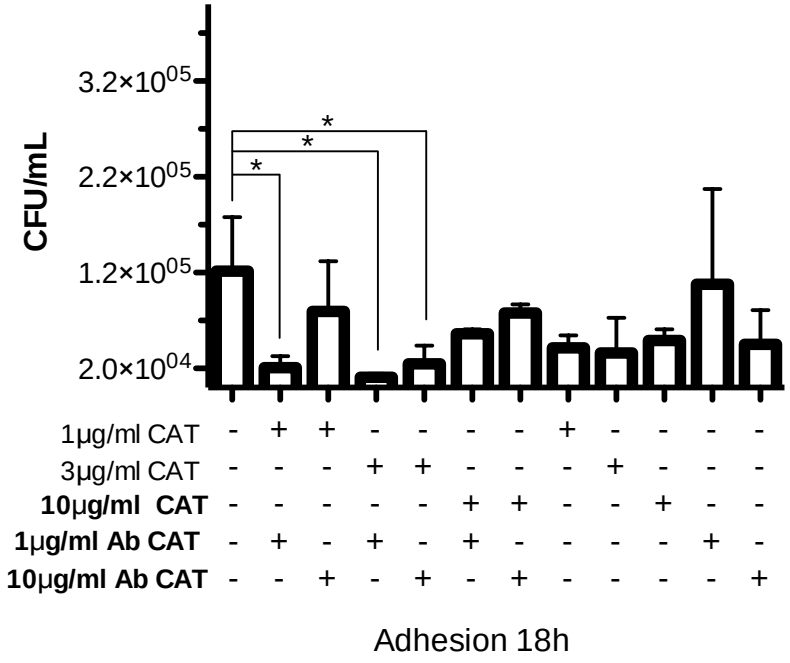


Fig 6 Catalase influences the adhesion of *C. neoformans* to A549 cells. Treatment of 10^4 fungal cells with anti-Cn_rCAT2 and of human cells with soluble Cn_rCAT2, caused a significant inhibition ($p < 0.05$) of the association between fungal and host cells. The inhibitory effect was not observed when the antibodyes against tubulin were used as control. Control systems consisted of A549 or *C. neoformans* cells that were not exposed to the antibody or to the Cn_rCAT2, respectively.

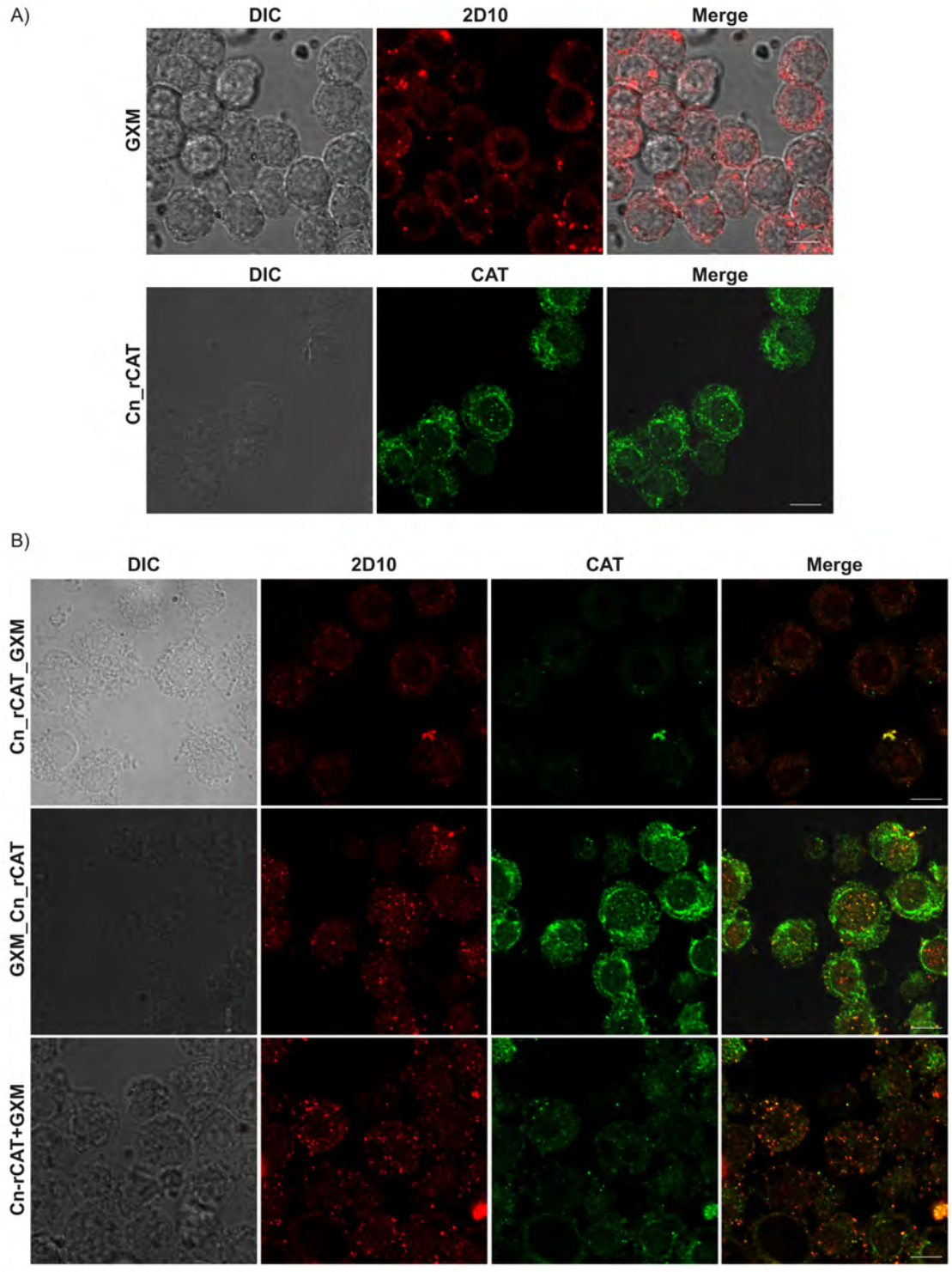


Fig 7 Cn_rCAT2 and GXM bind to different sites in J774.1 cells. A) 10µg/ml of Cn_rCAT2 (green fluorescence) was incubated for 60 min with J774.1 cells (Cn_rCAT2); 10µg/ml of GXM (red fluorescence) was incubated for 60 min with J774.1 (GXM); B) 10µg/ml of Cn_rCAT2 (green fluorescence) was incubated for 60 min for further addition of 10µg/ml of GXM (red fluorescence)

to the system (Cn_rCAT2_GXM); 10µg/ml of GXM (red fluorescence) was incubated for 60 min for further addition of 10µg/ml of Cn_rCAT2 (green fluorescence) to the system (GXM_Cn_rCAT2); simultaneous incubation of 10µg/ml of Cn_rCAT2 and 10µg/ml of GXM for 60 min with J774.1 cells (Cn_rCAT2+GXM**.** The scale bar corresponds to 10 µm.

Capítulo II

Artigo submetido para publicação no Periódico *Fungal Genetics and Biology*

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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[1] This author contributed with purification of His-tagged recombinant protein, anti-Hsp70 sera production.

[23] These authors contributed with purification of His-tagged recombinant protein, anti-Hsp70 sera production.

[4] This author contributed with assay of Cn_rHsp70 interaction with host cell.

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 on 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. However, the protein did not affect phagocytosis or fungal killing by mouse macrophages. 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.

Keywords

Hsp70; *Cryptococcus neoformans*; surface proteins; adhesion.

Highlights

► Hsp70 modulated the interaction of *C. neoformans* with A549 cells

► Hsp70 did not affect phagocytosis or fungal killing by mouse macrophages

► Hsp70 colocalized with GXM at the plasma membrane in mouse macrophages after exposure to both antigens

Abbreviations

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

1. Introduction

C. neoformans is an encapsulated yeast-like 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). Macrophages play multiple roles during cryptococcosis and may include fungal killing, polysaccharide sequestration, cytokine production and antigen presentation (Garcìa-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 mechanisms of transcription, 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 (Muthoff *et al*., 2007). In its membrane-bound form, Hsp70 can activate macrophages (Asea *et al*., 2000) and may be involved in mechanisms of cell adhesion, molecular trafficking, receptor expression and macromolecule internalization (Vega *et al*., 2008).

In Fungi, Hsps have been identified as the dominant antigens in a variety of models of systemic infections, including candidiasis (Eroles *et al.,* 1997), aspergillosis (Burnie and Matthews, 1991; Gomes *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*., 1999). Furthermore, Hsps localize to the cell surface of a number of fungal pathogens (reviewed in Nimrichter *et al*., 2005). 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 5,000 x *g* 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 3h. 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-Hsp70 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 *C. neoformans* rHsp70 antigenicity

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 (PVDF, GE Healthcare) 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.

2.5. Surface detection 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* ($5x10^5$ 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-Hsp70 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 mouse was used as 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).

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 to 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 (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 or co-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. In the pre-incubation systems, Cn-rHSP70 (0 - 25 µg/ml) was added to cell monolayers for 18 h. The medium was then replaced with fresh medium containing *C. neoformans* at a ratio of 10 fungal cells per phagocyte. In the co-incubation systems, fungal suspensions at density of 10^5 cells/well were supplemented with Cn_rHsp70 at eight different concentrations (0 - 25 µg/ml). In both systems, 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). Fungal survival and phagocytosis indices were determined in duplicate and statistically analyzed using Student's *t* test. The phagocytic production of NO was measured using the Griess Reagent (Sigma-Aldrich) (Rivera *et al*., 2002). This assay was performed in duplicate

and statistically analyzed using an ANOVA test followed by a Tukey post-hoc test.

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 (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.

3. Results

3.1. Cn_rHsp70 is recognized by sera from cryptococosis patients

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 3h. 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 recognized soluble Cn_rHsp70. This reactivity was abolished when the

pre-immune serum was incubated with soluble Cn_rHsp70. 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. 1B).

3.2. Hsp70 is associated with the capsule

ELISA using intact *C. neoformans* cells demonstrated that Hsp70 is in the cell surface (Fig. 2A). Binding was not observed in control fungal cells using polyclonal antiserum against β-tubulin produced in mouse.

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

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 to 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 Hsp70 was evaluated during this interaction. The addition of Cn_rHsp70 had no effects on either the association of *C. neoformans* with macrophages or fungal killing by the host cells (Figs. 4A and 4B). However, the pre-incubation of J774 cells with 10 µg/ml of Cn_rHsp70 was sufficient to decrease the level of NO production (Fig. 4B).

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. 5). 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 when no antibody was used to coat *C. neoformans*, 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. **Hsp70 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 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 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 (Figs. 6C and 6D) 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.

4. Discussion

Previous studies on 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. The mechanism by which Hsp70 localizes to the cell surface is unclear, although it was recently demonstrated that hydrogen bonds may promote interactions between the surface components of *C. neoformans* (Ramos *et al*., 2012). The *C. neoformans HSP70* gene lacks secretory signal sequences, suggesting that it 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 *H. 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 Garcìa and Zaragoza, 2012). Cn_rHsp70 did not influence phagocytosis or fungal killing in our model. However, macrophage exposure to Cn_rHsp70 resulted in decreased levels of NO. 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.

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. 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 *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). 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 complex association of Hsp70 with molecules involved in the interaction between host cells and *C. neoformans* as well the impact of such an association on the infection process.

Acknowledgments

This work was supported by grants from FINEP (Financiadora de Estudos e Projetos, Brazil), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil) and Ludwig Biotecnologia. We thank Dr. Arturo Casadevall for providing the monoclonal antibody anti-GXM (18B7). Automated DNA sequencing was performed at the Brazilian Genome Network at the Center of Biotechnology, CBiot-UFRGS-RS. We also thank the Electron Microscopy Center of the Federal University of Rio Grande do Sul (CME, UFRGS) for the confocal microscopy analysis and Henrique Biehl for technical assistance.

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Figures and legends

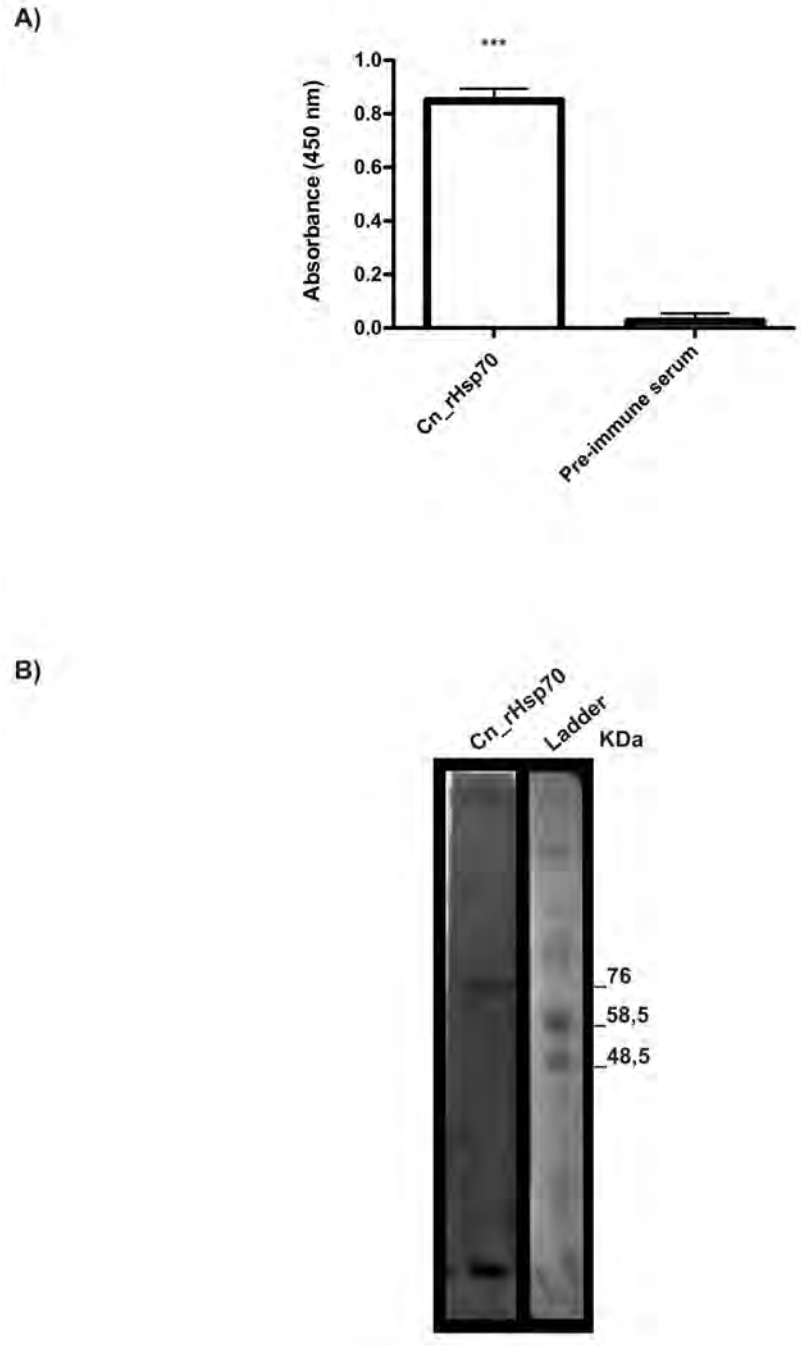

Fig. 1. Cn_rHsp70 is recognized by sera from immunized mice and patients with cryptococcosis. **A)** A polyclonal serum raised against *C. neoformans* Hsp70 interacts with Cn_rHsp70. The recombinant protein was used to coat 96-well polystyrene plates for ELISA. Positive reactions were observed when

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)** 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 on the right column.

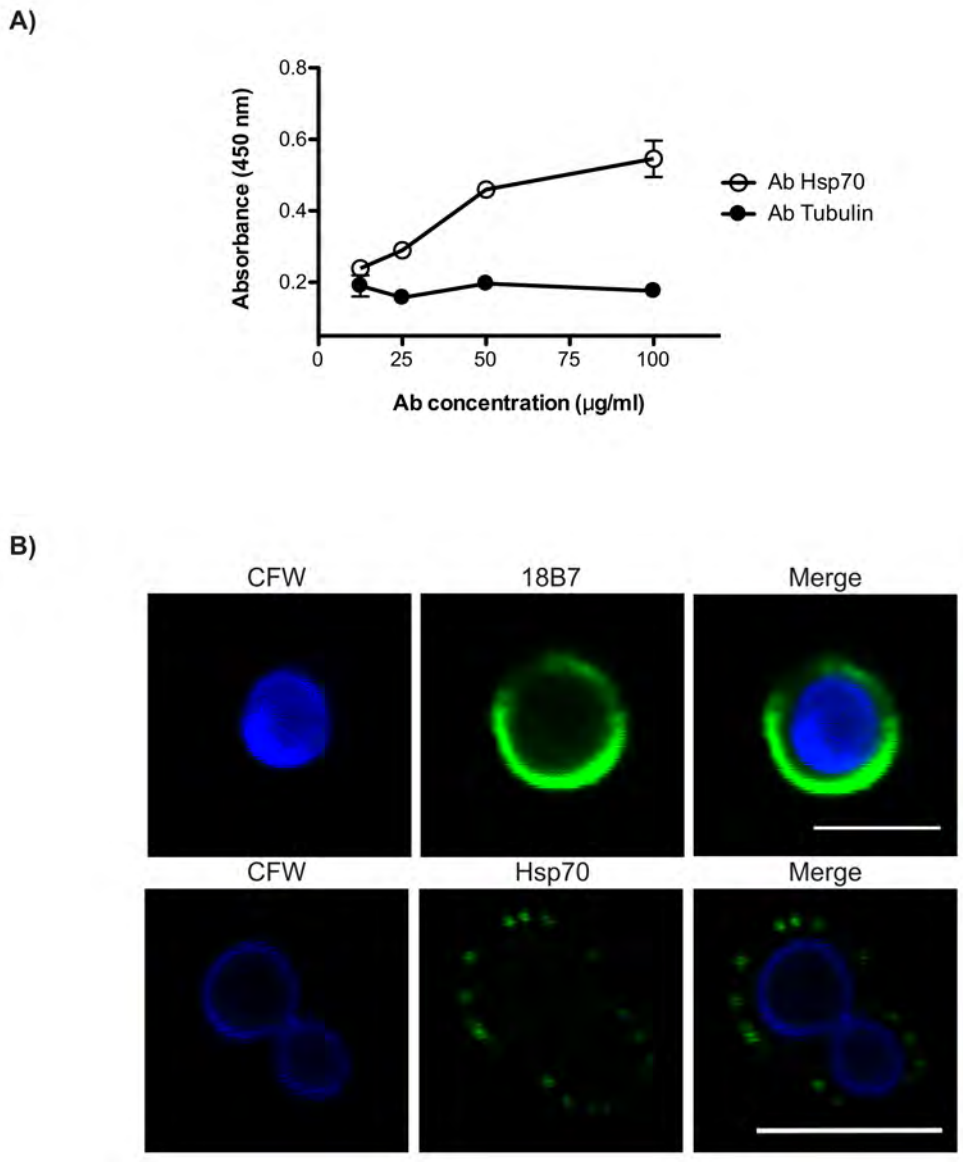

Fig. 2. Hsp70 is associated with the capsule. **A)** The anti-Hsp70 polyclonal serum detects Hsp70 of *C. neoformans.* ELISA using whole cells revealed efficient serum binding at concentrations greater than 25 µg/ml. An irrelevant antibody (β-tubulin) used as a control showed no binding to *C. neoformans* cells. 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 monoclonal antibody 18B7 (upper panel); Hsp70 appears in green (lower panel). The cell

wall was stained with calcofluor white (CFW- blue fluorescence). The scale bar corresponds to 5 μm.

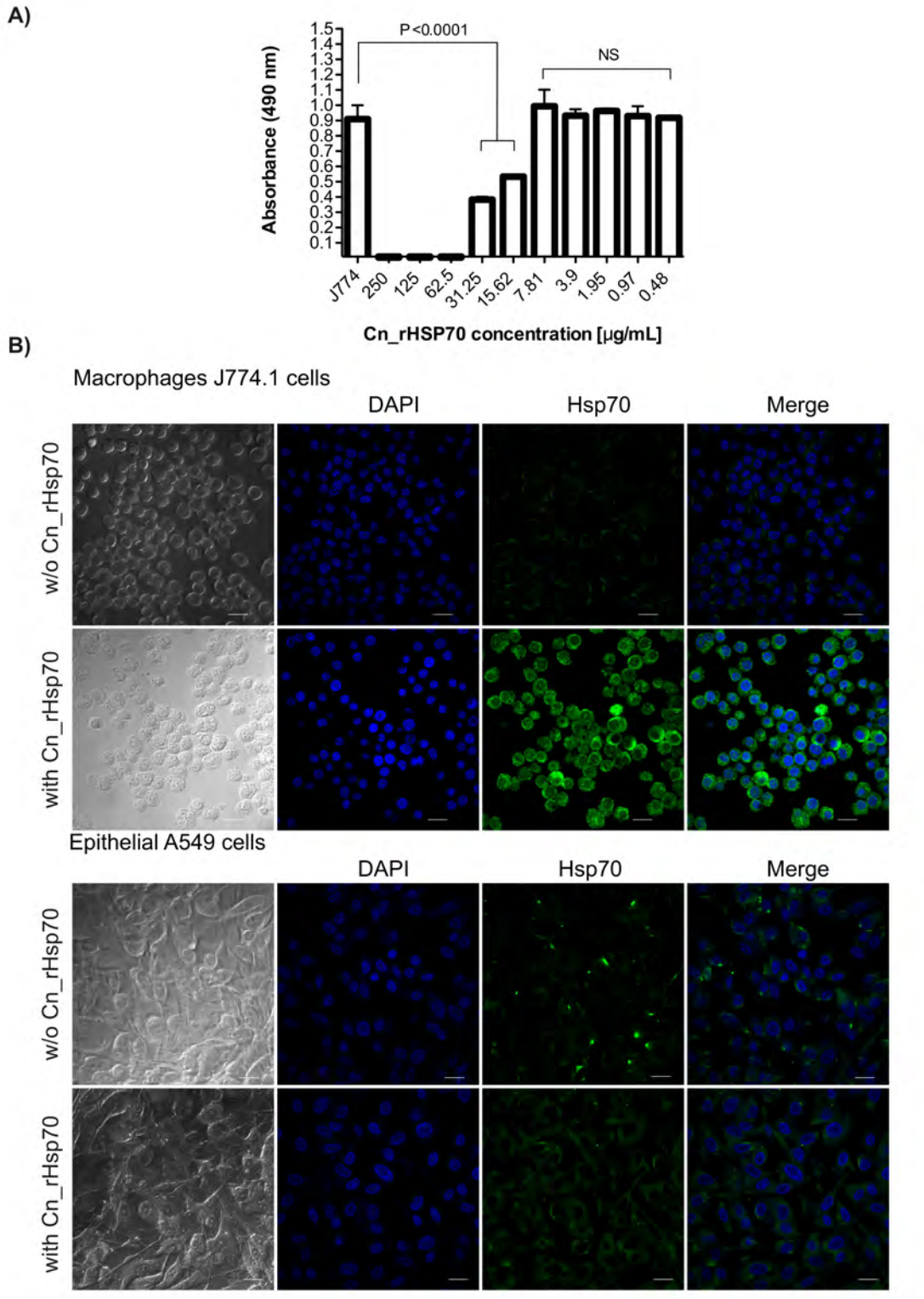

Fig. 3. Cn_rHsp70 binds to host cells**. A)** J774.1 cells were incubated with different concentrations of Cn_rHsp70 overnight followed by tetrazolium (XTT) reduction assays. Cell viability was affected by Cn_rHsp70 at concentrations

greater than 15.62 µg/ml. Asterisks denote *p* <0.05. **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). The scale bar corresponds to 7.3 µm.

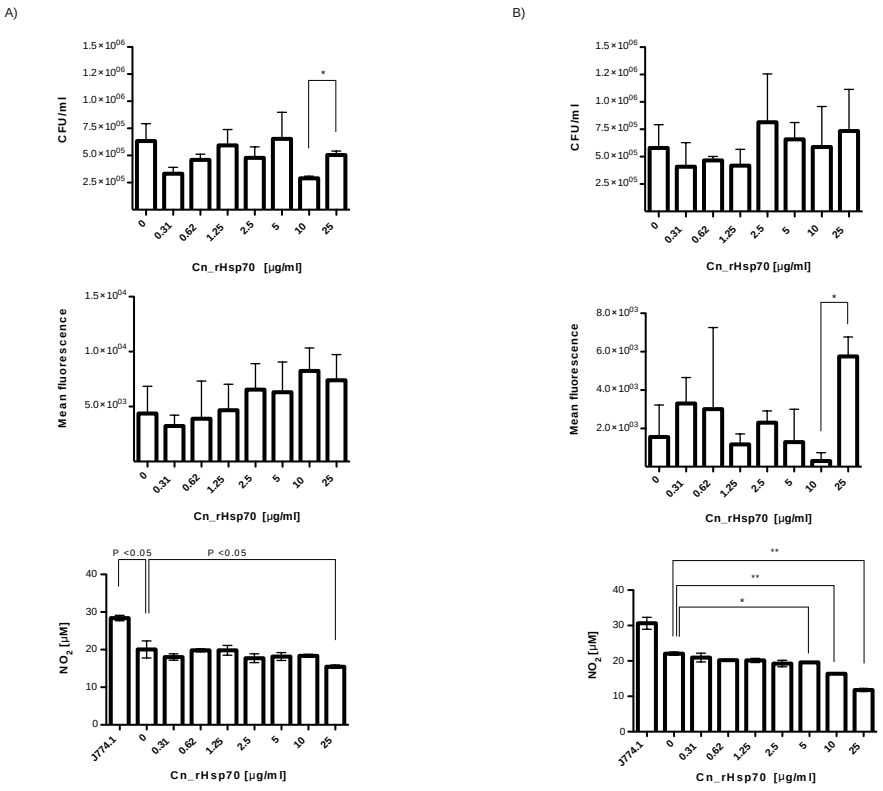

Fig. 4. Cn_rHsp70 has no influence on phagocytosis but induces NO reduction. **A)** The macrophages were incubated overnight with Cn_rHsp70 prior to interaction with *C. neoformans* cells**.** The fungicidal activity (upper panel), phagocytosis index (middle panel) and NO production (bottom panel) are shown. **B)** Macrophage exposure to Cn_rHsp70 during the interaction with *C. neoformans*. The fungicidal activity (upper panel), phagocytosis index (middle panel) and NO production (bottom panel) are shown. The results are representative of two independent experiments. Asterisks denote p <0.05.

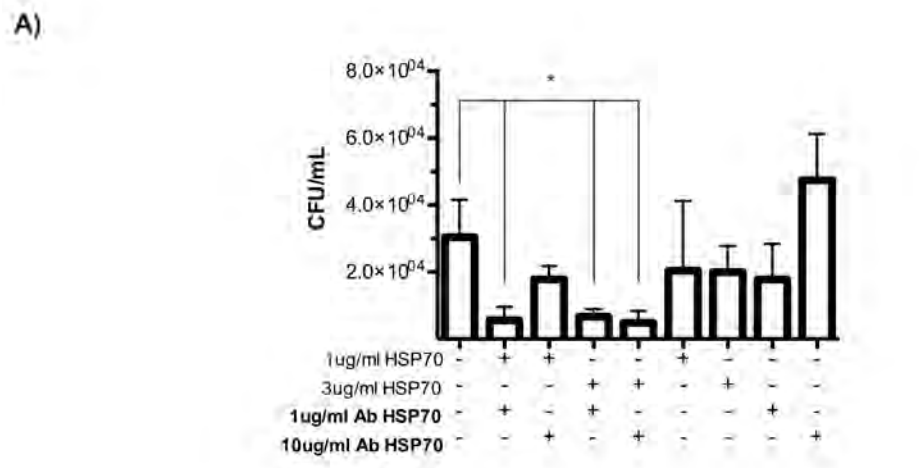

Fig. 5. Hsp70 modulates the adhesion of *C. neoformans* to A549 cells. The treatment of fungal cells with anti-Cn_rHsp70 and of human cells with soluble Cn_rHsp70 caused a significant inhibition (p <0.05) of the association between fungal and host cells. The inhibitory effect was not observed when an 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.

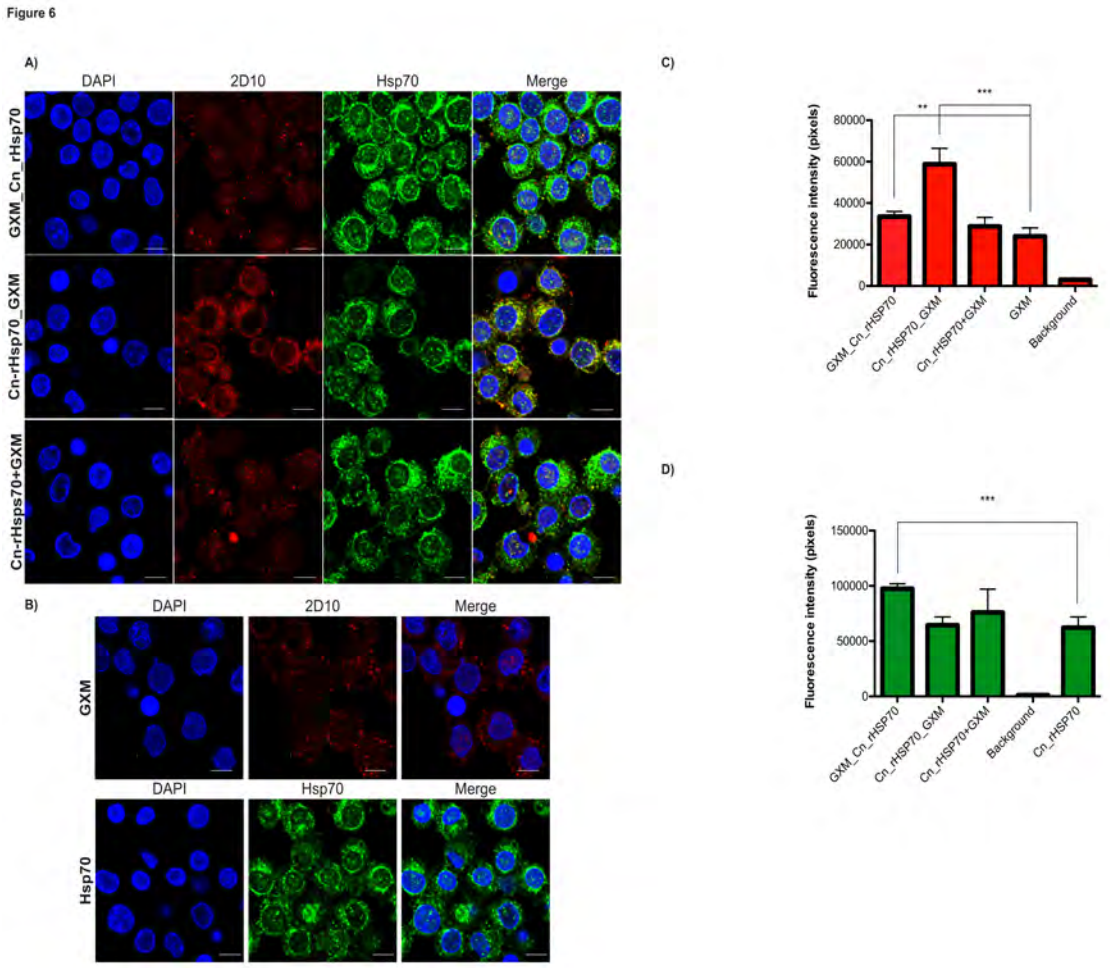

Fig. 6. Cn_rHsp70 and GXM colocalized in J774.1 cells. **A)** GXM (2D10, red fluorescence) was incubated for 60 min prior to the addition of Cn_rHSP70 (anti-Cn_rHsp70, green fluorescence) to the system (GXM_ Cn_rHsp70; upper panel). Cn_rHsp70 (anti-Cn_rHsp70, green fluorescence) was incubated for 60 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 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.

Capítulo III

Resultados Adicionais referentes ao Capítulo II não incorporados no manuscrito submetido.

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[1] This author contributed with purification of His-tagged recombinant protein, anti-Hsp70 sera production, assays involving Hsp70 effects on polysaccharide release, capsule formation and fungal viability.

[2] This author contributed with assays involving Hsp70 effects on polysaccharide release, capsule formation and fungal viability and the analysis of host receptor expression after Hsp70 exposure,

[3] This author contributed with assays involving Hsp70 effects on polysaccharide release, capsule formation and fungal viability.

[4] This author contributed with adhesion assays.

Supplemental Data

The results described below addresses additional experiments performed about the interaction of Hsp70 with host cells as well as the mechanism that involve GXM and Hsp70 binding to the receptor. The supplemental material is an attempt to understand the biological function of Hsp70 in *C. neoformans* cells and it relashioship with host cell.

Material and Methods

1.1 Hsp70 identification

To confirm identity of recombinant, Cn_rHsp70 protein separated by SDS-PAGE, excised from gel, alkylated and digested with trypsin, followed by extraction of peptides (Shevchenko *et al*., 1996). Peptides were identified by MALDI-TOF at the Unidade de Química de Proteínas e Espectrometria de Massas (Uniprote-MS), Cbiot, UFRGS.

1.2 Influence of Hsp70 with antifungals, melanization process and in vivo protection in mice infection

To test the synergistic effect of Cn_rHsp70 with antifungals, a disk diffusion methodology was performed following the CLSI M44-A disk diffusion method guidelines (CLSI, 2004). Briefly, the entire surface of Mueller-Hinton agar supplemented with 2% dextrose agar plates (150-mm plates) were inoculated simultaneously in three directions by using a swab dipped in the yeast inoculum suspensions of OD_{530} with range between 0.11 to 0.14. The inoculated agar was allowed to dry for 15 to 30 min, and the disks (fluconazole: 25 µg/disk, amphotericin B: 100 µg/disk; Cn_rHsp70: 10 µg/disk) were applied to the inoculated agar. The plates were incubated at 30°C for 48 h and the inhibition zone diameter was determined. All experiments were performed in triplicate and statistically analyzed using Student's *t* tests.

To verify if Cn_rHsp70 inhibit melanin production, cells were spotted on niger seed agar plates supplemented with 10 µg/ml of Cn_rHsp70. The plates

were incubated at 30°C and evaluated for 2 days.

The ability of Cn_rHsp70 to reduce lung and brain CFU burden was evaluated. Twenty micrograms of Cn_rHsp70 were administrated intraperitoneally in BALB/c mice (female, 4–6 weeks old) at day 1. Mice were infected intranasally with $2x10^5$ *C. neoformans* cells and immunized with 20 µg of Cn_rHsp70 at day 8. 24h post-infection, mice received 20 µg of Cn_rHsp70 and 1.5 mg/Kg amphotericin B by intraperitoneal administration. Mice received amphotericin B for 7 days. Two weeks after infection, mice were sacrificed and CFU burden determined. Data are representative of one experiment with 5 mice per group.

1.3 Hsp70 localization

The Hsp70 cytoplasmic localization was performed as described by Zámocky *et al*. 2013. Briefly, *C. neoformans* H99 cells were grown in YNB at 30 ^{o}C for 48h, washed with PBS and fixed in 4% paraformaldehyde. Cells were washed 5 times with PEM buffer (50 mM PIPES, 25 mM EDTA, 5 mM $MgSO_4$), cell wall was digested with digestion buffer [50 mg/ml Lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich, St. Louis, Missouri, USA), 5 mM EDTA, pH 7] at 37^{o}C for 5 min. Cells were washed with PEM buffer, immersed in cold methanol for 10 min and washed twice with PEM buffer. Then, cells were blocked with 1% BSA (Sigma-Aldrich) and incubated with the anti-Cn_rHsp70 polyclonal serum (1:50, v/v) for 1 h at 37^{o}C. After washing with PBS, the cells were incubated with Alexafluor 488-conjugated IgG (Invitrogen) for 1 h at 37 °C. The cells were again washed and mounted onto glass slides using a 50% glycerol/50% PBS/0.1 M *N*-propyl gallate solution. Cells were analyzed using Axioskop 40 (Zeiss) immunofluorescence microscope. Nucleus was stained with 0.25 µg/ml of 4',6-diamidino-2-phenylindole (DAPI).

The Snf7 and acapsular mutant of C. *neoformans* was analyzed by the immunofluorescence assay. Fungal cells from *Snf7* and CAP67 were grown in YNB at 30 ^{o}C for 48h, washed with PBS and fixed in 4% paraformaldehyde. Cells were blocked with 1% BSA (Sigma-Aldrich) and incubated with the anti-Cn_rHsp70 polyclonal serum (1:50, v/v) for 1 h at 37^{o}C. After washing with PBS, the cells were incubated with Alexafluor 488-conjugated IgG (Invitrogen

Corp., Carlsbad, California, USA) for 1 h at 37°C. The cells were again washed and mounted onto glass slides using a 50% glycerol/50% PBS/0.1 M *N*-propyl gallate solution. Stained cells were finally analyzed with an Olympus FluoView™ 1000 fluorescence microscope (Olympus Optical Co., Melville, New York, USA).

1.5 Effects of Hsp70 on polysaccharide extracellular release, capsule formation and fungal viability.

C. neoformans (10^6 cells / ml) were grown in DMEM to induce capsule formation for 48 h at 37°C (7.5% CO_2 atmosphere). The medium was supplemented with Hsp70 at concentrations varying from 5 to 250 µg/ml. Fungal cells were harvested by centrifugation, fixed in 4% glutaraldehyde for 1 h at room temperature and evaluated for capsule formation by counter-stained with India ink and immunofluorescence as previously described (Fonseca *et al*., 2009). Alternatively, the cells were plated onto YPD agar for CFU determination. GXM in the supernatants were determined by ELISA as described before (Casadevall *et al*. 1992).

1.6 Cytokines determination in host cells after rHsp70 exposure

J774.1 monolayers were washed twice in serum-free DMEM, placed in medium supplemented with various concentrations of Cn_rHsp70 (7.5, 15 and 30 µg/ml), and incubated for 18 h at 37°C (7.5% CO_2 atmosphere). As a positive control, macrophages were stimulated with 1 µg/ml lipopolysaccharide (LPS).

Total mRNA was isolated by Trizol (Invitrogen) for determination of relative transcript levels of genes that codes for IL-12, ARG1 and IL-5 cytokines. RNA was treated with DNAse (Promega) and was reverse-transcribed using M-MLV reverse transcriptase (Promega) using oligo-dT. qRT-PCR 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 ANOVA test. The specific primers are described in Table 1.

1.7 Co-localization of Cn_rHsp70 and GXM to J774 macrophage cells and A549 ephitelial cells

Cells were maintained as described above. A549 and J774.2 cells (10^6 cells/well- CellView Greiner Bio One) were double immunofluorescence stained with Cn_rHsp70 and GXM (10 µg/ml). Six systems were analysed: A) Cn_rHsp70 was incubated for 15 min, then GXM was added for 15 min; B) Cn_rHsp70 and GXM were co-incubated for 15 min; C) Cn_rHsp70 was incubated for 60 min, then GXM was added for 60 min; D) Cn_rHsp70 and GXM were incubated at the same time for 60 min. After washing, cells were fixed with 4% paraformaldehyde and blocked for 1 h (37°C) in PBS-1%BSA. Cells were washed 3 times with PBS and incubated with anti-HSP70 polyclonal serum or 2D10 mAb, a king gift of Arturo Casadevall (Albert Einstein College of Medicine of Yeshiva University, New York) for 1 h at 37°C following by incubation with Alexafluor 488 labeled anti-mouse IgG (Invitrogen) or 568 conjugated IgM anti-mouse. After washing, the second immunofluorescence 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.

1.8 Host receptor expression after rHSP70 exposure

J774.1 monolayers were washed twice in serum-free DMEM, placed in medium incubated with Cn_rHsp70, GXM, Cn_rHsp70_GXM, GXM_Cn_rHsp70 and incubated for 18 h at 37°C (7.5% CO_2 atmosphere). Real time of TLR2, TLR4 and CD40 expression was performed as described in item 1.6.

1.9 Binding of Cn_rHsp70 and GXM via TLR4

To verify the distribution of Cn_rHsp70 and GXM in mammalian cells, cells were maintained as described above. J774.1 cells (10^6 cells/well-CellView Greiner Bio One) were double immunofluorescence stained with Cn_rHsp70 (10 µg/ml) and GXM (10 µg/ml). To verify the if TLR4 was involved in HSP70 and GXM co-localization in J774.1 cells, TLR4 was blocked with 200 ng/ml of LPS before incubation with Cn_rHsp70 or GXM and double immunofluorescence was proceed as described above. Control systems did not receive LPS. Three systems were analyzed: A) Cn_rHsp70 was incubated for 60 min, then GXM was added and the incubation proceeded for more 60 min (Cn_rHsp70 _GXM); B) GXM was incubated for 60 min, then Cn_rHsp70 was added and the incubation proceeded for more 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 (37°C) in PBS-1%BSA. Cells were washed 3 times with PBS and incubated with anti- Cn_rHsp70 polyclonal serum or 2D10 mAb (a cortesy from Arturo Casadevall, Albert Einstein College of Medicine of Yeshiva University, New York) for 1 h at 37°C following by incubation with Alexafluor 488- labeled anti-mouse IgG (Invitrogen Corp.) or 568 -conjugated IgM anti-mouse. After washing, the second immunofluorescence was performed. Cells were incubated with 2D10 mAb or anti-HSP70 polyclonal serum, and then incubated with 568 conjugated IgM anti-mouse or Alexafluor 488 labeled anti-mouse IgG (Invitrogen) respectively. Stained cells were finally analyzed with an Olympus FluoView™ 1000 fluorescence microscope (Olympus Optical Co., Melville, New York, USA).

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

2. Results and Discussion

2.1 Cn_rHsp70 is recognized by serum antibodies

Expression vector (pET23d) containing Hsp70 sequence was confirmed by restricted digestion (Fig.1A). Plasmid was linearized with NcoI and XhoI enzymes and the Hsp70 sequence was released with double digestion using NcoI and XhoI. *E. coli* pLysS was transformed with resulting plasmid and induced with 1 mM IPTG for 3 h (Fig. 1B). Even at 1h induction, recombinant protein expression could be detected. Cn_rHsp70 was purified using affinity chromatography and eluted using increasing concentrations (50, 100, 150, 250 and 500 mM) of imidazole (Fig. 1C). The identity of the Cn_rHsp70 peptides was confirmed by MALDI-Tof spectrometry (Fig. 1D).

2.2 Hsp70 does not enhance antifungal effect in vitro and in vivo and interferes in melanin production

The antifungal activity of fluconazole and amphotericin B in association with Cn_rHsp70 was not increased in vitro against *C. albicans*, C. *parapsilosis* and *C. neoformans.* Inhibition zone does not have a significatively difference between disk diffusion agar containing fluconazole (25 µg/ml), amphotericin B (100 µg/ml) or co-incubated with 10 µg/ml of recombinant protein (Fig. 2A), demonstrating that Cn_rHsp70 does not have antifungal activity, as the incubation of only with the recombinant protein does not led to any inhibition zone.

Recombinant protein (10 µg/ml) incubated with agar niger did not interfered in the melanization (Fig. 2B) by *C. neoformans and C. gattii*. *C. albicans* was used as a control of melanization.

As Cn_rHsp70 does not possess an intrinsic antifungal activity, we speculated that it could be effective in the stimulation of immune response in mice infection. BALB/c mice were immunized with 20 µg/ml of Cn_rHsp70 and infected by intranasal inhalation with *C. neoformans* cells. Then, mice received 2 doses of protein and were treated with 1.5 mg/kg of amphotericin B, every day. Mice were sacrificed and CFU burden in the lungs (Fig. 2C) and brain (Fig. 2D) were determined. As demonstrated in Fig. 2C, mice that received Cn_rHsp70 treatment had no significatively decreased in CFU burden compared to the non-treated group control (control – H99). However,

mice treated with amphotericin B in association with Cn_rHsp70 had an increased in CFU burden compared to the group that were treated with amphotericin B alone (p <0.05). Relative to the brain burden, when mice were treated with amphotericin B, no fungal cell was detected. No difference was observed when mice were treated with Cn_rHsp70 compared to the group non-treated.

Our results demonstrated that recombinant Hsp70 protein does not have antifungal activity and can influence fluconazole and amphotericin B activity, since became ineffective the cryptococcosis treatment.

Hoshino *et al*. (2010) suggested that Hsp70 supress a melanin synthesis induced by UVB in melanocytes cells. So, the relationship of Hsp70 and melanin, lead us to evaluate if Hsp70 interferes in *C. neoformans* pigmentation. We observed that Hsp70 not interferes in melanization.

In *C. albicans* (Bromuro *et al.,* 1998), *H. capsulatum* (Allendoerfer *et al*., 1996) infections, Hsp70 are immunogenic, but not protective in murine models. These data lead us to investigated if Hsp70 from *C. neoformans* protect mice against criptococcosis, but the treatment used here not confered mice protection against *C. neoformans.*

2.3 Hsp70 is located on the capsular network besides cytoplasmic localization

The cytoplasmic localization was performed using protoplast cell (Fig. 3A). In addition, the cellular distribution of Hsp70 in Snf7 and acapsular mutant CAP67 were analyzed by confocal microscopy using the polyclonal serum raised to Cn_rHsp70 (Fig. 3B). Hsp70 was mainly distributed in the capsular network besides cytoplasmic localization.

As showed in Fig. 3B (upper panel), Hsp70 was found on the surface cell of *snf7* mutant. Snf7 protein is involved in many biological process, such as endocytosis, multivesicular body formation, unconventional secretion and has influence in *C. gattii* and *C. neoformans* virulence (Crestani *et al*., 2013, unpublished data). The presence of Hsp70 in extracellulary vesicles is contraditory (Rodrigues *et al*., 2008), due to the absence of signal sequence at the Hsp70 protein and the localization of Hsp70 in the Snf7 mutant. It has been reported that Hsp70 could be released to extracellular media by lysis of the cell, by vesicles or through an endolysosomes (Mambula *et al*., 2007). In

fact, the mechanism which Hsp70 from *C. neoformans* is secreted remained obscure and more studies are necessary to elucidate the process.

In conclusion, the presence of this protein on the surface could be the first contact with the host cell, being response to the recognition, adhesion, attachment or immune response stimulation to clearance the infection. We hypothetized that the surface conserved protein could be located outside the cell to fool the immune system allowing the progress of the infection.

2.4 Hsp70 affects fungal viability at high concentrations and extracellular release of GXM but not influence in capsule size

Capsule size was increased in *Ssa1* mutant from *C. neoformans*, a homolog of *HSP70* gene (Zhang *et al*., 2006). It is a strong evidence that Hsp70 and capsule are related or can interact. Besides, based on the fact that Hsp70 and GXM bind at the same sites on the host cell and the interaction of *C. neoformans* with macrophages mediates by capsule and other molecules are relevant to fungal virulence, led us to investigate if Cn_Hsp70 has effect on capsule formation and polysaccharide release to the extracellular space.

The co-incubation of Cn_rHsp70 and *C. neoformans* cells caused a altered morphology manifested with small cells clear since 50 µg/ml of protein (Fig. 4A). We also observed that the co-incubation caused a decreased in extracellular GXM release (Fig. 4B). CFU determination after treatment of fungal cells with Cn_rHsp70, revealed that recombinant protein had effect on cryptococcal viability (Fig. 4D). However, Cn_rHsp70 treated cells had no effect in capsular dimensions (Fig. 4C).

2.5 Co-localization of purified HSP70 and GXM to J774 macrophage cells and A549 ephitelial cells

C. neoformans binds to A549 cells (Merkel *et al.*, 1997; Barbosa *et al*., 2006) and this interaction can be mediated by GXM through CD14 receptor. These association lead us to investigate if Cn_rHsp70 bind to the host cell as well as co-localize with GXM. A549, a human type II alveolar epithelial cell-line were maintained and grown as described to J774.1 cells. A549 were double immunofluorescence stained with rHSP70 and GXM (10 µg/ml). Six

systems were analysed: A) Cn_rHsp70 was incubated for 15 min, then GXM was added for 15 min (Fig. 5A, upper panel); Cn_rHsp70 and GXM were co-incubated for 15 min (Fig. 5B, upper panel); C) Cn_rHsp70 was incubated for 60 min, then GXM was added for 60 min (Fig. 5A, bottom panel); Cn_rHsp70 and GXM were incubated at the same time for 60 min (Fig. 5B, bottom panel). As observed in Fig. 5A, when Cn_rHsp70 was incubated with A549 cells followed by GXM, a co-localization occurred. Also, we observed that, in 15 min, there is a litlle association of Cn_rHsp70 and GXM. However, in 60 min, this association increased. When both molecules were co-incubated, co-localization was not observed as well as when Cn_rHsp70 was incubated first (Fig. 5B).

2.6 rHSP70 stimulates IL-12 and IL-5 production by macrophages

Cytokines production was evaluated by qRT-PCR after incubation of the macrophages with Cn_rHsp70 (0-30 µg/ml). IL-12 was chosen in this assay due its ability to promote protection of mice against *C. neoformans* infection through induction fungicidal activity (Kawakami *et al*., 1999). IL-5 was selected for cytokine tests since it regulates eosinophil differentiation and the recruitment of macrophages into the lungs during *C. neoformans* infection (Huffnagle *et al*., 1998). In our model, IL-12 mRNA levels were significantly higher in macrophages stimulated by either LPS or Cn_rHsp70 in comparison with control systems ($P < 0.0001$) (Figure 6A). Interestingly, a similar profile was observed for IL-5 mRNA levels (Figure 6B). The mRNA levels for the cytokines IL-4, IL-6, IL-13 were also measured, but Cn_rHsp70 induced no alterations in the expression of these immunomodulatory molecules.

The increased production of IL-12 was previously associated with antifungal activity, protection and recruitment of macrophages against *C. neoformans* infection (Decken *et al*., 1998; Huffnagle *et al.*, 1998). On the other hand, IL-5 production is associated with eosinophil differentiation, that generated a nonprotective immune response causing lung pathology (Kawakami *et al*., 1999).

Thus, we suggested that the cytokines profile produced by macrophages incubated with Cn_rHsp70 can shift the balance on Th1 and Th2 immune response, and we believe that it also could happen in vivo.

2.7 Cn_rHsp70 and GXM co-localization on the surface of host cells is mediated by TLR4

We hypothesized that GXM and Hsp70 could also share the same cellular binding sites in macrophages. To evaluated which receptor could be involved in Hsp70/GXM recognition, the TLR-2, TLR-4 and CD40 expression was measured by qRT-PCR. A decreased was observed in TLR-2 expression when Hsp70 was pre-incubated with macrophages followed by GXM (Fig. 7A). When only Hsp70 was incubated with macrophages there was an increase in TLR-4 expression (Fig. 7B). When both molecules are simultaneously incubated, there was an increased in CD40 expression (Fig. 7C). When both antigens are simultaneously incubated with macrophages, the molecules are detected at the plasma membrane level, but co-localization does not occur (Fig. 8I, upper panel). Exposure of the mammalian cells to GXM followed by Cn_rHsp70 results in similar patterns of antigen binding to host cells (Fig. 8G, upper panel). When the cells are given Cn_rHsp70 followed by GXM, both antigens clearly co-localize (Fig. 8E, upper panel). The co-localization decreased when LPS was added, compared to the control (Fig. 8E, lower panel). However, when cells were incubated with Cn_rHsp70 only (Fig. 8C), there was a decrease of the binding, suggesting that *C. neoformans* Hsp70 may bind to macrophages via TLR4, since LPS is an antagonist of TLR4-dependent signaling (Schofflen *et al*., 2013). The fluorescence levels were quantified by ImageJ software as demonstrated in Fig. 8B, D, F, H, J. Using the Pearson correlation coefficient, positive values indicate that the distribution of the fluorophores coincide with 1.0 perfect match and zero representing that the fluorophores are randomly distributed. The Pearson's correlation coefficient found here was 0.84 without LPS indicating a high degree of co-localization.

A speculative mechanism of Hsp70 and GXM was elaborated to explain the interaction of Hsp70 associated with GXM. We believe that extracellular Hsp70 binds to the specific receptor, for instance, TLR4 receptor (Fig. 9; upper panel) and a structural change or receptor expression pattern can be change; GXM close contact with TLR4 and binds in it (middle panel), the recognition occurred and the yeast are internalize (lower panel)

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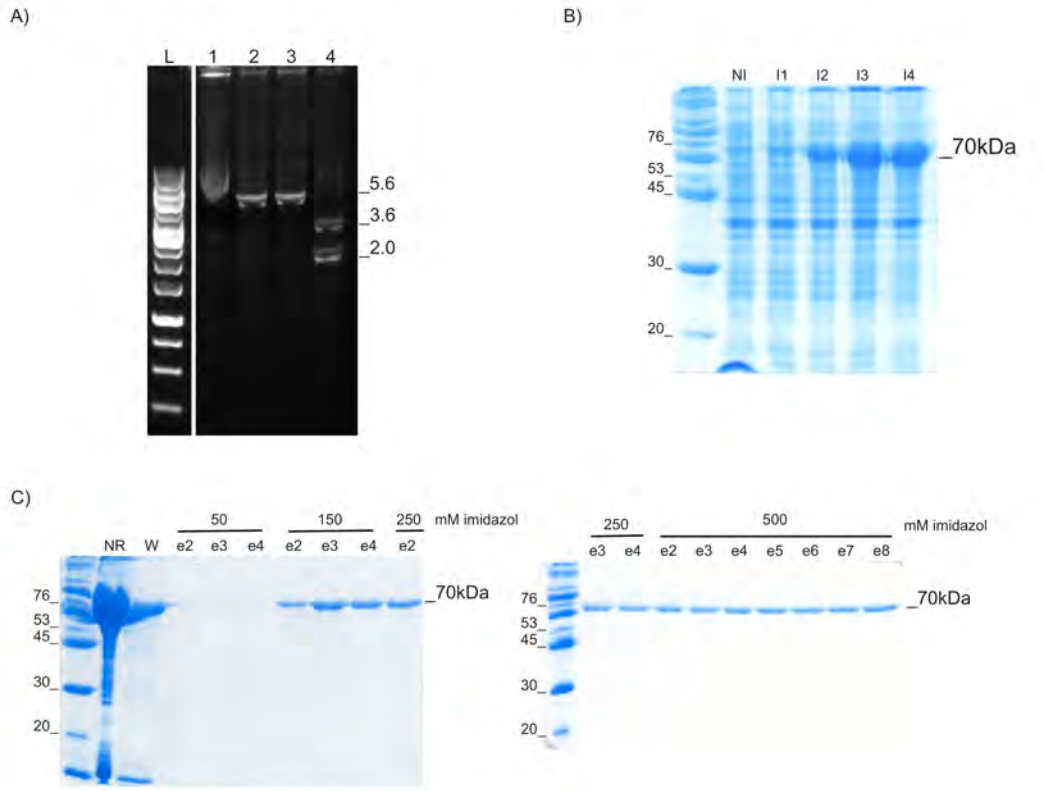
Figures and legends



D)

Protein name	Accession Number NCBI	Theoretical pI/MW (kDa)	Identification score	Sequence Coverage (%)
Heat shock protein	Q5K8W5	5.52/71900	202	19

Fig. 1: Cn_rHsp70 from *C. neoformans* expression and purification. **A)** Hsp70 sequence was cloned into pET23d vector and the restriction enzymes were used to confirm plasmid construct. L: 1kb ladder; 1: pET23dHsp70; 2: pET23dHsp70 linearized with NcoI; 3: pET23dHsp70 linearized with XhoI; 4: double digestion of pET23dHsp70 with NcoI and XhoI releasing a catalase sequence (1.5kb) and pET23d (3.6 kb). **B)** *E.coli* BL21(DE3)pLysS was transformed with pET23dHsp70, non-induced (NI) and induced with 0.1 M

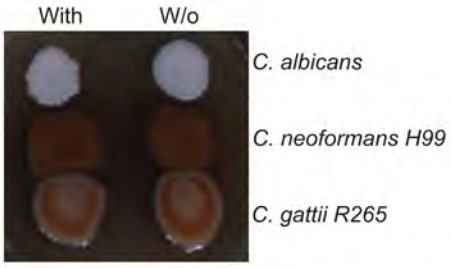
IPTG for 1 h(I1), 2 hrs (I2), 3 hrs (I3) and 4 hors (I4). A 70kDa band corresponding to catalase protein appeared starting 1 hour of induction. **C)** Recombinant protein was purified using increasing concentration of imidazole. NR: not retained; W: wash; e1, e2, e3 50: eluted 1, 2 and 3 with 50 mM imidazole respectively. e1, e2, e3 150: eluted 1, 2 and 3 with 150 mM imidazole respectively. e1, e2, e3 250: eluted 1, 2 and 3 with 50 mM imidazole respectively. e1, e2, e3, e4 and e5 500: eluted 1, 2 and 3 with 500 mM imidazole respectively. **D)** Hsp70 peptides were identified by MALDI-TOF and confirmed the identity of recombinant protein from *C. neoformans.*

A)

Microrganisms	Zone diameter (mm)		
	Candida albicans	*Candida parapsilosis*	*Cryptococcus neoformans*
Saline	0	0	0
HSP70	0	0	0
AB+HSP70	15,6 ± 2,07	21,2 ± 1,48	25,4 ± 1,67
Anfo	19,6 ± 2,7	22,6 ± 0,89	26,4 ± 2,70
Fluco + HSP70	0	38,6 ± 1,94	31 ± 7,10
Fluco	0	39,6 ± 1,14	28,4 ± 7,23

AB: Amphotericin B (100 µg/disk); Fluco: fluconazol (25 µg/disk); HSP70 (10 µg/disk)

B)



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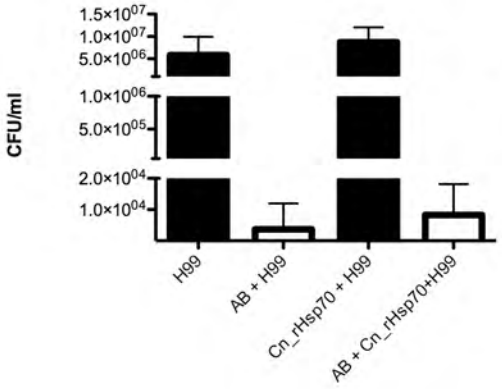

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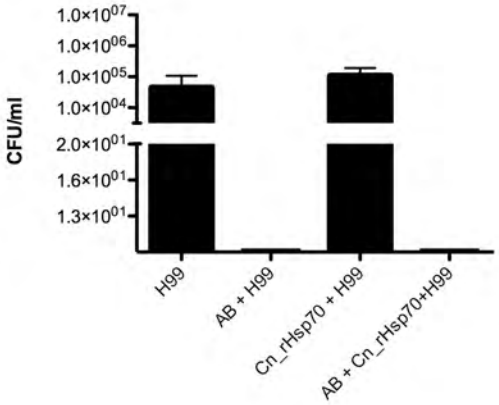


Fig. 2: Cn_rHsp70 does not have synergistic effect with fluconazole and amphotericin B in vitro and in vivo and does not interferes in melanin production. **A)** Inhibition zone of fluconazole and amphotericin B disks in association with 10 µg/ml of Cn_rHsp70 was measured to *C. albicans*, C. *parapsilosis* and *C. neoformans.* **B)** *C. neoformans* and *C. gattii* incubated

with Cn_rHsp70 on niger agar produced melanin. *C. albicans* was used as negative control. **C)** CFU burden in the mice lungs were evaluated after infection, and treatment with Cn_rHsp70 and amphotericin B. Asterisks denotes P <0.05. **D)** CFU burden in the mice brain were evaluated after infection, and treatment with Cn_rHsp70 and amphotericin B. No detectable cells were observed in the group that received amphotericin B. Asterisks denote P <0.05. Bars are representative of 6 mice infected per group.

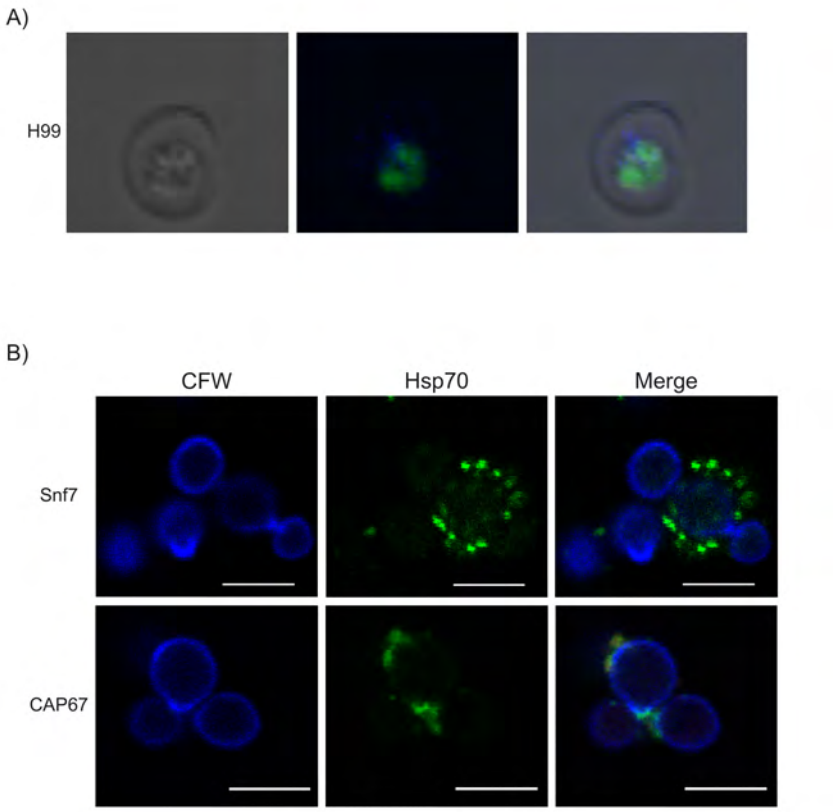


Fig. 3: The anti-Cn_rHsp70 polyclonal serum binds to the cell surface of *C. neoformans*. **A)** A cytoplasmic localization of Hsp70. The nuclei were stained with DAPI (blue fluorescence). **B)** Hsp70 localizes to the fungal cell surface in Snf7 mutant. Acapsular mutant CAP67 was stained and demonstrated capsular network localization of Hsp70. Hsp70 appears in green. The cell wall was stained with calcofluor white (blue fluorescence). The scale bar corresponds to 5 μm.

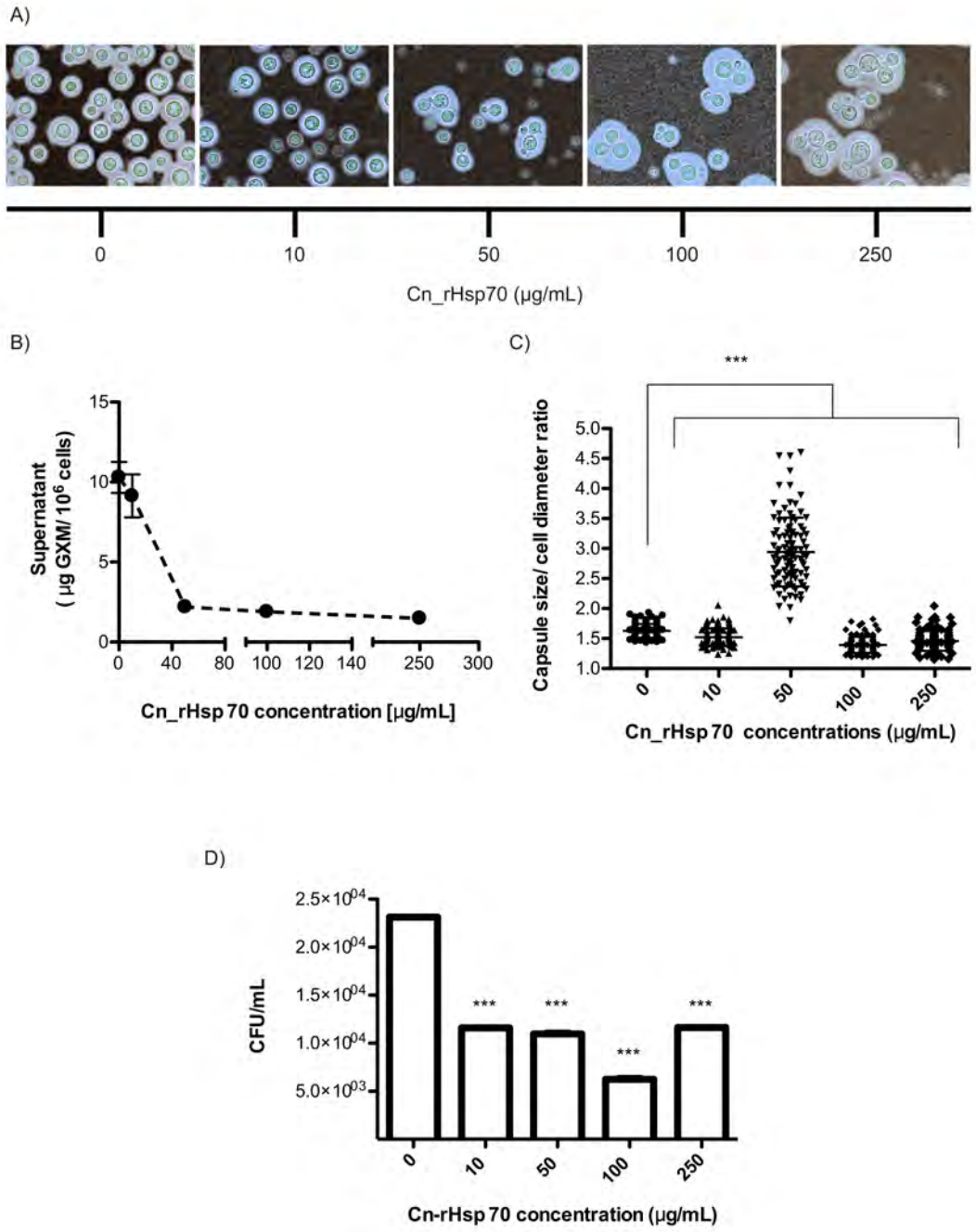


Fig. 4: Treatment of *C. neoformans* with Cn_Hsp70 affects polysaccharide secretion, cell viability but not capsule size. **A**) Cn_rHsp70 affects the morphology, but not capsule dimensions (**C**) of yeast cells incubated with increasing concentration of recombinant protein. **B**) The co-incubation of *C.neoformans* cells with Cn_rHsp70 followed by GXM determination in supernatants revealed a dose-dependent inhibition of polysaccharide extracellular release. **D**) Incubation of *C. neoformans* cells with increasing concentration of Cn_rHsp70 revealed CFU decreasing compared to no incubated cells.

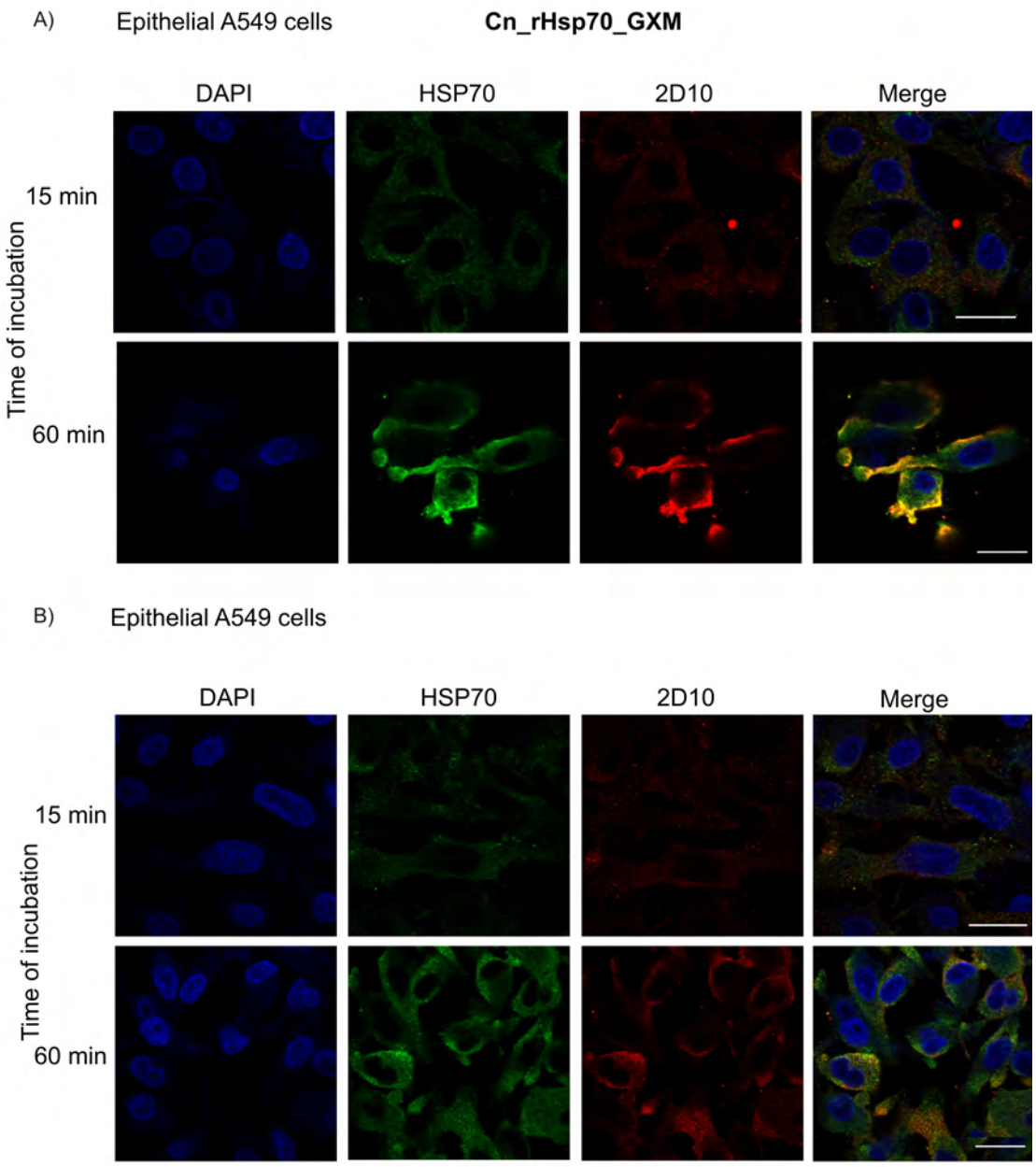


Fig. 5: Binding of Cn_rHsp70 to the surface of host cells. A) A549 cells were incubated with 10 µg/ml of Cn_rHSP70 for 15 (**A**) or 60 (**B**) minutes. The nucleus was stained with DAPI (blue fluorescence).

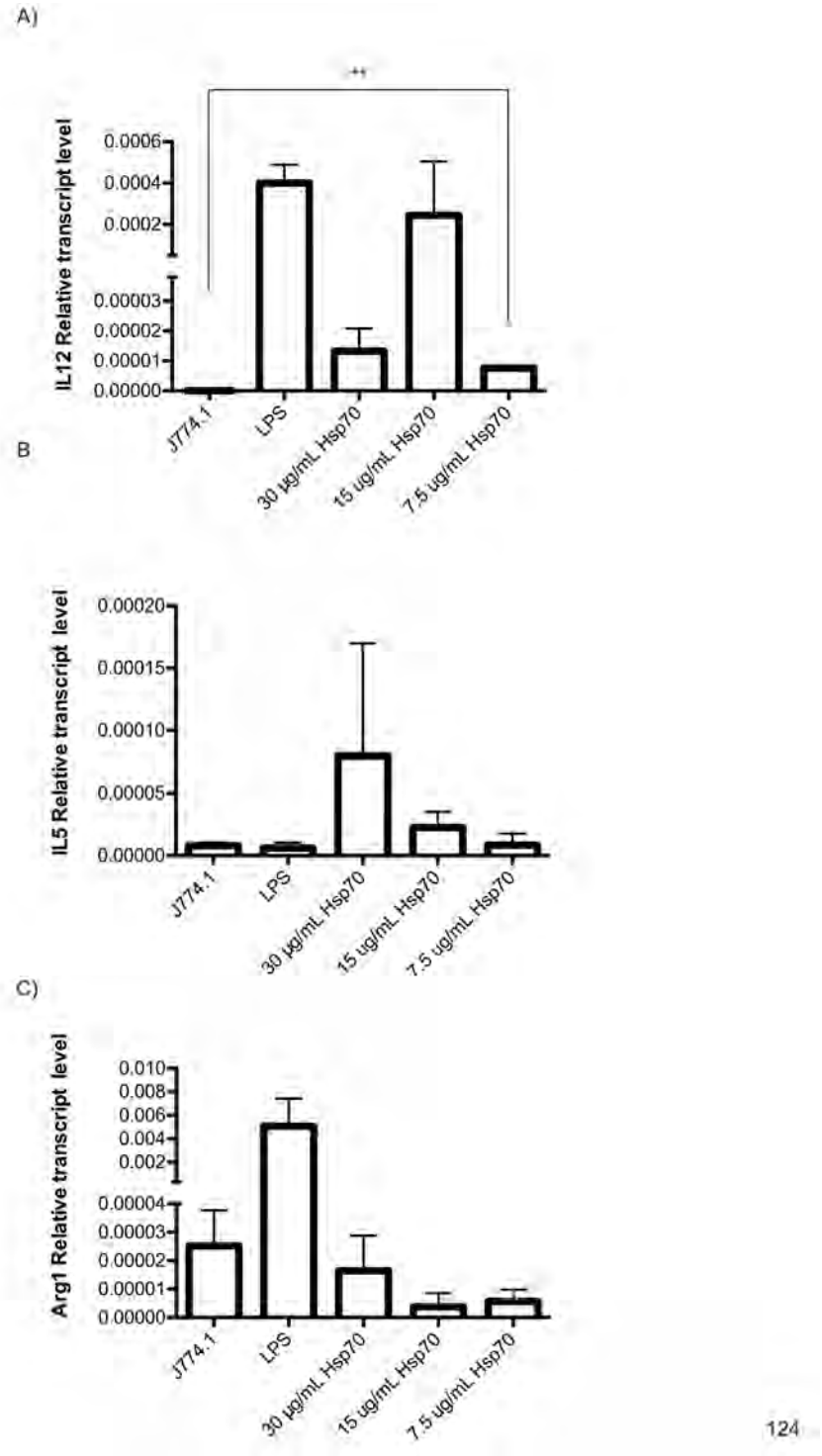


Fig. 6: Cytokine induction in macrophages stimulated with Cn_rHsp70. Macrophages were stimulated with 30, 15 and 7.5 µg/ml of Cn_rHsp70 for further extraction of total mRNA. The levels of IL-12 (**A**), IL-5 (**B**) and Arg1 (**C**) mRNAs were determined by qRT-PCR. The values of expression were standardized using values of obtained for the constitutive gene encoding actin. Data shown are representative of the results of two independent experiments.

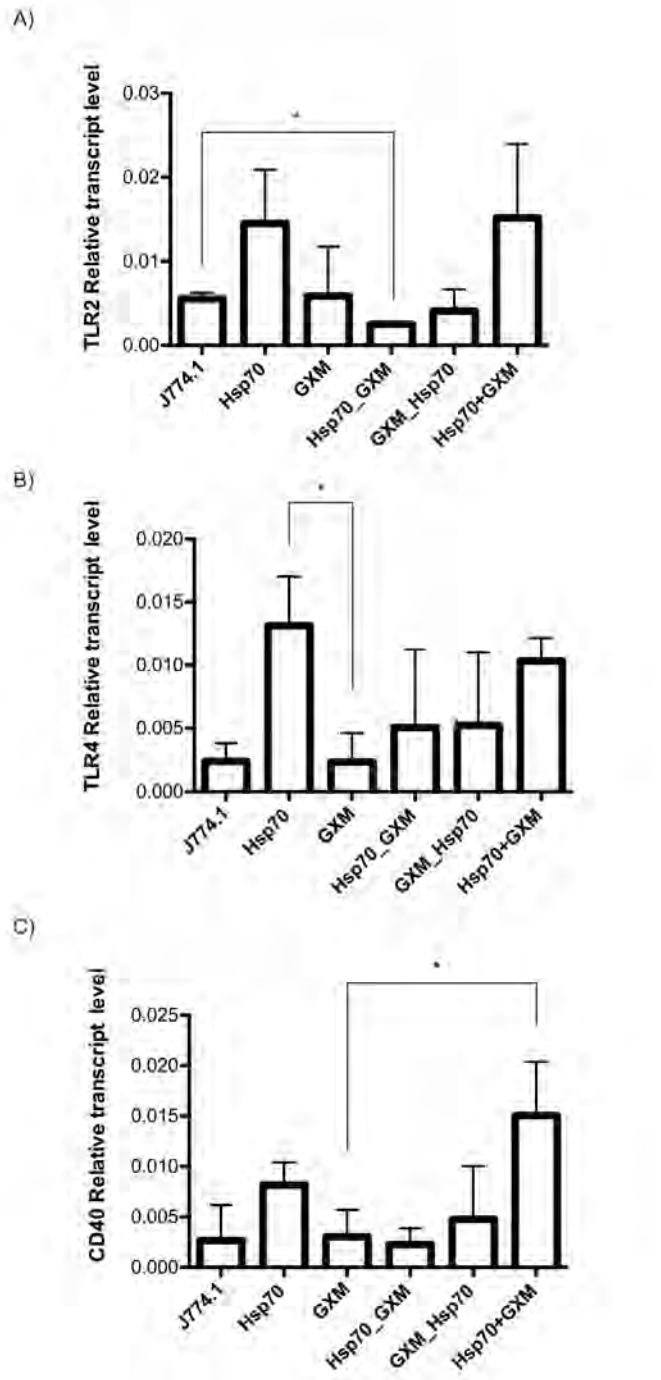
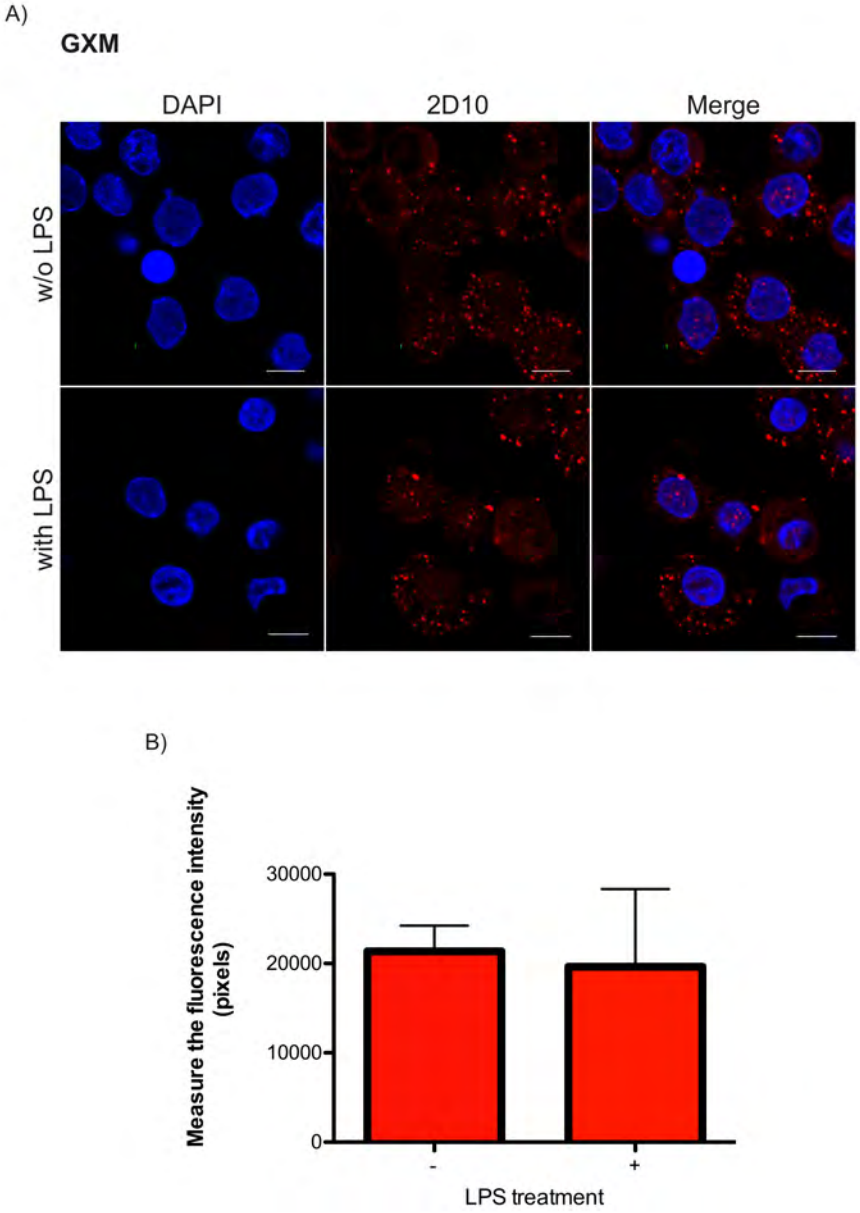


Fig. 7: Receptor expression in macrophages stimulated with Cn_rHsp70. Macrophages were stimulated with 30, 15 and 7.5 µg/ml of Cn_rHsp70 for further extraction of total mRNA. The levels of TLR2 (**A**), TLR4 (**B**) and CD40 (**C**) mRNAs were determined by qRT-PCR. The values of expression were standardized using values of obtained for the constitutive gene encoding actin. Data shown are representative of the results of three independent experiments.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GXM
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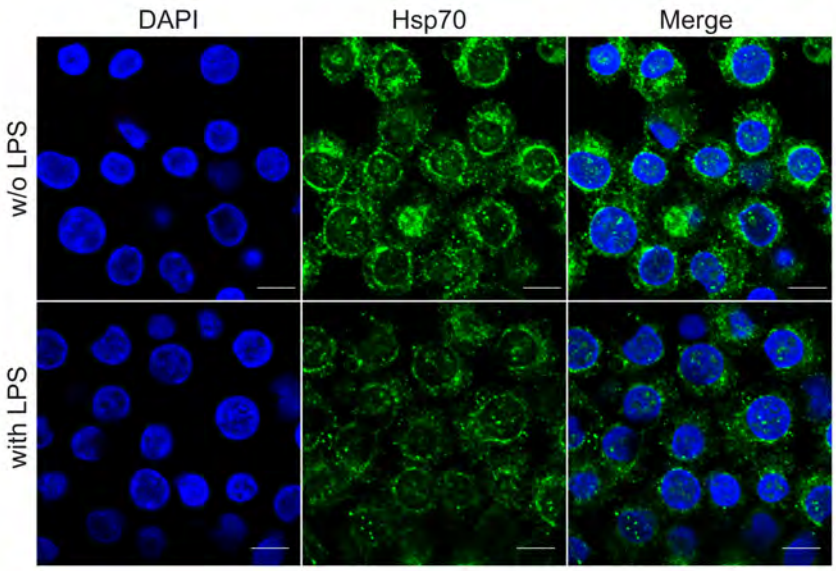
C)

Hsp70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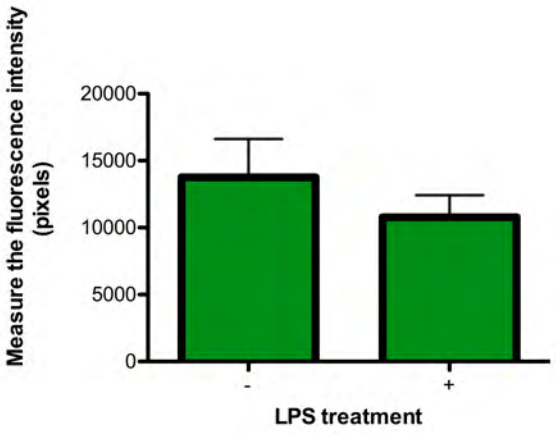
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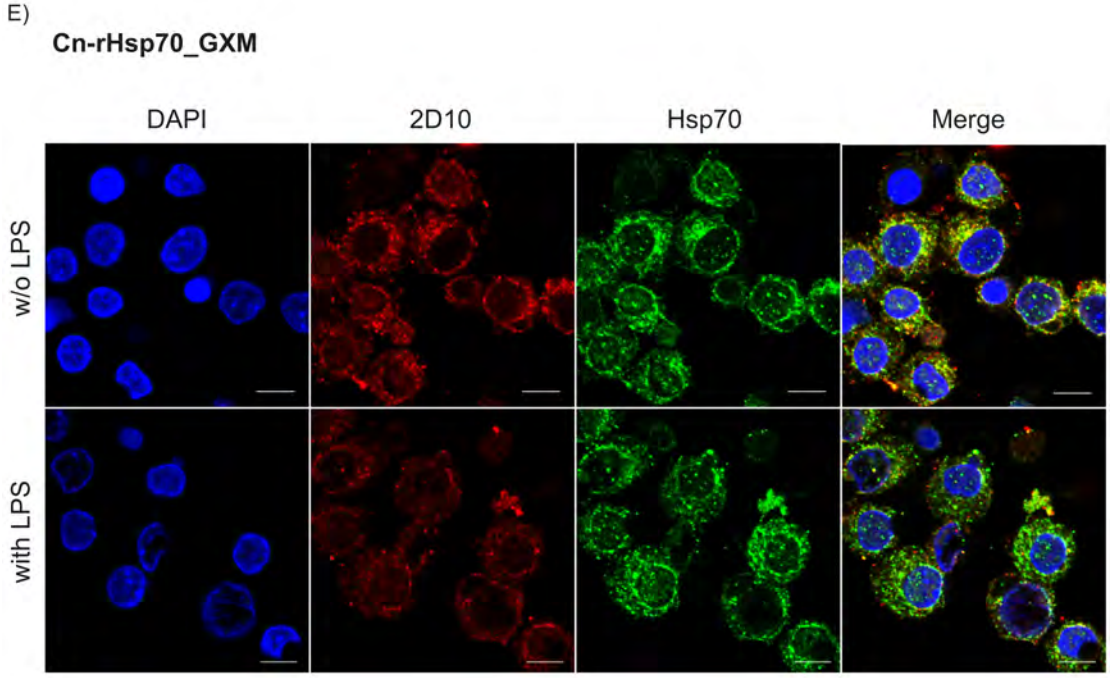
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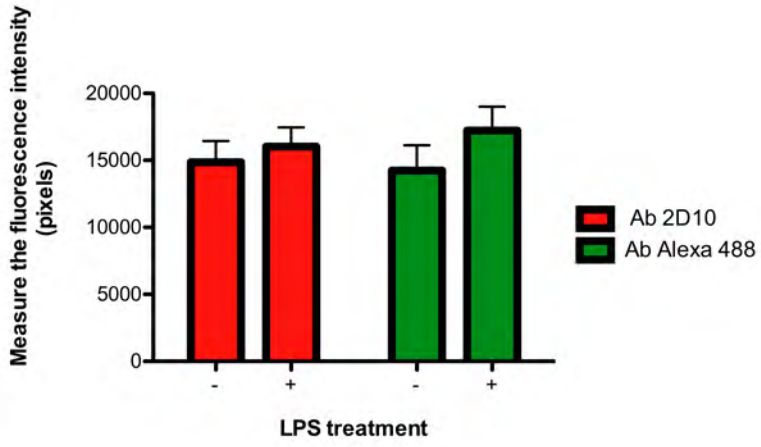
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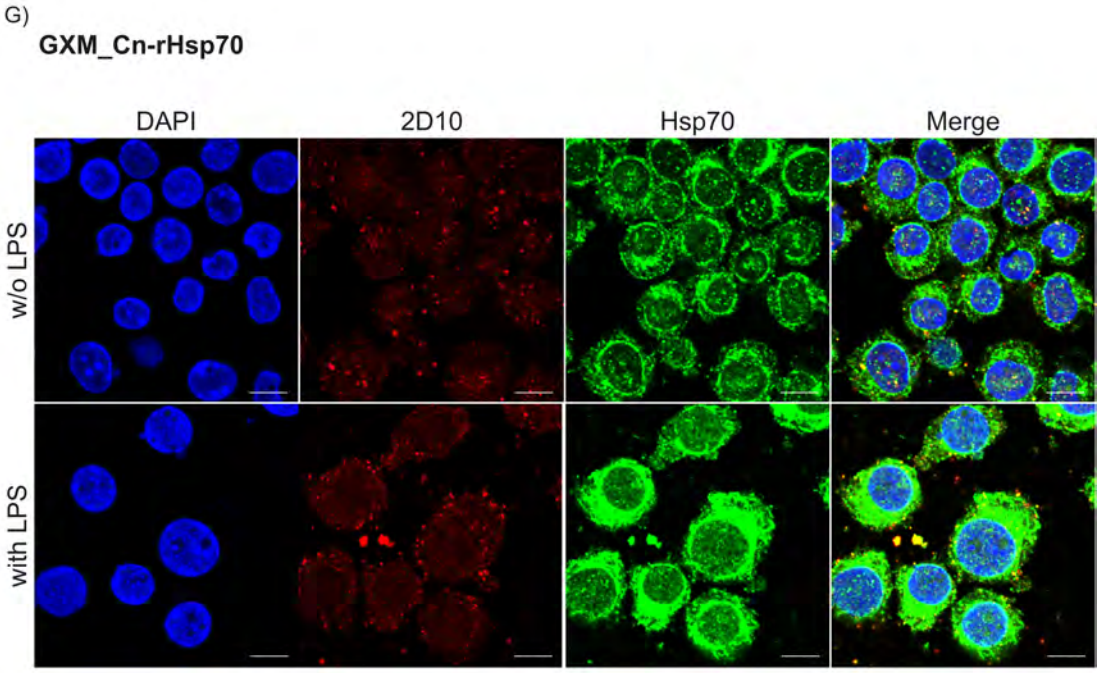
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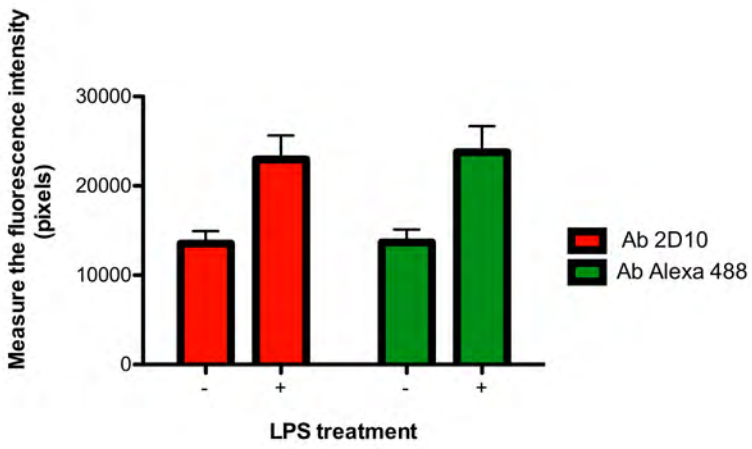
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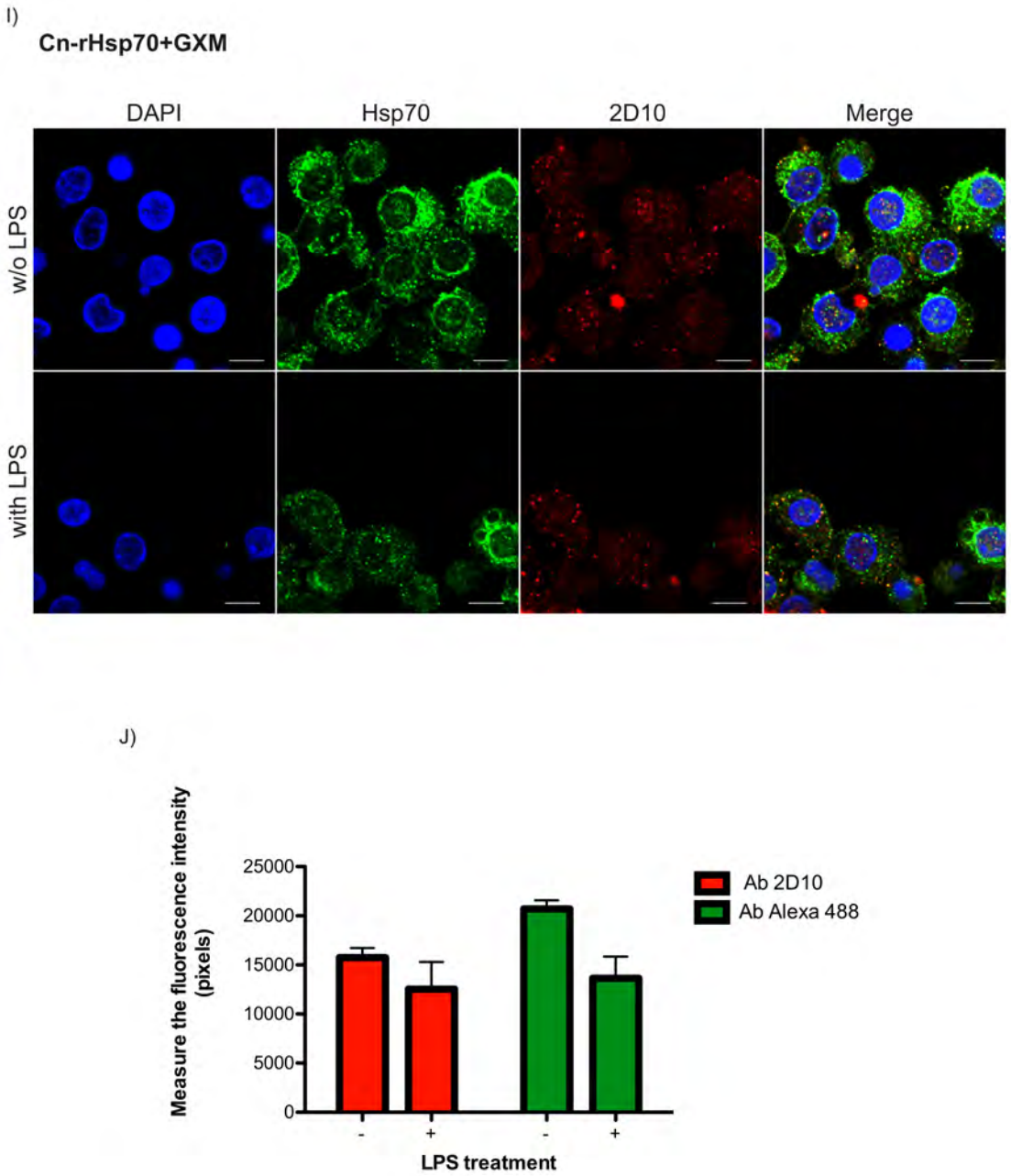

Fig. 8: Cn_rHsp70 and GXM bind in different sides in J774.1 cells. **A)** GXM (red fluorescence) was incubated for 60 min with J774.1 (GXM); **B)** Fluorescence quantification of the J774.1 cells incubated with LPS or without LPS by ImageJ software; **C)** Cn_rHsp70 (green fluorescence) was incubated for 60 min with J774.1 cells (Cn_rHsp70); **D)** Fluorescence quantification of the J774.1 cells incubated with LPS or without LPS by ImageJ software; **E)** Cn_rHsp70 (green fluorescence) was incubated for 60 min for further addition

of GXM (red fluorescence) to the system (Cn_rHsp70_GXM); **F)** Fluorescence quantification of the J774.1 cells incubated with LPS or without LPS by ImageJ software; **G)** GXM (red fluorescence) was incubated for 60 min for further addition of Cn_rHsp70 (green fluorescence) to the system (GXM_Cn_rHsp70); **H)** Fluorescence quantification of the J774.1 cells incubated with LPS or without LPS by ImageJ software; **I)** simultaneous incubation of Cn_rHsp70 and GXM for 60 min with J774.1 cells (Cn_rHsp70+GXM)**;** **J)** Fluorescence quantification of the J774.1 cells incubated with LPS or without LPS by ImageJ software. The scale bar corresponds to 10 μm.

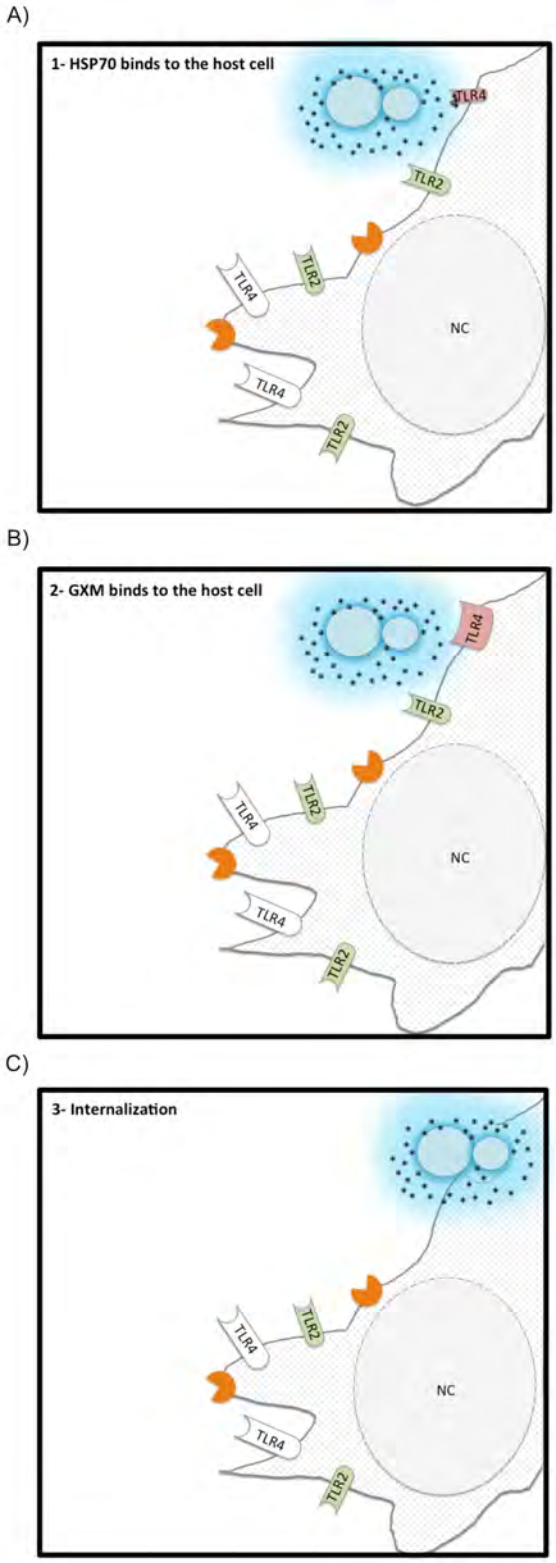

Fig. 9: A speculative mechanism of Hsp70 and GXM interaction. Hsp70 binds to the scpecific receptor (upper panel) and cause a structural change or receptor expression change on the cell; GXM close contact with the same receptor and binds in it (middle panel) allows fungal recognition and internalization (lower panel).

Capítulo IV

Artigo formatado para ser submetido ao período *Vaccine*.

The DNA vaccine expressing Hsp70 from *Cryptococcus neoformans* induces immune response, reduces fungal burden but does not improve mice survival in criptococcosis

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[12] These authors contributed with pulmonary infection and therapeutic treatment.

Abstract

Hsp70 is a conserved chaperone involved in many biological processes. Due to important function on the physiology between species, Hsp70 is widely studied. Several reports demonstrated that Hsp70 induces immune response in many diseases. However, some researchers have demonstrated the immunosuppressive effect of Hsp70 in human disease. Other members of the Hsp family had already been used in the treatment against fungal infection. Catalase is an enzyme that protects the organism against reactive oxygen species. Both proteins are also located on cell surface in *Cryptococcus neoformans*, encapsulated yeast that causes pulmonary disease in imunocompromised patient, which if untreated, may disseminate to the brain causing meningoencephalitis. Th1-Th2 cytokine balance influences host protective responses to *C. neoformans* and results of both host and pathogen characteristics. New approaches have been developed for the treatment of cryptococcosis but DNA vaccination has not been evaluated yet. The purpose of this study is to evaluate the therapeutic effect of DNA construction containing Hsp70 or catalase sequence, named pcDNAHsp70 and pcDNACAT, respectively, in mice infection. BALB/c mice were protected by pcDNAHsp70, but the vaccination did not induce Th1 immune response. On the other hand, C57BL/6J mice were not protected by pcDNAHsp70, although a Th1 immune response has been induced. pcDNACAT seems to be not effective in cryptococcosis infection even demonstrating an increased in phagocytosis assay and TNF-α production in macrophages transfected with plasmid. These results provide new prospects for therapy of cryptococcosis, and raised questions about the role of Hsp70 and catalase from *C. neoformans* in the infection.

Keywords

Hsp70, catalase, DNA vaccine, immune response

Highlights

- pcDNA stimulates immune response against *C. neoformans* infection.
- pcDNAHsp70 protected BALB/c mice but not induced Th1 immune response.
- pcDNAHsp70 did not protect C57BL/6J mice against cryptococcosis infection, but induced Th1 immune response.
- pcDNACAT does not protect mice against cryptococcosis infection.

1. Introduction

Cryptococcosis is a pulmonary disease that initiates by inhalation of *Cryptococcus neoformans* and can disseminate to central nervous system, causing meningitis [1]. Worldwide, cryptococcal meningitis affects about 957.900 people per year, resulting in 624.700 deaths [2]. The antifungal therapies are limited because of toxicity, low efficacy rates, high cost-effective and survival outcomes [3]. Therefore, new approaches that prevent or protects the host from the infection are required and become important for public health. Some studies have been evaluating the immunomodulation in therapeutic treatment of cryptococcosis infection using unmethylated CpG sequence [4,5], glucosylceramides [6], ß-glucans [7] and radiotherapy [8] yielded promising results in animal models. Furthermore, a passive immunization using monoclonal antibody against capsule components [9] and melanin [10] is effective to prevent criptococcosis dissemination.

When it comes to immune response to opportunist fungal pathogens, the Th1-cell-mediated immunity is critical for it elimination, mainly via $CD4^+$ and $CD8^+$ cells [11]. In this sense, the virulence factors produces by this pathogen drives the TNF-α production that modulates the IL-12 and IFN-γ expression [12]. Wozniak *et al*. [13] demonstrated that T-cell deficient mice generated protective immunity against *C. neoformans* immunization, indicating that an anti-cryptococcal vaccine to immune compromised patients could be efficient.

To fungal disease caused by others fungal cells, including *Coccidioides immitis* [14,15] and *Paracoccidioides brasiliensis* [16], urease and gp43 DNA

vaccination has been evaluated in murine models yielding promising results. Moreover, it has been reported that DNA vaccines were more effective than the recombinant protein vaccines [11].

Omics approaches have indicated some immunogenic proteins that may provide protective anti-cryptococcal immune responses [17,18]. Most proteins found are highly conserved, such as heat shock proteins, glycolytic enzymes [19] and catalases [20].

Heat shock proteins (Hsp) are a conserved chaperones family, identified in every organism, both prokaryotic and eukaryotic. Hsps are mainly induced by heat shock, oxidative stress and nutrient deprivation and promotes refolding of denatured proteins [21]. Hsp70 is a 70- kDa heat shock protein that has suppressive effect in autoimmune disease [22]. Additionally, Hsp70 has been evaluated for the ability to activate the host innate immune response, resulting in dendritic cell activation and maturation, activation of complement, and release of pro-inflammatory cytokines [23].

In this regard, heat shock proteins are known to be immunogenic and have been tested against *Candida albicans* [24,25]*, Histoplasma capstulatum* [26] and *P. brasiliensis* infections [27]. Members of heat shock 70 family have also been detected as immunodominant antigens in murine and human pulmonary cryptococcosis [28,29].

Catalase is an enzyme that hydrolyse hydrogen peroxide (H_2O_2) to oxygen and water, conferring resistance to oxidative mechanisms. Specifically, fungal catalases were subdivided in four clades (peroxisome, cytoplasmic, spore-specific and secreted catalases) and cluster into distinct groups of plants and algaes [30]. Although the enzymes that protect fungi against reactive oxygen species are related with pathogenicity, catalase is not considered a virulence factor in *Aspergillus fumigatus* [31] and *C. neoformans* [32].

Meanwhile, catalase was identified as a major diagnostic antigen in *H. capsulatum* that elicits both humoral and T-cell- mediated immune response [33]. Furthermore, this protein was used as antigen to vaccination in animal experiment and caused a reduction in *Helicobacter pylori* colonization [34].

The advantages provided by DNA vaccine, including low cost, simple administration, fast production and safety, lead us to study the possibility to

use the Hsp70 or catalase as target molecule of humoral as well as cellular immune response against *C. neoformans* infection.

2. Material and Methods

2.1 Fungal strain and culture media

C. neoformans strain H99 was maintained on YPD media (1% yeast extract, 2% peptone, 2% dextrose, and 1.5% Bacto agar). Yeast cells were grown for 48 h at 30 °C with shaking in YPD broth, collected by centrifugation, washed three times with sterile phosphate-buffered saline (PBS) and counted with an hemacytometer.

2.2 Plasmid constructions

The HSP70 (CNAG_05199.2) and catalase (CNAG_05256.2) gene sequences were obtained from the Broad Institute (www.broadinstitute.org). Total RNA was isolated with PureLink® RNA Mini Kit according to manufacturer's instruction (Invitrogen, CA, USA). Complementary DNA was synthesized from 1 µg of total RNA in the presence of oligo(dT)18 (Fermentas, MD, USA) and M-MLV (Invitrogen, CA, USA). A BamHI and XhoI site were added to the sense and antisense primers, respectively (Table 1). PCR product was cloned in pUC18, into SmaI site and subcloned into the pcDNA3.1(+) (Invitrogen, CA, USA) by directional insertion in the BamHI and XhoI sites. pcDNA3.1 (+) was kindly donated by Dr. Henrique Bunselmeyer Ferreira (Universidade Federal do Rio Grande do Sul, Brazil). The resulting plasmids were called pcDNAHsp70 or pcDNACAT, respectively. The confirmations of the inserts were done by restriction enzymes, PCR using specific primers and DNA sequencing.

2.3 Preparation of plasmid DNA

To prepare plasmid DNA for immunization, *Escherichia coli* XL1Blue cells were transformed by electroporation with the pcDNACAT, pcDNAHsp70 or pcDNA alone (control) and then cultured at 37°C in Luria broth supplemented with ampicillin (100 µg/ml). DNA for immunization was purified using the EndoFree Giga Kit (Qiagen, CA, USA) and was diluted in TE buffer

to the final concentration of 1 mg/ml.

2.4 Plasmid gene expression in mammalian cells

To evaluate the expression of pcDNAHsp70, pcDNACAT or pcDNA in mammalian cells, a transient-transfection assay was performed using Lipofectin (Invitrogen, CA, USA) and 500 ng of plasmid transfected into human type II alveolar epithelial cell line, A549. Cells 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. After 18, 24 and 48h of transfection, the cells were harvested, and total RNA or protein was isolated with Trizol for reverse transcription RT-PCR or Western blot, respectively.

2.4.1 RT-PCR with RNA extracted of epithelial cells

Genomic DNA contamination was removed by treatment with RQ1 RNAse- free DNAse (Promega, Madison, USA) and control PCR reactions were performed to confirm DNA removal. cDNA synthesis was conducted with M-MLV Reverse Transcriptase (Invitrogen, CA, USA) using 1µg of RNA and 3' BD SMART CDS Primer II A (Clontech Laboratories). PCR were accessed with 1µl of the first-strand cDNA using DNA Recombinant *Taq*-polymerase (Invitrogen, CA, USA). As internal control for RNA loading in the RT-PCR reactions, primers for the *β- actin* gene from human were employed. Hsp70 or catalase mRNA expression was evaluated using specific primers.

2.4.2 Western blot with protein extracted of epithelial cells

Total protein from transfected A549 cells was separated by SDS-PAGE, transferred to a polyvinylidene fluoride membrane (PVDF, GE Healthcare, *formerly Amersham Biosciences,* Uppsala Sweden) and probed with anti-Hsp70 or anti-CAT from *C. neoformans* at the dilution of 1:10. Detection was performed using an ECL-plus system (GE Healthcare, *formerly Amersham Biosciences,* Uppsala Sweden) according to the manufacturer's instructions.

2.5 Phagocytosis rates, killing or TNF-α production

The macrophage-like cell lineage RAW 264.7 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. Then were cultivated in 12-well plates (TPP, Switzerland) at a density of 10^6 cells/well at 37°C with 7.5% CO_2 to confluent monolayer. RAW 264.7 were transfected with pcDNA, pcDNACAT or pcDNAHsp70 using Lipofectin (Invitrogen, CA, USA) according to manufacturer's instruction. *C. neoformans* yeast cells (ratio of 10 fungal cells per phagocyte) were incubated 18h after the transfection to evaluate phagocytosis rates, killing or TNF-α production. Mammalian cells were incubated in the presence of *C. neoformans* for 18h 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 rates were performed removing infected macrophages from plates by scraping and fixed in 4% paraformaldehyde for further flow cytometry analysis using a GUAVA flow cytometer and processed with GUAVA Cytosoft (Millipore, Billerica, MA). To discriminate adhered and internalized yeasts, macrophages were treated for 10 min at room temperature with Trypan blue (200 µg/ml), which quenches the fluorescence of external FITC-labeled yeasts cells. Control consisted of macrophages that were not exposed to yeast cells.

The TNF-α production by macrophages in supernatant was evaluated using the Elisa Array System (Invitrogen, CA, USA) according to the manufacturer's instructions.

2.6 Immunization of mice with plasmid DNA

BALB/c mice (female, 4–8 weeks old) were obtained from Fundação Estadual de Produção e Pesquisa em Saúde (Porto Alegre, RS, Brazil) and maintained under standard laboratory conditions. C57BL6J mice (female, 4–6 weeks old) were obtained from Universidade Estadual de Campinas (Campinas, SP, Brazil) and maintained under the same conditions. All immunizations were performed intramuscularly in quadriceps muscle.

Female 6-8-week-old BALB/c mice were immunized intraperitoneally with 100 µg of plasmid (pcDNA, pcDNAHsp70 or pcDNACAT). Mice were boosted 2 and 4 weeks post immunization. Sera were then 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.

2.7 IgG detection after immunization with DNA vaccine

Enzyme-linked immunosorbent assays (ELISA) were used to examine whetter DNA vaccine against an Hsp70 and catalase stimulates antibody production. Briefly, a recombinant protein Hsp70 or catalase from *C. neoformans* was coated 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, St. Louis, Missouri, USA) in PBS for 1h at 37°C. The wells were washed three times with 0.1% Tween-20 in PBS and incubated with the sera. After 1h at 37°C, the plates were washed, incubated with peroxidase-labeled antibodies raised against to mouse IgG (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated for 1h at 37°C. Serologic reactions were measured by addition of TMB (Invitrogen Corp., Carlsbad, California, USA) and spectrophotometrically determined at 450 nm. Mice immunized with PBS were used as negative control.

2.8 Pulmonary infection and therapeutic treatment

BALB/c and C57BL/6J were anesthetized with ketamine (8 mg/kg) and xylazine (60 mg/kg) and infected intranasally with $2x10^5$ *C. neoformans* H99 cells. To BALB/c infection, five days after the infection, the treatment was initiated by intramuscularly route and the mice were immunized with 3 doses of 100 µg of vector at 2-days intervals (Fig. 1A). To C57BL/6J infection, two days after the infection, the treatment was initiated also by intramuscularly route and the mice were immunized with 3 (Fig.1B) or 5 doses (Fig. 1C) of 100 µg of vector at 2-day intervals. Mice were separated into five groups (10 animals per group): (1) mice immunized with 3 doses of 100 µg of empty vector pcDNA at 2-days intervals (pcDNA group); (2) mice immunized with 3

doses of pcDNSHsp70 at 2-days intervals (pcDNAHsp70 group); (3) mice immunized with 3 doses of pcDNACAT at 2-days intervals (pcDNACAT group); (4) mice immunized with saline (H99 group); (5) non-immunized and non-infected mice (control group). BALB/c mice treated with pcDNACAT were evaluated to the survival only.

Mice were sacrificed 12, 15 or 17 days after infection, the lungs, brain and spleen were removed. Weighed tissue sections were homogenized and then washed 3 times with PBS and suspended in 1 ml PBS. Brain and lungs were homogenized in 1 ml of sterile PBS, plated on YPD agar and CFU were determined following incubation at 30°C for 48h.

2.9 Histological analysis

Lungs and brain from BALB/c mice were fixed in 4% formalin, paraffin-embedded, sectioned in five micrometer and followed rehydration through a xylene-alcohol series to a final wash in PBS. Tissue sections were then stained using hematoxylin and eosin and analyzed with a Zeiss immunofluorescence microscope.

3.0 Survival assays

Survival assays were conducted as previously described [35]. BALB/c mice were infected via nasal inhalation as described above and the survival analysis was calculated by Gehan-Breslow-Wilcoxon test using GraphPad Prism Software.

3.2 Cytokine determination

Lungs and spleen were assayed for IFN-γ, IL-10, TNF-α production, using the Elisa Array System (Invitrogen, CA, USA) according to the manufacturer's instructions. To analyzed IL4, IL13, Arg1, lungs were harvested and homogenized in 1 ml of TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). RNA was quantified by Qubit (Invitrogen, CA, USA). The reaction mix was made using an M-MLV (Invitrogen, CA, USA). Real Time RT-PCR 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, CA, USA) 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 to the levels of transcription of β- actin gene, 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 [36], The primers used in the cytokines assays are listed in Table 1. For each cytokines, samples from individual mice (*n*=1) were run in triplicate.

4.0 Results

4.1 Plasmid construction, expression and interaction with mammalian cells

Catalase 2 and Hsp70 were successfully cloned in pcDNA3.1(+) plasmid (Fig. 2A, schematic representation). After purification by EndoFree Giga Kit, the constructions were tested for further evaluation of plasmid expression. As demonstrated in Figure 2B, the construction was confirmed by restriction enzymes. To pcDNACAT, a 1.5 kb fragment, corresponding to catalase sequence and 5.4kb, corresponding to the empty pcDNA3.1 vector, were released. Further, to pcDNAHsp70 confirmation, a 2 kb fragment, corresponding to Hsp70 sequence and 5.4kb, corresponding to pcDNA3.1 were released.

The mRNA expression was assayed by RT-PCR (Fig. 3A, 3D) using specific primers. Total RNA from transfected A549 cells was isolated 18, 24 or 48h after transfection to determine the expression of the mRNA. Epithelial cells A549 transfected with pcDNACAT, had an increased expression 18 h after transfection. After 24 and 48 h, there was a decreased of mRNA expression (Fig. 3A). On the other hand, transfected cells with pcDNAHsp70, shown an increase of expression occurring over time where the expression reached the highest at 48 hours after the transfection (Fig 3D). β- actin was used as internal control for RNA loading.

The protein expression was evaluated by Western blot using anti-CAT (Fig. 3B) or anti-Hsp70 antibodies (Fig. 3E) from *C. neoformans*. As

demonstrated in Fig. 3B and 3E, endogenous protein from eukaryotic cells are recognized by antibody against yeast catalase 2 (Fig. 3B) or Hsp70 (Fig. 3E) molecules, since polipeptides corresponding to catalase (56 kDa) and Hsp70 (72 kDa), respectively, were detected in A549 cells non-transfected and A549 cells transfected with the empty pcDNA vector.

To examine if pcDNACAT or pcDNAHsp70 stimulated IgG production, sera from immunized mice were evaluated by ELISA (Fig. 3C, 3F) against soluble recombinant catalase or Hsp70, respectively. As observed, the immunization with DNA vaccine did not produce IgG antibody compared to the controls (PBS, pcDNA).

In order to verify the effect of the transfection on the interactions between yeasts and macrophages, fungal survival assay, TNF-α production and phagocytosis rates were assayed using RAW 264.7 macrophages.

Fungal survival in macrophages transfected with pcDNACAT resulted in an increase of CFU (Fig. 4A). On the other hand, *C. neoformans* incubated with macrophages transfected with pcDNA or pcDNAHsp70 had no difference in CFU account compared to control. Hence, macrophages transfected with pcDNACAT was more efficient in phagocytosis compared to the other groups.

Macrophages transfected with pcDNA, pcDNACAT or pcDNAHsp70 produced significant increase of TNF-α level (Fig 4B) in supernatant compared to non-transfected group (H99).

Phagocytosis rates decreased in macrophages non-transfected compared to transfected cells (Fig. 4C). *C. neoformans* cells were phagocytized at similar rates by non-transfected macrophages (H99 -3.89%) and by pcDNA transfected macrophages (2.58%). When macrophages were transfected with pcDNACAT, yeast cells were more susceptible to phagocytosis (5.54%) than the control group (RAW). On the other hand, *C. neoformans* cells were phagocytized at similar rates by pcDNA transfected macrophages (2.46%) or pcDNAHsp70 (3.44%) compared to the non-transfected macrophages (H99 – 4.16%). Taken together, pcDNACAT and pcDNAHsp70 were expressed in mammalian cells but the immunization used was insufficient to produce IgG antibody. Furthermore, when macrophages

transfected with pcDNACAT were evaluated in the host cell interaction, an increase of phagocytosis and fungal survival were observed.

4.2 Histopathological, CFU and survival analyses

Lungs and brain sections from BALB/c mice showed the presence of numerous yeast cells by 15 days after infection in controls (H99 and pcDNA) and pcDNAHsp70 treated animals (Fig. 5). The histological analysis revealed no difference in the progression of the infection for all groups tested. The infected animals H99, pcDNA and pcDNAHsp70 presented the same pattern of brain and pulmonary injury.

However, there was a reduction of yeast cells observed by *C. neoformans* CFU determination in mice treated with pcDNA or pcDNAHsp70. Fig. 6A shows that animals treated with pcDNA showed pulmonary fungal burden similar to the pcDNAHsp70 group after 15 days of infection. CFU recovery from H99 mice was lower than that observed in pcDNA and pcDNAHsp70 groups ($p<0.05$). For the brain CFU determination, no difference was observed between mice treated with pcDNA or pcDNAHsp70 and H99 group (Fig. 6B).

Relative to mice survival, animals treated with pcDNA, pcDNACAT and non-treated had mean survival times of 23.5, 15 and 17 days respectively (Fig 7A). Mice treated with pcDNAHsp70 and non-treated had mean survival times of 20 and 20.5 days respectively (Fig. 7B). On the other hand, mice treated with pcDNA had mean survival of 16.5 days (Fig. 7B). The log-rank Mantel-Cox test (GraphPad Prism Software) revealed no significant difference between mortality rates caused by these three groups ($P<0.0001$).

When we used C57BL/6J mice, the number of lung CFU was significantly increased in pcDNA immunized animals compared to control H99 using fungal suspension at $2x10^3$ cells in 12 days post-infection ($p<0.05$) (Fig. 10A, black bars). Meanwhile, with an inoculum at $2x10^3$ cells in 17 days post-infection, there was no significantly difference among the groups (Fig. 10A, gray bars). When we evaluated $2x10^5$ cells after 12 days post-infection, there was no difference in CFU account (Fig. 10B, black bars), however, when $2x10^5$ cells after 17 days post-infection were tested, an increase occurred in

mice treated with pcDNA (p<0.05) compared to pcDNAHsp70 (Fig. 10B, gray bars). After 12 days post-infection, brain had no detectable fungal cells with both inoculum tested (data not showed). Notably, at 17 days post-infection, we observed a significantly reduction of cells recovery when $2x10^3$ cells were used to the infection in mice treated with pcDNA and pcDNAHsp70 with p values <0.05 (Fig. 10C, black bars). Nevertheless, the fungal load measured CFUs in mice receiving $2x10^5$ cells were the same in all groups tested (Fig. 10C, gray bars).

Taken together, these results indicated that the treatment strategy was not sufficient to avoid *C. neoformans* dissemination in BALB/c. Despite, pcDNA treatment confers protection to the mice through of reduction yeast burden in lungs, although it is not sufficient to avoid brain dissemination. In contrary, in C57BL/6J mice infection, pcDNA treatment did not confer a reduction of yeast burden in lungs, but in the brain, indicating that the treatment could prevent to the brain dissemination.

5.0 Cytokines production

BALB/c and C57BL/6J mice have different genetic backgrounds that strongly influence their response to infection by *C. neoformans* [37]. IL-10, IFN-γ and TNF-α were measured in the lungs and spleen of infected BALB/c mice (Fig. 8A, B, C). BALB/c mice responded to pcDNA immunization with significant increase in IFN-γ in the lung homogenate compared to H99 group (Fig. 8A, black bars). When IFN-γ level was evaluated in the spleen, there is an increased production of this cytokines in the pcDNA compared to non-treated or pcDNAHsp70 treated animals (Fig. 8A, gray bars). For IL-10 and TNF-α production in the lungs, BALB/c mice treated and non-treated showed the same cytokines levels (Fig. 8B,C, black bars). In contrast, mice treated with empty pcDNA vector, increased the IL-10 and TNF-α level in the spleen compared to the non-treated group (H99) and pcDNAHsp70 (Fig. 8B,C, gray bars).

To evaluate the IL-4, IL-13 cytokines production and Arg1 expression in BALB/c mice, qRT-PCR was performed using lungs from mice non-treated (H99), treated with pcDNA or pcDNAHsp70. Fig. 9 showed that pcDNAHsp70

treatment increased IL-13 production compared to the control H99 and pcDNA, and the same was not observed with IL-4 and Arg1 expression.

Furthermore, the cytokines production (IFN-γ, TNF-α, IL-10 and IL-17) were measured in the lungs of infected C57BL/6J mice (Fig. 11) including the treatment with pcDNACAT that not was evaluated to the BALB/c mice. C57BL/6J mice produced significantly more IFN-γ after immunization with pcDNAHsp70 in comparison to treatment with pcDNA alone and with not immunized mice H99 ($p<0.05$). The treatment with pcDNAHsp70 and pcDNACAT increased the TNF-α production compared to the H99 group ($p<0.05$). Moreover, C57BL/6J treated with pcDNAHsp70 increased the IL-10 (the same was observed to pcDNA group) and IL-17 levels relative to the H99 non-treated group ($p<0.05$).

6.0 Discussion

We have presented evidence that immunization of BALB/c mice with mammalian empty plasmid expression vector provides protection against a challenge of *C. neoformans*. Our initial selection of Hsp70 and catalase as a vaccine candidate was based on the results from the identification of immunodominant *C. neoformans* proteins, detected in serum from cryptococcosis patients. They are originally thought to be exclusively intracellularly proteins. However, these proteins can be released or secreted from cells by non-classical secretory pathway [38,39] and thus could elicit immune response in the host cell.

Hsp60 and Hsp70 from bacteria are highly immunogenic, inducing antibody production and T-cell activation [27]. Extensive works have been done to evaluate the protective potential of Hsps in fungal diseases. For example, Hsp60 confers protection to BALB/c mice against paracoccidioidomycosis and histoplasmosis infections [40,41]. In contrast, Hsp60 isolated from *C. immintis* was less effective as a protective antigen [14,15]. To invasive candidiasis, there is a commercial monoclonal antibody against Hsp90 that successfully protects against the infection [24,25].

Besides the studies with proteins as protective antigen, many efforts have been focused in DNA vaccine development. Therapeutic and

prophylactic vaccine using Hsp65 from *P. brasiliensis* has shown efficiency against the infection [42,43]. Intent to evaluate DNA vaccine expression, a RT-PCR and Western blot were performed using A549 epithelial cells transfected with plasmids. Curiously, pcDNACAT have increase expression at 18h after transfection and pcDNAHsp70 increases the expression in the course of time, reaching the highest expression at 48 h post-transfection. This difference in plasmid expression may have influence the efficacy of DNA vaccine and determine the therapeutic strategy against fungal disease.

Neither pcDNAHsp70 nor pcDNACAT elicited the humoral immune response by IgG production in immunized mice. Wozniak *et al*. [44] demonstrated that B-cell deficient mice were able to resolve second challenge with *C. neoformans*, indicating that B-cell-mediate immunity is not essential to *C. neoformans* clearance. As several variables could influences in the DNA vaccines efficacy, including the amount of DNA administered and the route of DNA administration, we speculated that cells transfected by intramuscularly route expressed low amount of antigen to be presented to lymphocytes, then generating no detectable antibody.

The interaction of the macrophages with *C. neoformans* cells is well described [45,46], so we have evaluated the phagocytosis events after macrophage transfection with pcDNA, pcDNACAT or pcDNAHsp70. Catalase appears to influence the phagocytosis process, but the role of this protein in this interaction needs to be elucidated. Chaturvedi *et al*. [47] demonstrated that catalase inhibits killing of *C. neoformans* by neutrophils cells through scavenging reactive oxygen intermediated. Transfected macrophages produced a significant amount of TNF-α, according to described by Yasuda *et al*. [48]

Despite Hsp70 and catalase were identified in the sera patients and previous studies demonstrated the cell surface localization, the apparently do not confer protection to BALB/c mice as also been reporter by Lopes *et al*. [49] for histoplasmosis infection. The treatment with empty pcDNA vector was sufficient to reduce CFU burden, so the Hsp70 sequence cloned into pcDNA made no difference in clearance of infection. To pcDNACAT immunization, the sequence of catalase showed the same results that the one obtained for

non-treated mice. We speculate that catalase could support yeast cell to evade immune response, since suppressed pcDNA empty effect. Regarding to survival assay, any treatment increase mice survival. As CFU determination to the BALB/c mice were done 5 days after the last immunization, probably pcDNA contained the progress of the infection. To evaluate survival curve, mice were given just 3 immunization and remained few days without received any dose of plasmid until their death.

It is well known that cytokines are generated during the infection and, once released, play a critical role in host defense to a pathogen. To date is consistently revealed that the protective response against pulmonary *C. neoformans* infection was associated with Th1-type cells, predominantly IFN-γ and IL-12 production [50,51]. Consistent with these believe, an increase of IFN-γ, TNF-α but not a decrease of IL-10 production were observed in the lungs of BALB/c mice treated with pcDNA. Furthermore, a reduction of Th2 cytokines, IL-4 and IL-13, that are associated with exacerbation of the disease in animal models, was also observed in the same group of mice [52,53]. Thus, the induction of protective immunity by pcDNA immunization appears to require the induction of Th1-type cell mediated immune responses, since a Th2 response results of both, host and pathogen characteristics. In contrast, our data point to that Hsp70 induces a Th2 response, since IL-4 and IL-13 expression were increased compared to controls groups. Both IL-4 and IL-13 induce Th2-mediated immune mechanisms in *C. neoformans*, producing an exacerbation of disease. IL-13 is also associated with alternatively activated macrophages in pulmonary criptococcosis [54,55].

Hsp70 modulates an inflammatory response via T-cells induction [56]. Meanwhile, some authors had focused attention to suppressive function of Hsp70 [57,58]. Despite, pcDNAHsp70 vaccination does not increase IFN-γ and TNF-α production, interestingly, the production of IL-10 was significantly decreased compared to others immunized mice. Arg1 is expressed in macrophages by exogenous stimuli including IL-4 and IL-13. Since iNOS is a source of fungicidal NO, Arg1 competes with iNOS for arginine, depleting fungicidal activity of nitric oxide [59]. It was curious that mice treated with pcDNAHsp70 expressed less Arg1 than mice non-treated or treated with

pcDNA, since had an increased in IL-4 and IL-13 expression. IL-5, IL-6, IL-12, iNOS cytokines production were evaluated but were not detected.

Some works demonstrated that BALB/c and C57BL/6J mice show different responses to solve the criptococcosis pulmonary infection [44,58] and both species are classically used in murine immune response. So we attempted to evaluate the therapeutic treatment using DNA vaccine in C57BL/6J mice. It is very important to use different challenge doses, to assess the effects of host factors on susceptibility to infection. By the way, we evaluated two challenge doses (10^3 and 10^5) and time course of infection, to verify any difference on *C. neoformans* clearance. Both challenges showed no difference in lungs burden, 12 and 17 days post-infection even in the brain of mice challenged with 10^5 cells. However, mice challenged with 10^3 yeast cells, demonstrated a significantly decrease in brain fungal burden. Then, we evaluated the cytokines profile of mice infected with 10^3 yeast cells at 17 days post-infection. As expected, mice treated with pcDNAHsp70 produced more IFN-γ and TNF-α than control (H99). Surprisingly, the same was not observed to mice treated with the empty pcDNA vector, which also had less CFU than the control. The pcDNAHsp70 treatment also increased IL-17 production, which contributes to immune defenses against *C. neoformans* infection [59]. In addition, pcDNAHsp70 induced IL-10 production, which have been related to suppressive response of Hsp70 [22].

Comparison of the results using pcDNACAT and pcDNAHsp70 in C57BL/6J mice, demonstrated that Hsp70 could be a more potent antigen than catalase, since catalase showed same pattern of cytokines profile that controls.

It is very important to consider, that empty pcDNA plasmid is a strong stimulator of the immune system against *C. neoformans* infection due to the reduced pulmonary fungal burden and induced protective cytokines in BALB/c mice. When susceptive mice C57BL/6J were evaluated, empty pcDNA vector did not induce protective defense in the lungs, but avoid brain dissemination. When we look to Hsp70 effects, the treatment with pcDNAHsp70 avoided brain dissemination, independent of the Hsp70 sequence, but also induced a range profile of cytokines that could protect mice against *C. neoformans*

infection.

Taken together, our studies show that although pcDNAHsp70 decreased fungal burden in BALB/c mice, it does not induce protective cytokines production, but develops a Th2-cell mediate immunity, suggesting that Hsp70 could act as suppressive molecule in *C. neoformans* infection using resistance model. In contrast, when we used susceptible mice, it seems that Hsp70 can promotes Th1 cell response to avoid brain dissemination.

Hence, the functional characterization of Hsp70 from *C. neoformans* and its relationship with host cells becomes to extreme importance to evaluate the potential use of this protein in vaccine approaches.

Acknowledgments

This work was supported by grants from FINEP (Financiadora de Estudos e Projetos, Brazil), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Brazil), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Brazil) and Ludwig Biotecnologia. The authors thank Dr. Henrique Bunselmeyer Ferreira (Universidade Federal do Rio Grande do Sul, Brazil for providing the pcDNA3.1(+) plasmid. Automated DNA sequencing was performed at the facilities of the Brazilian Genome Network at the Center of Biotechnology, CBiot-UFRGS-RS.

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Figures and Legends

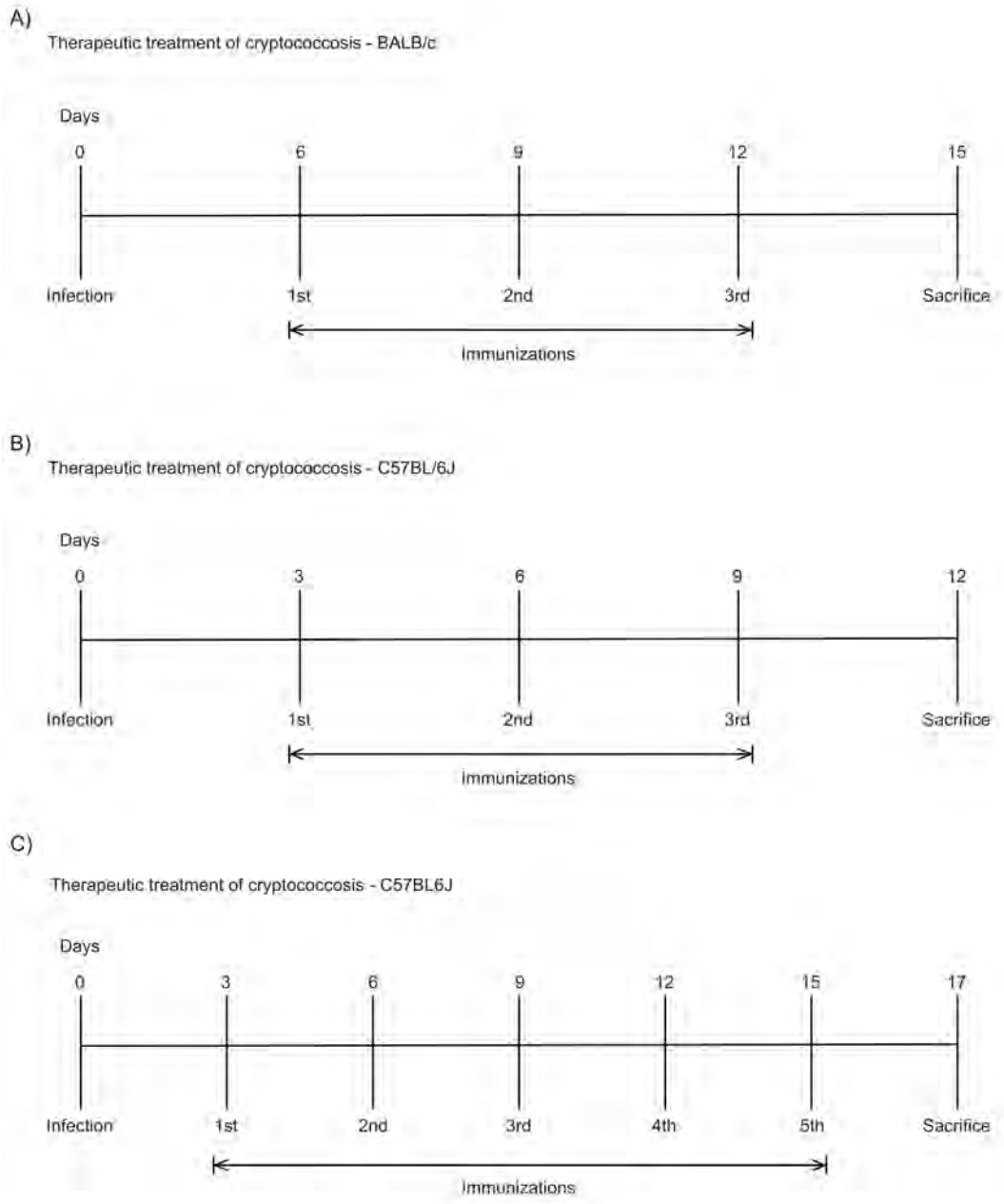


Figure 1. Schematic representation of therapeutic treatment. A) Five days after infection, BALB/c mice received 3 doses of DNA vaccine at 2-days intervals and sacrificed 15 days later. **B)** Two days after infection (mice infected with $2x10^3$ or $2x10^5$ cells) C57BL/6J mice received 3 doses of DNA vaccine at 2-days intervals and sacrificed 12 days later. **C)** Two days after infection (mice infected with $2x10^3$ or $2x10^5$ cells), C57BL/6J mice received 5 doses of DNA vaccine at 2-days intervals and sacrificed 17 days later.

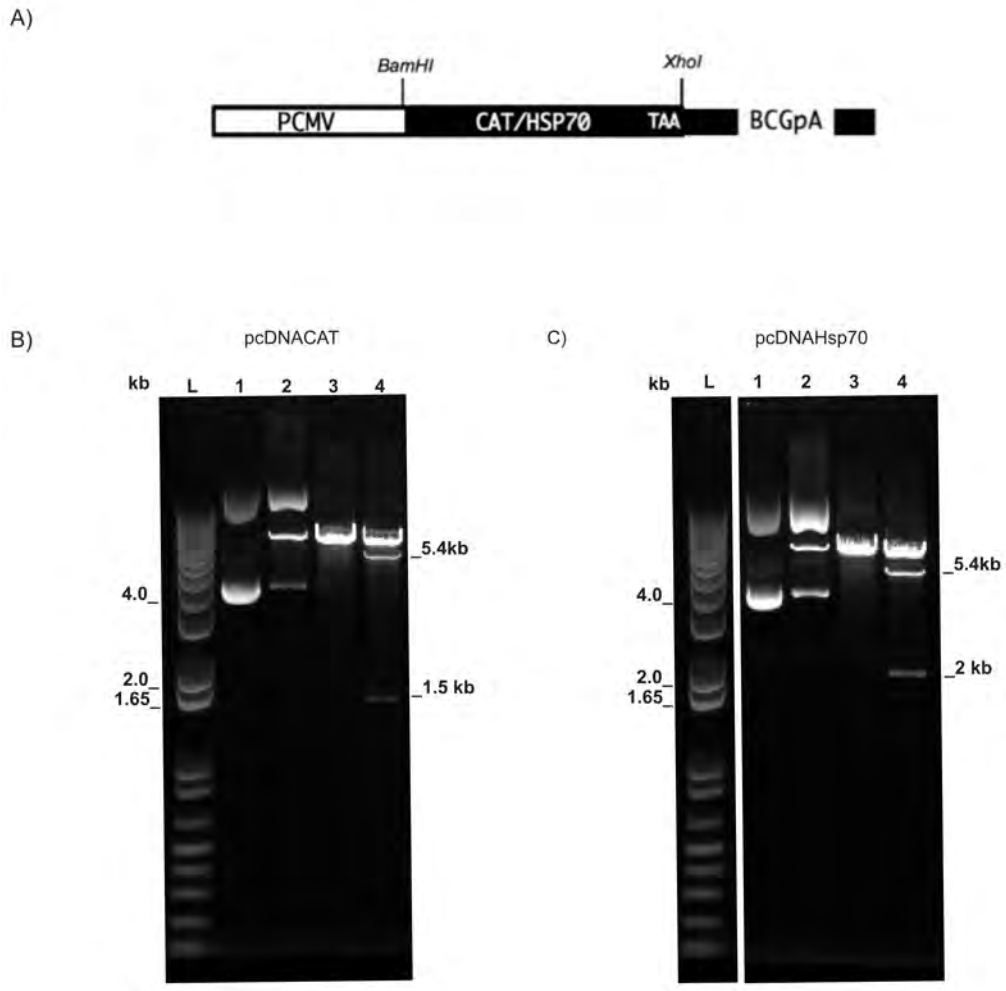


Figure 2. DNA vaccine assembly and confirmation. A) Schematic representation of DNA vaccine plasmid containing HSP70 or catalase gene cloned between BamHI and XhoI into pcDNA3.1(+) vector. **B)** DNA vaccine pcDNACAT plasmid confirmation by restriction enzymes. L:1kb plus ladder; 1: pcDNACAT; 2: pcDNACAT digested with BamHI; 3: pcDNACAT digested with XhoI; 4: pcDNACAT double digested with BamHI and XhoI. **C)** DNA vaccine pcDNAHsp70 plasmid confirmation by restriction enzymes. L:1kb plus ladder; 1: pcDNAHsp70; 2: pcDNAHsp70 digested with BamHI; 3: pcDNAHsp70 digested with XhoI; 4: pcDNAHsp70 double digested with BamHI and XhoI.

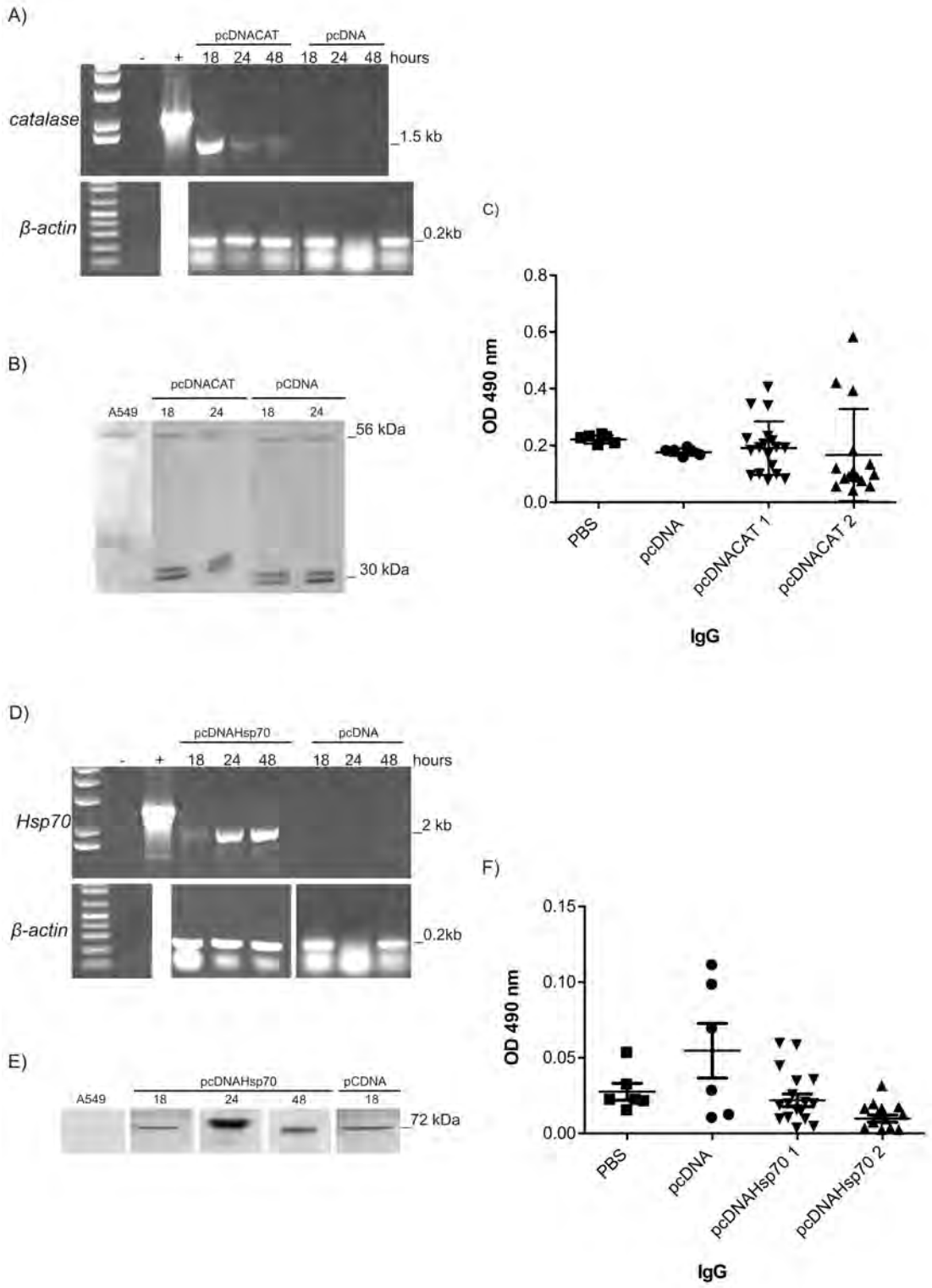


Figure 3. DNA vaccine expression in mammalian cells. A) Catalase and β-actin (internal control) mRNA expression in A549 cells transfected with pcDNACAT and pcDNA plasmids. -:negative control without template; +: positive control with DNA from *C. neoformans*; 18: 18 hours after A549 transfection with pcDNACAT or pcDNA; 24: 24 hours after A549 transfection

with pcDNACAT or pcDNA; 48 hours after A549 transfection with pcDNACAT or pcDNA. **B)** Catalase protein expression in A549 cells transfected with pcDNACAT or pcDNA plasmids. A549: A549 cells non-transfected; pcDNACAT 18: 18 hours after A549 transfection with pcDNACAT; 24: 24 hours after A549 transfection with pcDNACAT; pcDNA 18: 18 hours after A549 transfection with pcDNA; 24: 24 hours after A549 transfection with pcDNA. **C)** IgG production after immunization with PBS, pcDNA, pcDNACAT. pcDNACAT1 corresponds to mouse 1 immunized with pcDNACAT; pcDNACAT2 corresponds to mouse 2 immunized with pcDNACAT. **D)** Hsp70 and β- actin (internal control) mRNA expression in A549 cells transfected with pcDNAHsp70 and pcDNA plasmids. -:negative control without template; +: positive control with DNA from *C. neoformans*; 18: 18 hours after A549 transfection with pcDNAHsp70 or pcDNA; 24: 24 hours after A549 transfection with pcDNAHsp70 or pcDNA; 48 hours after A549 transfection with pcDNAHsp70 or pcDNA. **E)** Hsp70 protein expression in A549 cells transfected with pcDNAHsp70 or pcDNA plasmids. A549: A549 cells non-transfected; pcDNAHsp70 18: 18 hours after A549 transfection with pcDNAHsp70; 24: 24 hours after A549 transfection with pcDNAHsp70; 48: 48 hours after A549 transfection with pcDNAHsp70; pcDNA 18: 18 hours after A549 transfection with pcDNA. **F)** IgG production after immunization with PBS, pcDNA, pcDNAHsp70. pcDNAHsp70 1 corresponds to mouse 1 immunized with pcDNAHsp70; pcDNAHsp70 2 corresponds to mouse 2 immunized with pcDNAHsp70.

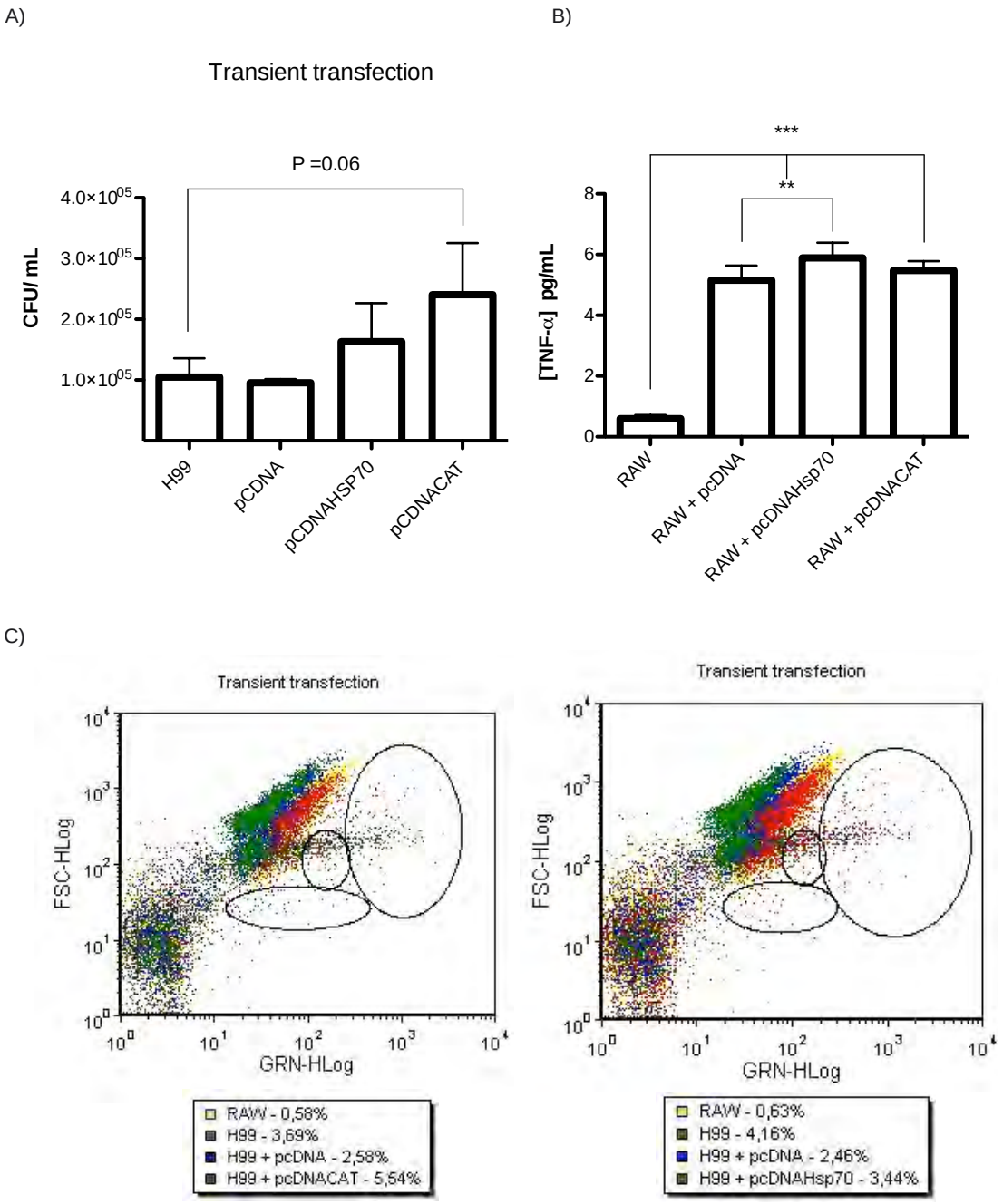


Figure 4. Macrophage cell interaction with *C. neoformans* after transient transfection with pcDNA, pcDNACAT and pcDNAHsp70. RAW 264.7 cells were transfected with pcDNA, pcDNACAT or pcDNAHsp70 and assayed for fungal survival, TNF-α production and phagocytosis rates. **A)** Fungal survival by viable yeast cells recovery from macrophages lysed 18 hours after incubation. **B)** TNF-α production after transient transfection and interaction with yeast cells. **C)** Phagocytosis rates after transient transfection and interaction with yeast cells. Left panel indicates phagocytosis rates of macrophages transfected with pcDNACAT. Right panel indicates

phagocytosis rates of macrophages transfected with pcDNAHsp70. Macrophages non-transfected and incubated with *C. neoformans* cells were used as control. H99 represents macrophages non-transfected and incubated with *C. neoformans* H99 cells. pcDNA represents macrophages transfected with pcDNA and incubated with *C. neoformans* H99 cells. pcDNACAT represents macrophages transfected with pcDNACAT and incubated with *C. neoformans* H99 cells. pcDNAHsp70 represents macrophages transfected with pcDNAHsp70 and incubated with *C. neoformans* H99 cells.

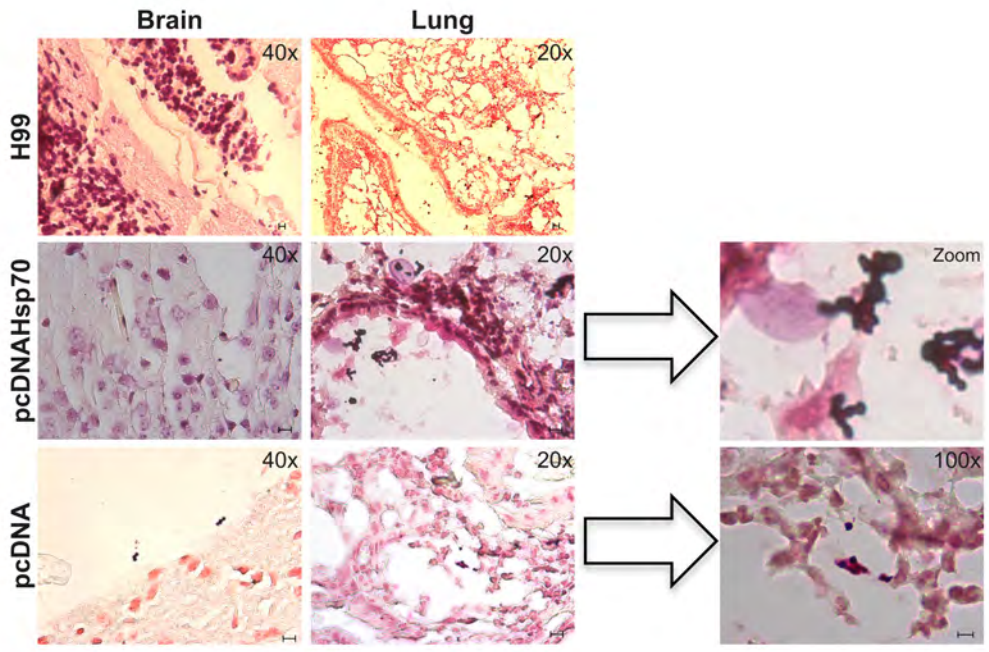


Figure 5. Histopathology of lungs from intranasally infected BALB/c mice. Animals were infected with *C. neoformans* for 5 days, treated with or without vectors carrying Hsp70 sequence according to schematic representation (Fig.1A), and sacrificed 15 days after the initial infection. Infected mice and non- treated (H99), treated with pcDNAHsp70 or pcDNA. Haematoxylin-eosin staining; original magnification, 40X for the brain and 20X for the lungs. Arrows indicate yeast cells.

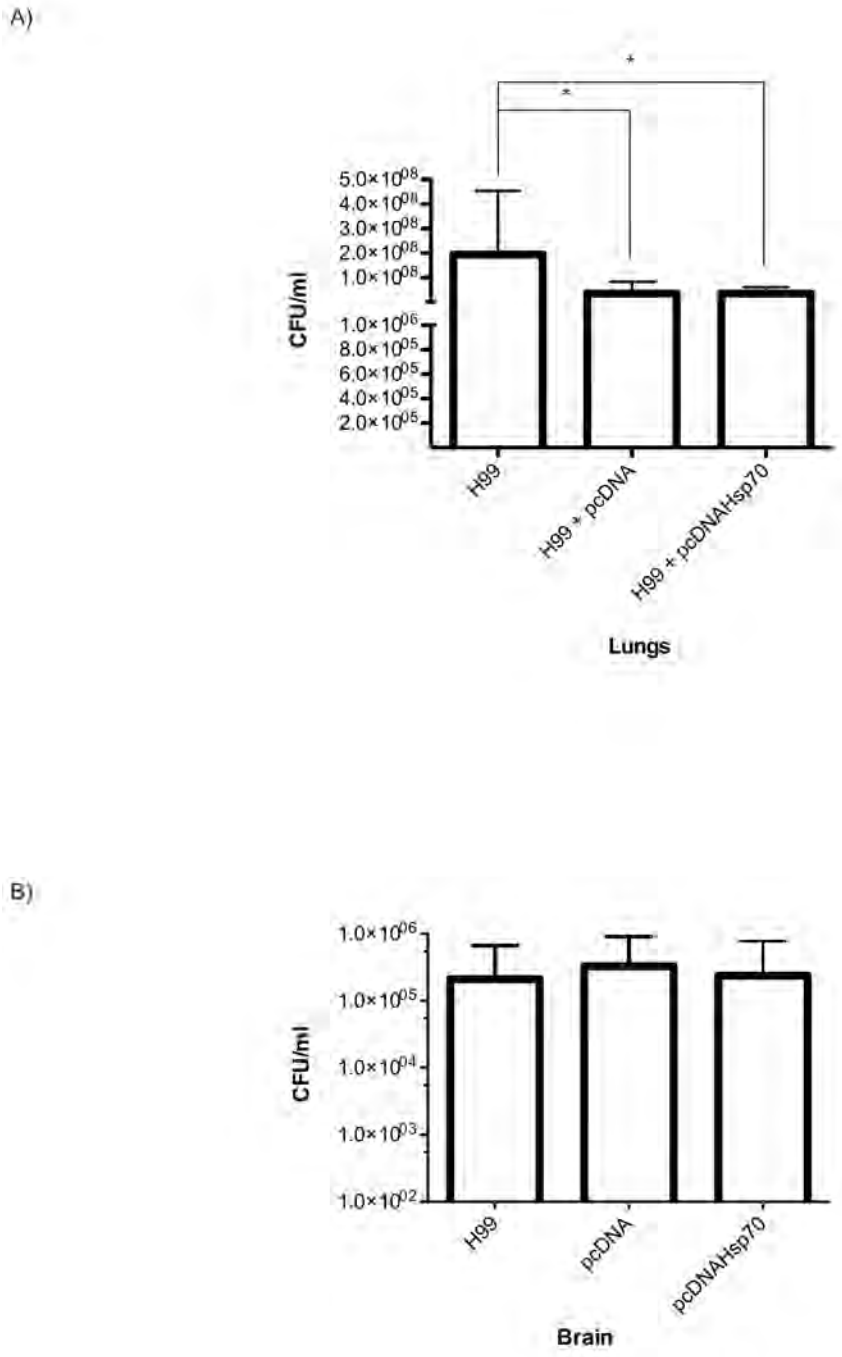
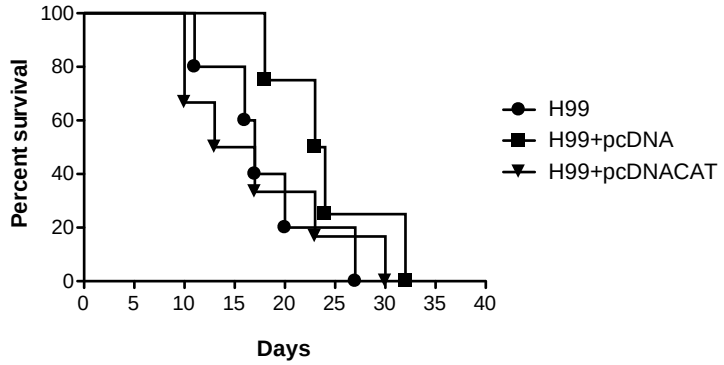


Figure 6. Effect of pcDNA in therapeutic treatment of criptococose in BALB/c mice. BALB/c mice infected intranasally with 2X10^5 yeast cells and treated 5 days after infection. Control mice were treated with PBS. Mice were sacrificed 15 days after infection. Each bar represents the average counts and standard deviations of CFU in lungs **(A)** and brain **(B)** from 10 animals in each group. Data are representative of two independent experiments.

A)



B)

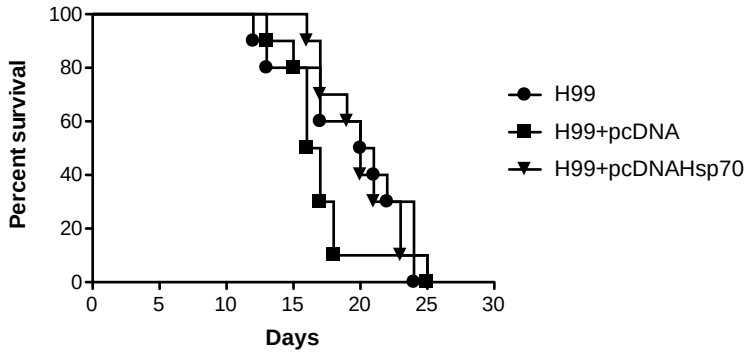


Figure 7. Therapeutic effect of pcDNA on mice survival. A) Mice were treated with 100 µg/mouse pcDNA or pcDNACAT, on days 6, 9 and 12 days after infection and survival curve was determined. Mice were treated with 100 µg/mouse pcDNA or pcDNAHsp70, on days 6, 9 and 12 days after infection and the number of dead mice was noted daily. **B)** Alternatively, mice were treated with 100 µg/mouse pcDNA or pcDNAHsp70, on days 6, 9 and 12 days after infection and the number of dead mice was noted daily.

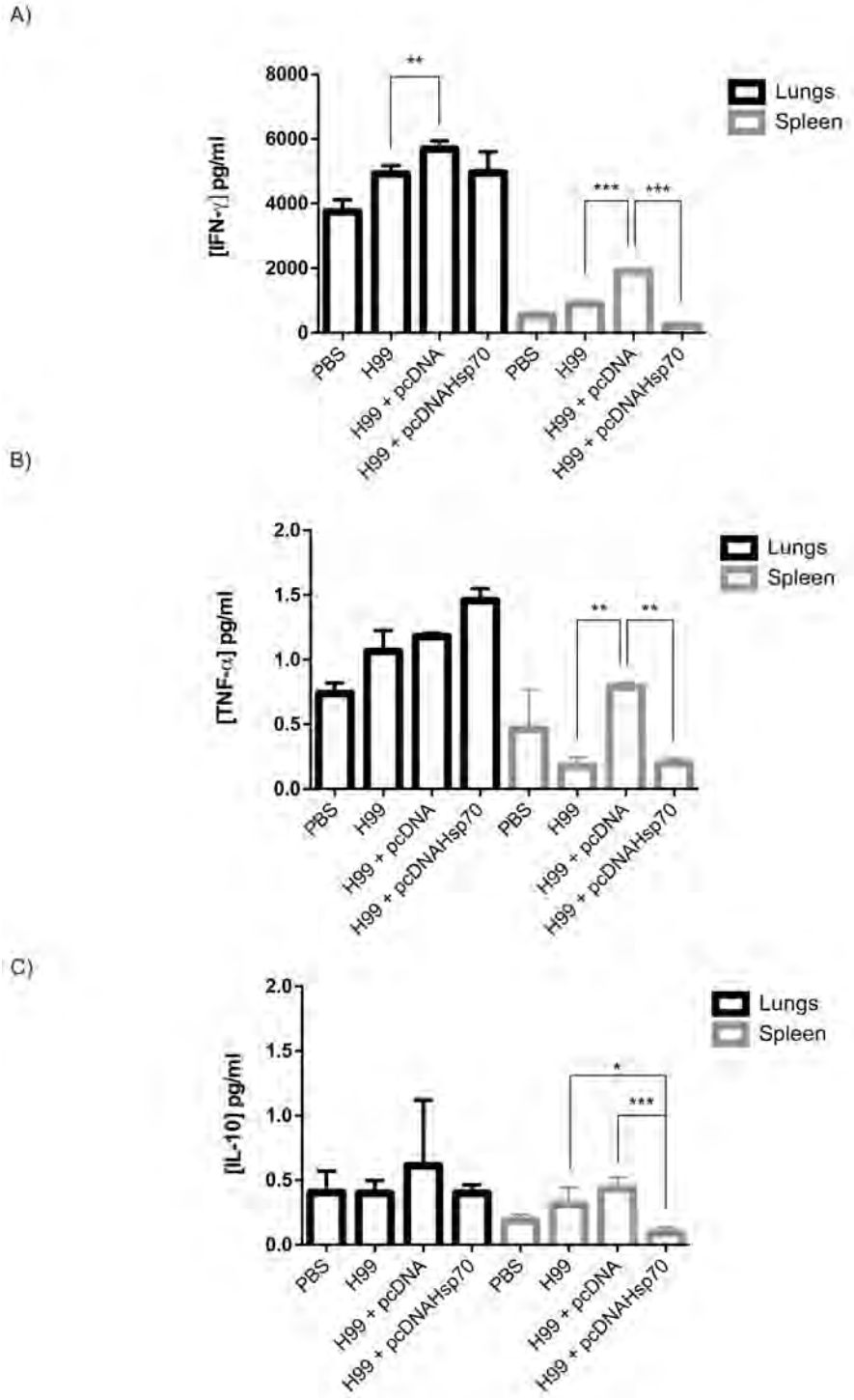


Figure 8. Therapeutic effect of pcDNA on the production of cytokines in BALB/c mice after cryptococcal infection. Mice infected intranasally with *C. neoformans* H99 strain were treated with 100 µg of pcDNA, pcDNAHsp70 on days 6, 9 and 12 days after infection. Controls mice were treated with PBS (H99) or were non-infected (PBS). The concentrations of interleukin IFN-γ **(A)**, interferon TNF-α **(B)** and IL-10 **(C)** in lungs (left panels) and spleen (right panels) homogenates were measured on days 15 post-infection.

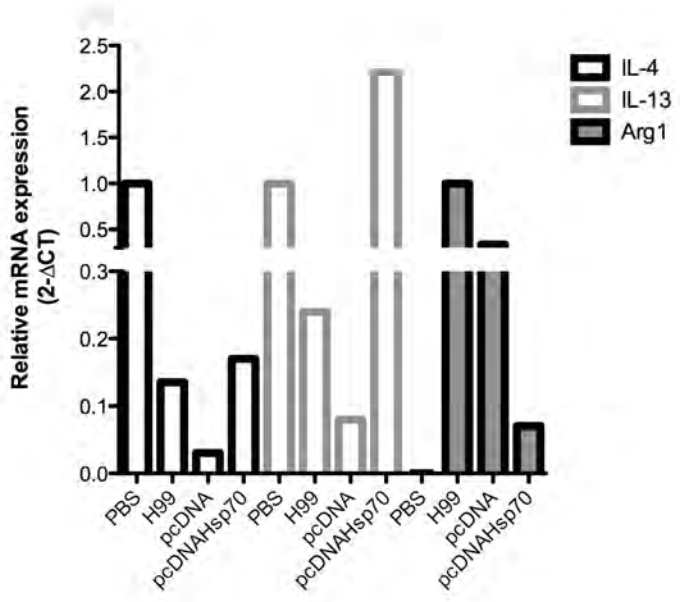


Figure 9. Therapeutic effect of pcDNA on the IL-13, IL-4 and Arg1 production in BALB/c mice after cryptococcal infection. A) IL-4, IL-13 and Arg1 mRNA expression in lungs. RNA was extracted from organ homogenates after 15 days after intranasal infection and expression of cytokines was evaluated by real-time RT-PCR.

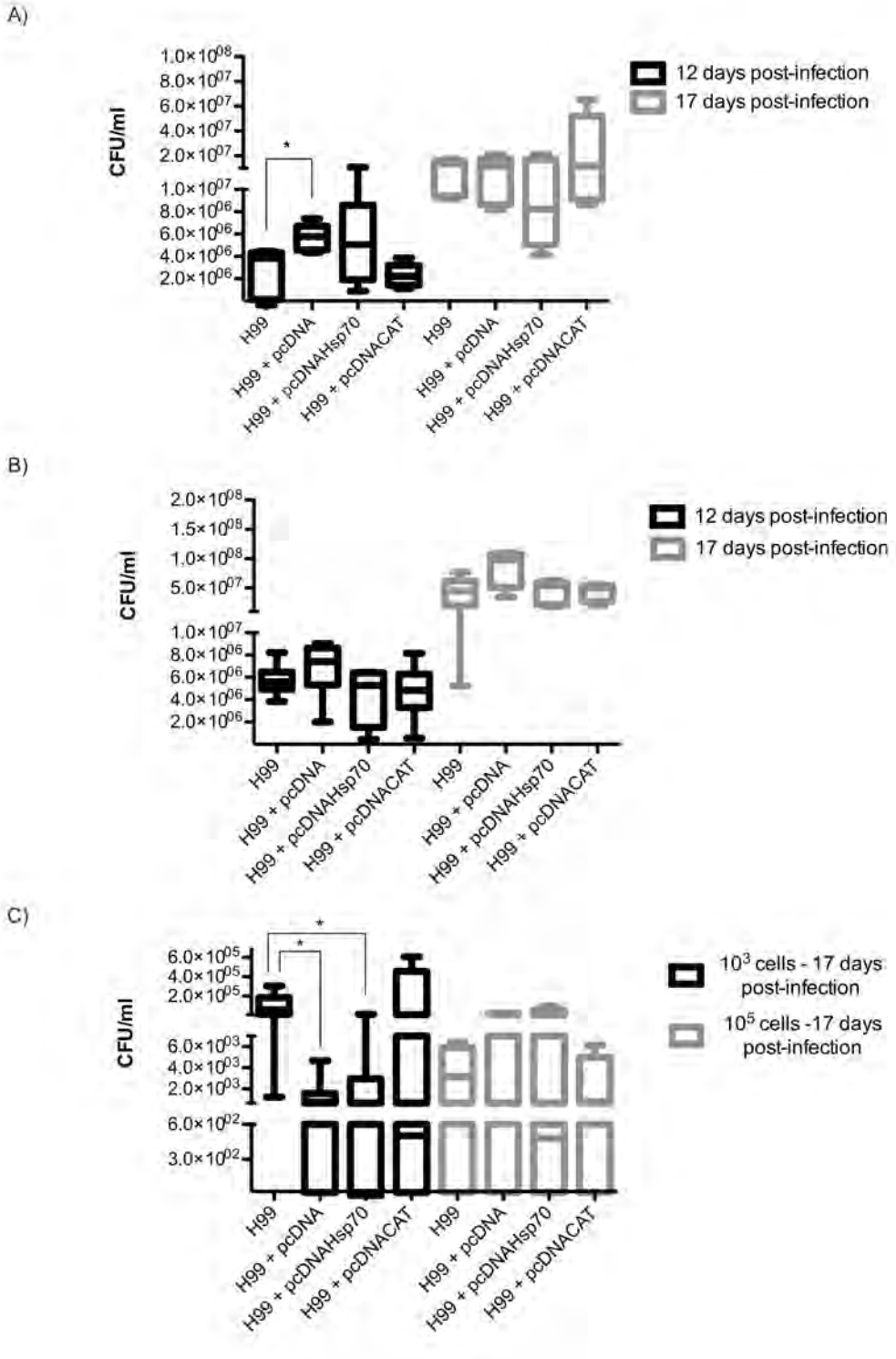


Figure 10. Effect of pcDNA in therapeutic treatment of criptococose in C57BL/6J mice. Mice infected intranasally with $2X10^3$ or $2X10^5$ yeast cells and treated 2 days after infection. Control mice were treated with PBS. Mice were sacrificed 12 or 17 days after infection. **A)** CFUs are from lungs of mice infected intranasally with $2X10^3$ yeast cells, subjected to immunization with

vectors containing pcDNA, pcDNAHsp70 and pcDNACAT, sacrificied 12 days (left panels) or 17 days post-infection (right panels). **B)** CFUs are from lungs of mice infected intranasally with $2X10^5$ yeast cells, subjected to immunization with vectors containing pcDNA, pcDNAHsp70 and pcDNACAT, sacrificied 12 days (left panels) or s 17 days post-infection (right panels). **C)** CFUs are from brain of mice infected intranasally with $2X10^3$ yeast cells, subjected to immunization with vectors containing pcDNA, pcDNAHsp70 and pcDNACAT and sacrificied 17 days post-infection.

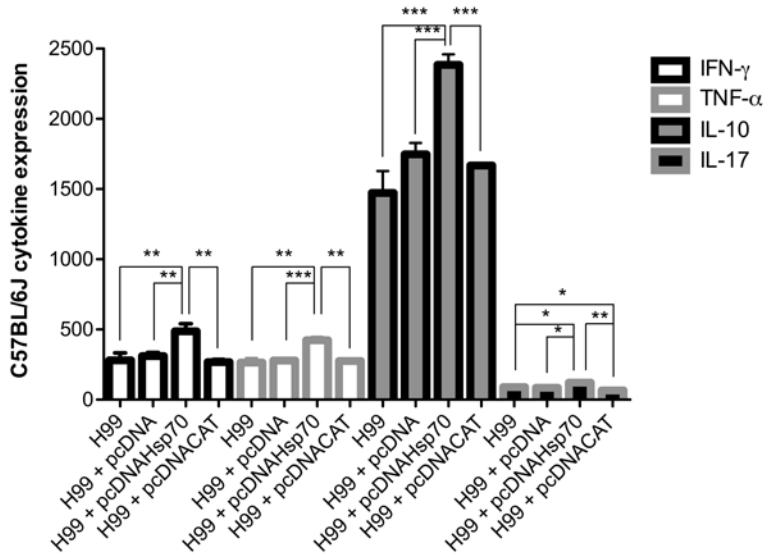


Figure 11. Therapeutic effect of pcDNA on the production of cytokines in C57BL/6J mice after cryptococcal infection. Mice infected intranasally with $2X10^3$ yeast cells were treated with 100 µg of pcDNA, pcDNAHsp70 or pcDNACAT on days 3, 6, 9 or 12 days after infection. Controls mice were treated with PBS (H99) or were non-infected (PBS). The concentrations of interleukin IFN-γ, TNF-α, IL-10 and IL-17 in lungs homogenates from mice were measured on days 17 post-infection.

4.0 DISCUSSÃO

Muitas proteínas expressas pela célula fúngica estão envolvidas na manutenção da homeostase e na defesa contra estresse, seja ele oxidativo/nitrosativo ou causado por mudança de temperatura (Gessler *et al*., 2007). Nesse sentido, a levedura *C. neoformans* precisa adaptar-se às condições ambientais para burlar o sistema imune do hospedeiro e conseguir estabelecer a infecção (Brown *et al*., 2007). O sistema de defesa antioxidativo de muitos fungos patogênicos possui muitas moléculas com funções sobrepostas e compensatórias, evitando assim, danos na célula causada por espécies reativas (Holdbrook *et al*., 2013).

A levedura *C. neoformans* possui um arsenal de fatores de virulência que favorecem o estabelecimento da infecção em pacientes imunocomprometidos, tornando-o um patógeno oportunista. A adaptação desta levedura para sobreviver no ambiente intracelular do hospedeiro está intimamente relacionada com o processo de evolução que este microrganismos sofreu no seu habitat natural (Steenbergen *et al*., 2001; Chrisman *et al*., 2010; Derengowski *et al*., 2013).

Muitas proteínas conservadas, que estão envolvidas na regulação metabólica ou em resposta ao estresse, possuem funções biológicas adicionais nas quais podem influenciar na virulência de microrganismos. Estas proteínas são chamadas de proteínas *moonlighting* e podem exercer estas funções adicionais quando encontradas em localizações diferentes das normalmente encontradas (Henderson *et al*., 2011). Proteínas de choque térmico possuem funções de chaperona e foram identificadas como as principais proteínas *moonlighting* em bactérias. Elas podem ativar monócitos

e a produção de citocinas, serem proteínas de superfície envolvidas na ligação à macrófagos e plasminogênio, dentre outras funções (Henderson *et al*., 2011). Proteínas *moonligthing* já foram bem caracterizadas em leveduras e estão, principalmente envolvidas no processo da glicólise e na transcricão de genes (Gancedo & Flores, 2008; Flores & Gancedo, 2011). Apesar da importância na biologia dos microrganismos, proteínas Hsps e catalases são pouco estudadas no modelo *C. neoformans*. Além disso, nenhum proteína *moonlighting* foi descrita para este fungo patogênico.

Muitos estudos têm utilizado ferramentas proteômicas para identificar os genes envolvidos no processo de infecção de *Cryptococcus,* bem como na identificação de potenciais antígenos para uma nova abordagem terapêutica (Young *et al.,* 2009; Crestani *et al*., 2012; Upadhya *et al*., 2013; Martins *et al*., 2013).

Uma análise proteômica realizada pelo nosso grupo, identificou duas proteínas de *C. neoformans*, catalase (CNAG_05256.2) e Hsp70 (CNAG_05199.2) (Polese, M., 2009), cujos anticorpos estavam presentes no soro de pacientes com criptococose. Nesse contexto, o presente trabalho foi desenvolvido com o objetivo principal de estudar a função biológica de uma Hsp70 e de uma catalase de *C. neoformans* e avaliar o efeito terapêutico destas proteínas no tratamento da criptococose.

Giles *e colaboradores* (2006) construíram mutantes nulos da família inteira de de genes que codificam para catalase em *C. neoformans* e não observaram atenuação da virulência em modelo murino. Além disso, os mutantes gerados não apresentaram sensibilidade à estresse oxidativo

exógeno, indicando um mecanismo complexo e compensatório de defesa antioxidante nesta levedura.

O anticorpo policlonal gerado a partir da proteína recombinante, Cn_rCAT2, foi utilizada para identificar a localização desta proteína nas células de *C. neoformans.* As análises de imunofluorescência demonstraram uma localização na parede celular da levedura e esta enzima localizada na superfície possui atividade de catalase, o que pode auxiliar no mecanismo de defesa antioxidativo. A proteína Hsp70 também foi localizada na superfície celular, porém associada a cápsula polissacarídica. Ambas as proteínas não possuem peptídeo sinal e portanto, parecem ser transportadas para o meio extracelular através de vesículas. Estes dados são confirmados pela identificação destas duas proteínas nas vesículas secretadas por mecanismos não-convencionais em *C. neoformans* (Rodrigues *et al*., 2008). Além disso, catalase e Hsp70 já foram identificadas na superfície celular de fungos patogênicos (Long *et al*., 2003; Nogueira *et al*., 2010; Lopes *et al*., 2010).

A interação de ambas as proteínas com células epiteliais foi avaliada para verificar se estas proteínas influenciam no processo de adesão das células da levedura ao hospedeiro. A proteína Hsp70 está envolvida no processo de adesão de *C. neoformans* à célula epitelial, indicando uma função adicional desta chaperona no processo de infecção. Portanto, este trabalho indica, pela primeira vez, a existência de uma proteína *moonlighting* em *C. neoformans* que está envolvida na adesão, podendo assim, influenciar no processo de infecção. A catalase 2 parece influenciar no processo da adesão e não influenciar na ativação dos macrófagos; já macrófagos

incubados com a proteína recombinante Hsp70 produzem menos NO indicando que a presença da proteína na superfície celular pode modular negativamente a resposta imune do hospedeiro.

A GXM, principal componente da cápsula polissacarídica, é reconhecida pelos receptores CD14, TLR2, and TLR4 *in vitro* (Levitz, 2002; Yauch *et al*., 2004; Monari *et al*., 2005). Já foi descrito que TLR2 e TLR4 também reconhecem Hsp70 (Thèriault *et al*., 2005). Nesse sentido, fomos avaliar a interação da GXM com Hsp70 ou catalase 2 à superfície de macrófagos e células epiteliais. GXM e catalase 2 não compartilham o mesmo sítio de ligação em macrófagos. Porém, GXM e Hsp70 são capazes de dividir a ligação pelo mesmo receptor em ambos os tipo celulares testados. Pelos experimentos de imunofluorescência, fica evidente que a ligação de Hsp70 facilita a ligação de GXM na superfície celular do hospedeiro. Esta interação parece ocorrer através de TLR4, pois quando o antagonista deste receptor é utilizado, a co-localização diminui.

O tamanho da cápsula polissacarídica, um dos principais fatores de virulência, é modulado pelo ambiente em que a célula do fungo se encontra (O'Meara & Alspaugh, 2012). Por outro lado, mutantes nulos para o gene *SSA1* de *C. neoformans*, que codificam para uma Hsp70, possuem um fenótipo de cápsula aumentada e possuem a virulência atenuada. *SSA1* é um gene homólogo ao gene que codifica para uma Hsp70 (Zhang *et al*., 2006). Portanto, acreditamos que a proteína Hsp70 modula o tamanho da cápsula polissacarídica, influenciando assim, na virulência de *C. neoformans.* A proteína recombinante altera a viabilidade de células da levedura, o que pode levar à alteração do tamanho da cápsula e da secreção de GXM no ambiente

extracelular. Além disso, também foram observadas alterações da morfologia da levedura e a presença de células menores.

Hsp60 e Hsp70 são proteínas imunogênicas que ativam imunidade celular mediada por células T (Zügel & Kaufmann, 1999). Muitos trabalhos avaliam o uso destas proteínas bem como dos anticorpos específicos para estes antígenos no tratamento de infecções fúngicas (Gomez *et al*., 1995; Matthews *et al*., 2003; de Bastos *et al.,* 2008). Porém, em algumas infecções causadas por fungos patogênicos, Hsp parece não conferir proteção (Li *et al*., 2001). Outra abordagem que está sendo utilizada no tratamendo de infecções fúngicas é a utilização de vacina de DNA (Ribeiro *et al.*, 2009; Ribeiro *et al*., 2010). A vacina de DNA contendo o gene da Hsp70 e da catalase foi utilizada neste estudo no tratamento terapêutico de camundongos infectados com células de *C. neoformans.*

Células de macrófagos transfectadas com o plasmídeo pcDNACAT foram capazes de fagocitar mais células da levedura e estas permaneceram viáveis dentro do fagócito quando comparadas com o controle. Este resultado é um indicativo de que a catalase quando expressa dentro da célula pode promover a proteção do fungo contra as espécies reativas de oxigênio. Além disso, a transfecção dos macrófagos com os plasmídeos pcDNA ativou as células do hospedeiro a produzir TNF-α.

Demonstramos que o tratamento terapêutico utilizando o plasmídeo pcDNA e pcDNAHsp70 reduziu a concentração de levedura encontrada nos pulmões de camundongos BALB/c infectados com *C. neoformans*, apesar de não aumentar a sobrevivência dos animais. Além disso, pcDNAHsp70 não induziu à resposta celular mediada por células Th1, sugerindo que ela pode

agir como uma molécula imunosupressora como descrito por Borges e colaboradores (2012).

Nosso estudo permitiu identificar novas proteínas possivelmente envolvidas no processo de infecção causados por *C. neoformans.* Essas proteínas parecem desempenhar funções adicionais que contribuem para o estabelecimento da infecção e podem ajudar a levedura a burlar o sistema imune do hospedeiro favorecendo o desenvolvimento da doença. Entender o mecanismo pelo qual estas proteínas contribuem para o processo de infecção é de suma importância uma vez que podem tornar-se alvo de um tratamento alternativo contra criptococose.

5.0 CONCLUSÃO

- A enzima catalase está localizada na superfície celular de *C. neoformans*, principalmente depositada na parede celular e possui atividade de degradação de peróxido de hidrogênio. Esta proteína é reconhecida pelo soro de pacientes com criptococose.
- A proteína recombinante catalase, Cn_rCAT não possui atividade antifúngica, não influencia na produção do pigmento melanina e não confere proteção em modelos animais de criptococose.
- Catalase é capaz de se ligar à superfície de macrófagos, porém em sítios de ligação diferentes do polissacarídeo capsular, GXM.
- Catalase pode influenciar no processo de adesão da levedura à células epiteliais.
- A proteína recombinante Cn_rHsp70 é reconhecida pelo soro de pacientes com criptococose.
- Hsp70 está localizada na superfície da célula de *C. neoformans*, principalmente associada à capsula polissacarídica.
- Cn_rHsp70 não influencia no processo de fagocitose de células de levedura por macrófagos.
- Cn_rHsp70 é capaz de se ligar à superfície de macrófagos com alta afinidade, já para células epiteliais, Cn_rHsp70 se liga com menor afinidade. Porém, esta proteína influencia no processo de adesão das células de levedura as mesmas células epiteliais.
- Cn_rHsp70 parece favorecer a ligação de GXM nas células de macrófagos, tendo ligação nos mesmos sítios que este polissacarídeo.

- Cn_rHsp70 altera a viabilidade celular quando em contato com as células de levedura, o diâmetro da cápsula e influencia na secreção do polissacarídeo para o ambiente extracelular.
- O tratamento terapêutico utilizando vacina de DNA contendo as sequências de catalase e Hsp70 não conferiram proteção em modelos animais.
- Cn_rHsp70 não possui atividade antifúngica, não interfere na melanização das células do fungo e não confere proteção quando associada à anfotericina B no tratamento de animais infectados com *C. neoformans.*
- Hsp70 foi localizada na superfície dos mutantes Snf7, porém a marcação foi diminuída nos mutantes acapsulares CAP67, indicando sua associação com a cápsula polissacarídica.
- Cn_rHsp70 induz a produção de IL-12 e IL-5 por macrófagos.
- Cn_rHsp70 parece se ligar à superfície dos macrófagos via TLR2 e TLR4.
- Células de macrófagos transfectadas com o plasmídeo pcDNACAT apresentaram um aumento no número de células de *C. neoformans* fagocitadas e viáveis em relação aos controles, indicando um provável mecanismo de proteção da célula do fungo pelo plasmídeo. Além disso, a transfecção dos macrófagos com os plasmídeos pcDNA, pcDNACAT ou pcDNAHsp70 foi capaz de ativar macrófagos a produzir TNF-α.
- Apesar de não conferir proteção, camundongos tratados com o plasmídeo pcDNAHsp70 tiveram um aumento na produção de IFN-γ.

- O padrão de resposta de camundongos C57BL/6J infectados com *C. neoformans* e tratados com as vacinas de DNA foi diferente do observado para a linhagem BALB/c. O tratamento com o plasmídeo pcDNA e pcDNAHsp70 não foi suficiente para conter a infecção pulmonar, porém reduziu o número de células fúngicas no cérebro.

6.0 PERSPECTIVAS

- Avaliar a atividade da catalase localizada na superfície em cultivos contendo peróxido de hidrogênio e em células opsonizadas com anticorpo anti-CAT. Com este experimento espera-se aumentar a atividade da enzima devido ao aumento da expressão da catalase localizada na superfície celular. A opsonização da célula de *C. neoformans* será utilizada como controle da atividade, onde espera-se que haja um bloqueio desta quando as células forem tratadas com o anticorpo.
- Contruir uma linhagem mutante de *C. neoformans*, contendo uma proteína de fusão da catalase com uma proteína fluorescente e avaliar as condições de cultivo em que esta catalase é expressa.
- Avaliar a função da catalase fusionada com a proteína fluorescente em infecções experimentais de criptococose.
- Identificar o mecanismo pelo qual Cn_rHsp70 influencia na viabilidade celular, no tamanho do diâmentro capsular e liberação de GXM no sobrenadante. Para isso, células de levedura tratadas com a proteína recombinante serão analisadas quanto à expressão de genes envolvidos na formação da cápsula polissacarídica.
- Identificar qual é o receptor que medeia a ligação de ambas as moléculas, GXM e Hsp70.
- Ensaios de interação proteína/proteína para verificar quais as proteínas interagem com Hsp70.

- Realizar experimentos de competição de ligação entre GXM e Hsp70 no respectivo receptor para tentar entender como ocorre o reconhecimento de Hsp70 e/ou GXM no processo de adesão e internalização.
- Caracterizar o tipo de células T que são ativadas no tratamento com pcDNA vazio.
- Avaliar a associação do plasmídeo pcDNA vazio com anfotericina B no tratamento de camundongos infectados com células de *C. neoformans*.

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