Universidade de Brasília
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Análise da expressão gênica de macrófagos murinos infectados por fungos patogênicos (*Paracoccidioides brasiliensis e Histoplasma capsulatum*)

Simoneide Souza Silva

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Análise da expressão gênica de macrófagos murinos infectados por fungos patogênicos (*Paracoccidioides brasiliensis e Histoplasma capsulatum*)

Tese de doutorado apresentada ao Programa de Pós Graduação em Biologia Molecular para obtenção de título de Doutor em Ciências Biológicas - Biologia Molecular.

Orientada: Simoneide Souza Silva

Orientadora: Maria Sueli Soares Felipe

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ºC	Graus Celsius
BSA	Albumina de soro bovino
cDNA	Seqüência de DNA complementar
cm	Centímetro
DNA	Ácido desoxirribonucléico
DNAse	Desoxirribonuclease
dNTP	Desoxirribonucleotídeos 5'-trifosfato
DTT	Ditiotreitol
EDTA	Ácido etilenodiaminotetracético
EST	Expressed Sequence Tag
g	Grama
G	Força gravitacional
H	Hora
L	Litro
M	Molar
mg	Miligrama
min	Minutos
mL	Mililitro
mM	Milimolar
MM	Massa molecular
Mn	Magnésio
mRNA	Ácido ribonucléico mensageiro
ηg	Nanograma
pb	Pares de bases
PCR	Reação em cadeia da polimerase
pH	Potencial hidrogeniônico
pmol	picomoles
p/v	peso/volume
RNA	Ácido ribonucléico
RNAse	Ribonuclease

rpm	Rotações por minuto
RT	Transcrição reversa
RT-PCR	Transcrição Reversa- Reação em cadeia da polimerase
SDS	Sódio dodecil sulfato
TA	Temperatura ambiente
TE	Tampão Tris-EDTA
TEB	Tampão Tris-EDTA-borato
Tris	Tri(hidroximetil)aminometano
UE	Unidade enzimática
UV	Radiação ultravioleta
v	Volume
V	Volts
YPD	Meio de cultura Yeast-Peptone-Dextrose (levedura-peptona-açúcar dextrose)
μg	Micrograma
μL	Microlitro
μm	Micrômetro
μM	Micromolar
%	Por cento

RESUMO

Paracoccidioides brasiliensis e *Histoplasma capsulatum* são os agentes etiológicos da Paracoccidioidomicose e Histoplasmose, respectivamente. *Paracoccidioides brasiliensis* causa uma micose sistêmica em humanos com maior incidência na América Latina enquanto *Histoplasma capsulatum* pode ser encontrado principalmente nos Estados Unidos. A regulação gênica global de genes envolvidos na resposta inata contra estes fungos é pouco conhecida. No presente trabalho foi investigado o perfil transcricional de macrófagos infectados com *P. brasiliensis* e *Histoplasma capsulatum*. O RNA total de macrófagos infectados e não infectados foi extraído, hibridizado contra microarranjos de cDNA e analisados. A abordagem cinética usada neste trabalho para analisar o perfil transcricional de macrófagos murinos durante a interação tanto com *P. brasiliensis* quanto por *H. capsulatum* proporcionou um melhor entendimento dos eventos que culminam na ativação coordenada e temporal de moléculas de defesa durante esta interação. Os resultados encontrados neste trabalho indicam que as leveduras destes patógenos induzem nos macrófagos uma modulação gênica dinâmica de moléculas que podem favorecer inicialmente a sobrevivência destes fungos no interior dos fagócitos. Por outro lado, observou-se nesta interação que os macrófagos parecem responder induzindo genes relacionados a atividade fungicida, possivelmente na tentativa de conter a disseminação do fungo. Adicionalmente, considerando as análises simultâneas de expressão gênica diferencial para o *P. brasiliensis,* descrito por nosso grupo em trabalho anterior, após 6 horas de infecção, foi proposto um modelo de interação para o *P. brasiliensis*-macrófago. Dessa forma, a indução da expressão de genes pelo fungo importantes para sua adaptação no microambiente do macrófago aliado a indução de moléculas do macrófago relacionados a quimiotaxia e fagocitose sugere que a invasão de novas células pelo fungo pode ser uma estratégia favorável para esse patógeno se manter viável e se multiplicar no hospedeiro.

ABSTRACT

Paracoccidioides brasiliensis and *Histoplasma capsulatum* are the thermo-dimorphic fungi that cause the Paracoccidioidomycosis and Histoplasmose, respectively. *Paracoccidioides brasiliensis* causes a human systemic mycosis with high incidence in Latin America and *Histoplasma capsulatum* is specially found in the United States. Little is known about the global regulation of genes involved in the innate immune host response to this fungus. In the presente work was investigated the kinetic profile of peritoneal macrophage infected with *P. brasiliensis* and *Histoplasma capsulatum*. Total RNA from infected and non infected macrophages was extracted, hybridized onto arrays and analyzed. The results showed in this work to suggest which the pathogens have been induced in macrophages a dynamic gene modulation that can create favorable conditions for fungal persistence. By the other hand the macrophages to induced genes related with fungicide activity, probably in a tentative of elimination of the fungus. In addition, considering the simultaneous analyses of differential gene expression for the *P. brasiliensis* reported before by our group, at six hours post infection, was proposed a model at molecular level for the *P. brasiliensis*-macrophage early interaction. In this regard, the induction of gene expression by *P. brasiliensis* specially related which its adaptation in macrophage microenvironment of macrophage probably is a strategy developed by this fungus for its viability into the host cells.

1. INTRODUÇÃO

1.1.   Os fungos dimórficos *Paracoccidioides brasiliensis* e *Histoplasma capsulatum*

Paracoccidioides brasiliensis e *Histoplasma capsulatum* pertencem ao grupo dos fungos dimórficos que apresentam ampla distribuição geográfica e podem causar doenças significativas que variam de uma infecção transitória a uma micose sistêmica de tratamento prolongado. Estes fungos apresentam-se como micélio a 22-25ºC e se diferenciam em células leveduriformes a 35-37ºC que constituem a forma patogênica dos mesmos. O mecanismo de transição dimórfica dependente de temperatura é fundamental para o estabelecimento da infecção por estes fungos, sendo desencadeado por uma série de fatores relacionados à interação patógeno – hospedeiro (Restrepo, 1986; Odds *et al.*, 2001; Gow *et al.*, 2002).

A infecção por esses fungos se dá pela inalação de propágulos em suspensão que vão se alojar predominantemente nos pulmões. Ao atingir o alvéolo pulmonar, o fungo transforma-se em célula leveduriforme adaptada à temperatura corporal (Restrepo, 1986; Medoff *et al.*, 1987; Kimberlin *et al.*, 1981; Newman *et al.*, 1990). Uma vez no organismo o fungo pode ser completamente destruído ou então persistir e multiplicar produzindo lesão tecidual. A evolução e as conseqüências da infecção vão depender da interação de fatores relacionados ao fungo, como virulência e composição antigênica; e do hospedeiro, como características genéticas e imunidade e ao meio ambiente (Calich *et al.*, 1985 e 1987).

O granuloma é a lesão fundamental causada por *P. brasiliensis* e *H. capsulatum* e assim como em outras doenças infecciosas (tuberculose e hanseníase) é o resultado de uma reação de hipersensibilidade tardia (DTH) contra os antígenos do agente infeccioso (De Brito & Franco, 1994; Romani, 1997). O termo granuloma se traduz morfologicamente, por coleção focal, mais ou menos compacta e organizada de fagócitos mononucleares e suas células derivadas, células epitelióides e células gigantes multinucleadas, acompanhada ou não de elementos acessórios como linfócitos, plasmócitos, eosinófilos, neutrófilos, mastócitos, fibroblastos e necrose (Kobayashi *et al.*, 2001; Williams & Williams, 1983). Um granuloma bem formado consiste de uma interface inflamatória envolvida por matriz extracelular que contém tanto células inflamatórias quanto o patógeno. A vantagem da formação do granuloma para o hospedeiro é que ele isola a inflamação, protege o tecido saudável ao redor, controla o crescimento do patógeno e previne disseminação sistêmica. Ao mesmo tempo o microrganismo também pode se beneficiar da localização do granuloma. Por se tratar de um

microambiente isolado, o granuloma apresenta um ecossistema especial para o patógeno no hospedeiro. A lesão granulomatosa pode ser um reservatório do qual o patógeno sobrevivente emerge para reativar a infecção após um longo período de latência rompida por falhas no sistema imune (Flynn & Chan, 2001; Chan & Flynn, 2004). Os componentes da parede celular do fungo parecem ser importantes no desencadeamento dessa resposta inflamatória. Frações de *P. brasiliensis* constituídas de quitina e β-glucana, injetadas de forma subcutânea em camundongos, induzem acúmulo de macrófagos, diferenciação de células epitelióides e persistência de granulomas bem formados. A β-glucana parece ser o principal componente responsável por essas ações, já que a quitina isoladamente não induz migração leucocitária ou formação de granuloma (Figueiredo *et al.*, 1986).

1.2.   *Paracoccidioides brasiliensis* e a Paracoccidioidomicose

P. brasiliensis é o agente responsável por causar a Paracoccidioidomicose (PCM), uma micose endêmica na América Latina. A PCM possui uma distribuição geográfica não uniforme (Restrepo *et al.*, 1994; Manns *et al.*, 1996), estendendo-se do México até a Argentina (Restrepo *et al.*, 1985), sendo que a região de maior prevalência é a América do Sul e os países com maior incidência são o Brasil, Venezuela e Colômbia, não havendo registros desta doença em países como Nicarágua, Guiana, Chile e Suriname (Restrepo *et al.*, 1994; Wanke & Londero, 1994). Estima-se que 10 milhões de pessoas tiveram contato com o fungo, sendo que provavelmente apenas 2% desenvolvem algum tipo de forma sintomática (McEwn *et al.*, 1995). O Brasil é o país que apresenta o maior número de pacientes notificados (80% dos casos), sendo as áreas mais afetadas observadas em São Paulo, Goiás, Minas Gerais e Mato Grosso (Coutinho *et al.*, 2002). No entanto, a determinação do local de aquisição da PCM, sua real distribuição e diagnóstico são comprometidos devido ao fato de ser uma infecção de longo período de latência (média de 15,3 anos) e de notificação não obrigatória (Brummer *et al.*, 1993).

Este fungo foi primeiramente isolado e cultivado por Adolfo Lutz em 1908, a partir de amostras colhidas de pacientes na cidade de São Paulo (Lacaz, 1994). Almeida (1930), após comparação com *Coccidioides immitis*, classificou o fungo como do gênero *Paracoccidioides* e da espécie *brasiliensis*. Posteriormente a classificação do fungo como pertencente ao filo Ascomycota, mesmo não se conhecendo a fase teleomórfica foi realizada por Leclerc *et al* (1994) através de comparação entre seqüências de DNA da subunidade ribossomal maior

(28S) de dermatófitos e fungos dimórficos. Através de análise filogenéticas dos genes que codificam para os RNAs ribossomais 26S (Gueho *et al.*, 1997), 5,8S (Peterson & Sigler, 1998) e 18S (Bialek *et al.*, 2000) de fungos dimórficos, *P. brasiliensis* foi classificado juntamente com os gêneros *Histoplasma, Emmonsia* e *Blastomyces*. Dessa forma, este foi incluído no Reino Fungi; Divisão Ascomycota; Subdivisão Euascomycete; Classe Plectomycete; Ordem Onygenales; Família Onygenacea; Subfamília Onygenaceae anamórficos; Gênero *Paracoccidioides*; Espécie *brasiliensis.*

Desde a sua descrição em 1908 por Adolfo Lutz, o fungo *P. brasiliensis* vem sendo considerado como uma espécie única, no entanto existe uma grande diversidade genética entre diferentes isolados de *P. brasiliensis*. Além disto, existe uma alta variação nos níveis de virulência desses isolados em modelos experimentais estabelecidos (Singer-Vermes *et al.*, 1989). Análises por marcadores RAPD (Randomly Amplified Polymorphic DNA) têm demonstrado fortes evidências de alta variabilidade entre isolados de *P. brasiliensis*, mostrando uma possível correlação com a virulência, distribuição geográfica e resistência a drogas (Soares *et al.*, 1995; Calcagno *et al.*, 1998; Molinari-Madlum *et al.*, 1999; Motta *et al.*, 2002; Hahn *et al.*, 2003). Recentemente, Matute *et al.* (2006) propuseram com base em estudos filogenéticos por MLST (Multi Lócus Sequencing Type) de 65 isolados de *P. brasiliensis*, a existência de três diferentes espécies crípticas decorrentes de dois eventos independentes de especiação, correlacionados ao padrão de distribuição dessas potenciais espécies.

A ploidia de *P.brasiliensis* ainda não é definida precisamente. Cano *et al.* (1998) e Montoya *et al*, (1999) sugerem que é um organismo diplóide, com 4 ou 5 cromossomos de tamanhos que variam de 2 a 10Mb, com tamanho do genoma estimado entre 20 a 23 Mb. Feitosa *et al*. (2003) utilizando as técnicas de eletroforese em campo pulsado e hibridização do DNA compararam o cariótipo de 12 isolados de *P. brasiliensis* de diferentes regiões geográficas, sendo que os resultados sugerem a existência de isolados haplóides, diplóides e ainda aneuplóides, indicando a grande variabilidade genética deste fungo. Recentemente, Almeida *et al*. (2007) avaliaram a ploidia e o tamanho do genoma de 10 isolados de *P. brasiliensis* utilizando citometria de fluxo. Estes autores relataram um genoma de tamanho estimado de 26 a 35Mb sendo que, ao contrário do observado por Feitosa *et al*. (2003), todos os isolados analisados apresentaram-se haplóides ou aneuplóides.

Embora o *P. brasiliensis* tenha sido isolado ocasionalmente do solo, seu habitat natural ainda não foi determinado precisamente (Silva-Vergara *et al.*, 1998). Por muito tempo acreditou-se que o homem fosse o único hospedeiro deste fungo. Entretanto, recentemente foi relatado um caso de infecção de um cão adulto doméstico, no qual foi verificada a formação de granuloma epitelióide, lesão característica da manifestação clínica da PCM (Ricci *et al*, 2004). A presença de *P.brasiliensis* tem sido confirmada também em tatus da espécie *Dasypus novemcinctus* infectando pulmões, baço, fígado e/ou nódulos linfáticos, com formação de granulomas em torno de células de leveduras no baço ou pulmões (Bagagli *et al.*, 1998; Silva-Vergara & Martinez, 1999).

A PCM é classificada, de acordo com sua história natural e condição clínica do paciente, nas formas aguda ou subaguda e crônica. A forma aguda ou subaguda, em geral, se desenvolve a partir de lesão primária não detectada, afeta jovens de ambos os sexos e progride rapidamente com disseminação linfática e hematogênica envolvendo baço, fígado, linfonodos e medula óssea (Franco *et al.*, 1987). A forma crônica é a mais comum e se desenvolve a partir do complexo primário ou da reativação de foco quiescente. Progride lentamente, afeta, em geral, indivíduos adultos do sexo masculino com mais de 30 anos de idade e, a maior parte dos casos, tem início nos pulmões. Pode permanecer localizada (forma unifocal) ou sofrer disseminação para outros órgãos ou tecidos (forma multifocal). A incidência da PCM predomina em adultos do sexo masculino (80 a 90% dos casos), chegando a uma taxa homem: mulher de 78:1 casos, porém esses dados não apresentam relação com o grau de exposição dos mesmos, uma vez que estudos indicam que ambos os sexos apresentam contato similar com o patógeno (Restrepo *et al.*, 1984). Estes autores sugeriram o papel protetor do hormônio feminino β-estradiol, uma vez que este hormônio inibe a transição *in vitro* e *in vivo* de *P. brasiliensis* de micélio ou conídios para levedura (Restrepo *et al.*, 1984; Salazar *et al.*, 1988, Aristizbal *et al.* 1998 e 2002).

1.3. *Histoplasma capsulatum* e a Histoplasmose

Samuel T. Darling observou e descreveu, em 1905, uma enfermidade sistêmica de evolução fatal, caracterizada pela presença de grande número de parasitos no interior de macrófagos e histiócitos, ao qual propôs a denominação *H. capsulatum* sendo que a natureza fúngica do mesmo só foi confirmada em 1932 por De Monbreum (De Mombreun, 1934; Bradsher, 1996).

H. capsulatum possui o tamanho do genoma estimado em 25Mb (Carr & Shearer 1998). Até recentemente acreditava-se que havia três variedades de *H. capsulatum*, var. capsulatum, var. duboisii e var. farciminosum, que confere variações nas manifestações clínicias e distribuição geográfica (Ajello, 1983). Isolados de *H. capsulatum* representando as três variedades oriundas de seis continentes revelou a existência de sete grupos geneticamente separados que podem ser reconhecidos como epécies filogenéticas (Kasuga *et al.*, 2003), evidenciando fortemente a grande variabilidade genética existente em diferentes isolados deste patógeno.

Histoplasma capsulatum é o agente causador da histoplasmose. Esta micose tem sido assinalada nas Américas, Europa, Ásia, Oceania e África, porém, como área endêmica ela se apresenta nos EUA no vale do Mississipi, Ohio, Kansas Missouri e Montes Apalaches, onde é mais freqüente do que em todos os outros locais do mundo (Ainsworth & Austwick, 1973). No Brasil o *H. capsulatum* já foi isolado do solo, fezes de morcegos, forros de habitação, e de animais silvestres, domésticos e do homem (Araújo, 1970; Londero & Ramos, 1978). Por algum tempo acreditou-se que este fungo tivesse como reservatório, roedores silvestres, entretanto sabe-se hoje que esses roedores também desenvolvem a histoplasmose. As fezes de aves (pombos e galinhas) e de morcegos são substratos em que o fungo não só se mantém, como prolifera, sendo importantes marcadores em regiões ou locais onde há numerosos casos da doença. Em virtude disso a doença também tem sido cognominada de "doença dos criadores de pombos", "doença dos criadores de frangos" e "doença das cavernas", neste último caso referindo-se à infecção de espeleólogos que exploram cavernas em que há morcegos (Londero & Ramos, 1978; Rippon, 1988).

A histoplasmose pode ser dividida nas seguintes formas clínicas: infecção primária assintomática e infecção pulmonar aguda; doença pulmonar obstrutiva crônica (DPOC); histoplasmose disseminada aguda e subaguda (Kauffman, 2007). A infecção primária assintomática representa a maior parte das infecções não determinando alterações clínicas no hospedeiro. A infecção pulmonar apresenta um amplo espectro de manifestações clínicas, desde casos que simulam uma gripe até pneumopatias agudas graves, com insuficiência respiratória. Na histoplasmose pulmonar crônica cavitária a forma clínica é idêntica à tuberculose avançada do adulto acometendo principalmente homens acima de 50 anos. A histoplasmose disseminada aguda ocorre na primeira infância, em algumas zonas endêmicas e em pacientes com grave comprometimento da imunidade celular, especialmente leucose,

linfomas e AIDS. A evolução para a morte ocorre na totalidade dos casos em um período de dois a seis meses. Já a histoplasmose disseminada subaguda, é semelhante à forma aguda, só se diferenciando por sua evolução mais prolongada. Não existem relatos na literatura que demonstrem uma incidência diferencial de histoplasmose em indivíduos de sexos diferentes, como ocorre para o caso de infecção fúngica causada por *P.brasiliensis*.

1.4. Genes de *P. brasiliensis* e *H. capsulatum* potencialmente envolvidos na interação patógeno- hospedeiro

Os fungos dimórficos usam uma variedade de moléculas de superfície para se ligar a matriz extracelular da célula hospedeira e estabelecer infecção (Lengeler *et al*., 2000). Os mecanismos moleculares envolvidos desde o primeiro contato com o agente infeccioso até os estágios subseqüentes da doença ainda são pouco conhecidos. Acredita-se que os fungos causadores de micoses sistêmicas utilizem mecanismos de patogenicidade, como capacidade de adesão, colonização, disseminação, sobrevivência em ambientes hostis e escape dos mecanismos de resposta imune para se estabelecer no hospedeiro e causar doença (Franco, 1987; Kurokawa *et al*., 1998). Os possíveis mecanismos responsáveis por determinar a patogenicidade e virulência de *P. brasiliensis* e *H. capsulatum* vêm sendo investigados extensivamente por meio de experimentos de interação *ex vivo* destes patógenos com células em cultura (Popi *et al*., 2002; Grosso *et al*., 2003; Andreotti *et al.* 2005; Barbosa *et al.* 2006; Moreira *et al*., 2004; Mendes-Giannini *et al*., 2006; Derengowiski *et al*., 2007; Fernandes, *et al*., 2007; Porta *et al*., 1999) e de experimentos que utilizam ferramentas moleculares de *"high throughtput"* tais como microarranjos de cDNA, ruptura gênica, interferência de RNA, entre outros (Marion *et al*., 2006; Rappleye & Goldman, 2006; Idnurm *et al*., 2004; Felipe *et al*., 2005, Goldman *et al*., 2003; Bailão *et al.* 2006; Tavares *et al*., 2007; Hwang *et al.* 2003; Nittler *et al*., 2005). Estes trabalhos têm gerado um volume considerável de informações e permitido um melhor entendimento da patobiologia destes fungos.

- *P. brasiliensis*

Componentes da parede celular de leveduras de *P. brasiliensis* têm sido caracterizados como importantes fatores de virulência e/ou patogenicidade. O conteúdo de α-1,3-glucana das formas de levedura de *P. brasiliensis* e de fungos dimórficos em geral tem sido considerado como um fator de virulência (San-Blas & San-Blas, 1977; Klimpel & Goldman, 1988). A

diminuição da α-1,3-glucana é observada após passagens sucessivas das leveduras em meio de cultura favorecendo a exposição da β-glucana presente na parede celular do fungo (Klimpel & Goldman, 1988). A β-glucana é um importante imunomodulador na resposta contra infecções fúngicas induzindo a secreção de TNF-α que potencializa a resposta inflamatória e eliminação do patógeno (Figueiredo *et al.*, 1993).

A colonização eficiente dos tecidos por fungos patogênicos é um evento complexo, que envolve geralmente adesinas codificadas pelo patógeno. A glicoproteína imunodominante de 43 kDa (*gp43*) tem sido descrita como uma importante adesina determinante da patogenicidade de *P. brasiliensis* (Carvalho *et al.*, 2005; Cisalpino *et al.*, 1996). A *gp43* diminui a fagocitose e a atividade microbicida de células fagocitárias (Popi *et al.*, 2002), sendo encontrada na superfície do fungo, em sobrenadantes de cultura e em soros de pacientes portadores da doença (Blotta & Camargo, 1993; Mendes Gianini *et al.*, 1989). Outra glicoproteína de *P. brasiliensis*, a gp70, diminui a regulação da função de macrófagos *in vitro* e elimina a formação de granuloma no pulmão, sugerindo seu envolvimento no estabelecimento do fungo e progressão de lesões na infecção primária (Grosso *et al.*, 2003). Recentemente, Andreotti *et al.* (2005) isolaram uma adesina de *P. brasiliensis* de 30kDa com a capacidade de se ligar a laminina. Esta proteína foi mais expressa em isolados de *P. brasiliensis* que possuem uma maior capacidade de adesão. Ainda neste mesmo trabalho foi verificado que o tratamento de células epiteliais em cultura com as adesinas de laminina de *P. brasiliensis* de 30 e 43 kDa, inibem a adesão deste fungo às células epiteliais. Outras duas proteínas de superfície celular de *P. brasiliensis* (32 e 19 kDa) interagem com várias proteínas da matriz extracelular, tais como laminina, fibronectina e fibrinogênio (Gonzalez *et al.*, 2005a; Gonzalez *et al.*, 2005b). Em trabalhos recentes, Barbosa *et al.* (2006) avaliaram a expressão da gliceraldeído-3-fosfato desidrogenase (GAPDH) recombinante de *P. brasiliensis* e verificaram que a expressão desta proteína em leveduras é importante para a adesão do fungo às células hospedeiras. A GAPDH se localiza na camada mais externa da parede celular e interage com proteínas da matriz extracelular, tais como laminina, fibronectin, provavelmente desencadeando uma resposta na célula hospedeira no estágio inicial da infecção. A paracoccina, uma adesina de *P. brasiliensis* recentemente descrita, interage com laminina de uma forma dose dependente. Esta adesina, o segundo maior componente reconhecido por anticorpos do soro de pacientes com PCM, foi identificada como sendo uma lectina ligante de

N-acetiglucosamina que interage com laminina presente na matriz extracelular e estimula a liberação de mediadores imunológicos (Coltri *et al.*, 2006).

Nos últimos anos, pesquisas moleculares têm demonstrado a importância de enzimas hidrolíticas em processos de infecção, causando degeneração à célula do hospedeiro e providenciando nutrientes ao patógeno em um ambiente onde o nutriente se encontra de forma restrita (Hogan *et al.*, 1996; Schaller, *et al.*, 2000). Uma serina proteinase extracelular SH-dependente tem sido caracterizada da fase de levedura de *P. brasiliensis*; é capaz de clivar componentes da membrana basal da matriz extracelular, incluindo laminina, fibronectina, colágeno tipo IV e proteoglicanos da membrana basal *in vitro*, sendo assim potencialmente relevante na disseminação do fungo (Carmona *et al.*, 1995; Puccia, *et al.*, 1998).

A melanina também tem sido indicada como um fator de proteção do *P. brasiliensis*. Gomez *et al.* (2001) verificaram que conídios e leveduras de *P. brasiliensis* podem produzir melanina tanto *in vitro* quanto em infecção de macrófagos murinos. Além disso, a presença de melanina aumenta a resistência deste fungo a compostos antifúngicos e o protege da fagocitose (Silva *et al.*, 2006). Outras diferentes moléculas têm sido associadas à patogênese de *P. brasiliensis*. A catalase P multifuncional de 61 kDa de *P. brasiliensis* foi superexpressa na forma de levedura e induzida pela adição de H_2O_2 exógeno; esses dados sugerem uma provável função de proteção de *P. brasiliensis* contra radicais de oxigênio durante a infecção (Moreira *et al.*, 2004). A expressão gênica de um membro da família das proteínas flavodoxina-like, *pby20,* foi detectada em altos níveis na fase de levedura de *P. brasiliensis*. Outros membros desta família de proteínas estão intimamente relacionados ao estresse térmico e oxidativo em distintos sistemas biológicos, o que sugere que *pby20* pode realizar uma importante função na detoxificação durante o parasitismo intracelular deste fungo (Daher *et al.*, 2005). Carvalho *et al.* (2003) analisaram o efeito de inibidores de componentes da via de transdução de sinal Ca^{2+}/ calmodulina em células de *P. brasiliensis* com o objetivo de verificar a possível função desta via na diferenciação morfogenética deste fungo. Neste sentido, os autores verificaram que a diferenciação foi inibida por drogas que se ligam tanto a calmodulina quanto a fosfodiesterase dependente de Ca^{2+}/calmodulina sugerindo que a calmodulina realiza uma importante função durante a transição morfológica de *P. brasiliensis* de micélio para levedura. Os autores sugerem ainda que considerando a função da Ca^{2+}/ calmodulina sobre a via de sinalização de P. *brasiliensis*, estudos estruturais envolvendo

inibidores de calmodulina são fundamentais para o desenho de novas drogas de potencial terapêutico no tratamento da PCM.

As recentes análises do perfil transcricional das formas de micélio e levedura de *P. brasiliensis* tem revelado importantes informações sobre o padrão de expressão diferencial de ambas as formas deste fungo e idenficado novos genes candidatos que podem desempenhar uma importante função na virulência e/ou patogênese de *P. brasiliensis* (Andrade *et al.*, 2005; Bailão *et al.*, 2006; Felipe *et al.*, 2005; Goldman *et al.*, 2003; Marques *et al.*, 2004; Nunes *et al.*, 2005). Recentemente, Felipe *et al.* (2003 e 2005) e Goldman *et al.* (2003) analisaram 6.022 (isolado P01) e 4.692 (isolado Pb18) sequências transcritas de *P. brasiliensis*, respectivamente. Esses projetos tiveram como principal objetivo mapear o transcriptoma na condição de cultivo *in vitro* das formas de micélio e levedura de *P. brasiliensis.* Através dos dados gerados por estes trabalhos foi possível realizar a identificação e categorização de genes ortólogos descritos para outros fungos patogênicos como aqueles relacionados à virulência, resistência a drogas, alvos para drogas, dimorfismo, vias de sinalização celular entre outros (Felipe *et al.*, 2003 e 2005; Goldman *et al.*, 2003). Do total de PbAESTs obtidas pelo projeto transcriptoma de *P. brasiliensis* desenvolvido por Felipe *et al.* (2003 e 2005), 68,3% mostraram similaridade com seqüências já descritas de outros organismos, depositadas em bancos de dados. Análises utilizando ferramentas de bioinformática permitiram categorizá-las em 12 grupos, de acordo com a provável função, como mostrado na Figura 1.
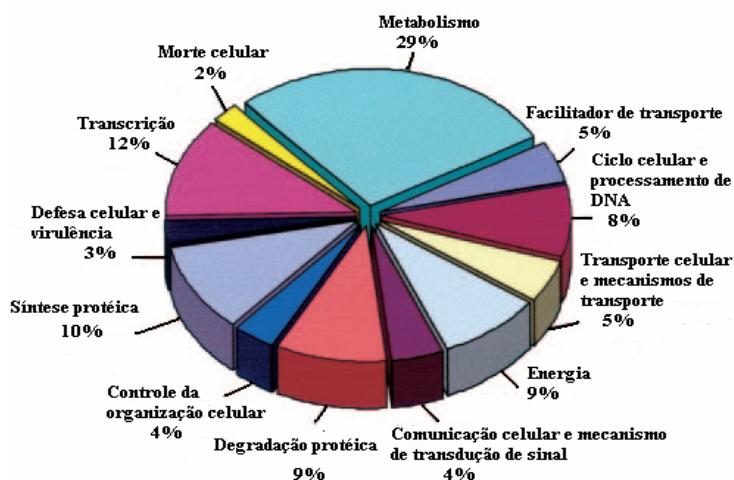
Figura 1: Principais categorias estabelecidas para os transcritos identificados pelo Projeto Genoma Funcional e Diferencial de *P. brasiliensis* (Felipe *et al.* 2003 e 2005). As PbAESTs que apresentaram similaridade com seqüências depositadas em bancos de dados foram categorizadas em 12 grupos de acordo com suas prováveis funções.

Após analisar os dados obtidos pelo projeto transcriptoma de *P. brasiliensis* (Felipe *et al.* 2003 e 2005), de forma mais aprofundada em alguns aspectos de interesse da patogênese, Tavares *et al.* (2005) categorizaram 30 prováveis genes de virulência nas seguintes classes: metabolismo, parede celular, detoxificação, fatores secretados e outros. Os genes *icl1* e *mls1* codificando respectivamente, isocitrato liase e malato sintase do ciclo do glioxalato são potencialmente correlacionados com virulência devido a sua ativação em condições de escassez de carbono. Outros genes relacionados à virulência foram identificados tais como *ade2* (fosforibosilaminoimidazole carboxilase), *nmt1* (N-miristoiltransferase) e *fas2* (subunidade α da enzima ácido graxo sintase), *tps1* (trealose-6-fosfato sintase), envovidos no metabolismo de nucleotídeos, lipídeos e glicose, respectivamente. Entre os genes do segundo grupo estão àqueles necessários para a integridade da parede celular durante o crescimento vegetativo e diferenciação de *P. brasiliensis* e inclue: *ags1* (α-glucana sintase), *chs3 (*quitina sintase 3)*, gna1 (*glucosamina-6-fosfato acetiltransferase), *pmt1* (manosil transferase), *mnt1 (*α-1,2-manosiltransferase), *phr1* e *phr2* (1,3-β-glucanosiltransferases). O terceiro grupo compreende os genes de virulência do patógeno que funciona na detoxificação de radicais oxidativos tais como: Cu/Zn superóxido dismutase (*sod1*), tiol peroxidase (*tsa1*) oxidase alternativa (*aox1*) e catalase (*cat1*). Os fatores de virulência potencialmente secretados incluem genes codificando proteinases, fosfolipases e urease e também foram apontados por Tavares *et al.* (2005) como importantes neste patógeno.

A partir dos dados obtidos pelo projeto transcriptoma de *P. brasiliensis* (Felipe *et al.*, 2005) foi construído um microarranjo parcial de cDNA em náilon contendo 1152 genes de *P. brasiliensis.* Este microarranjo foi utilizado para comparar a expressão diferencial das formas de micélio e levedura cultivados *in vitro* e permitiu a identificação de genes que em outros fungos patogênicos são comprovadamente relacionados ao dimorfismo e a virulência. Além disso, foi possível identificar genes de expressão diferencial que são potenciais alvos para o desenho de novas drogas antifúngicas (Felipe *et al.*, 2005; Andrade *et al.*, 2006). A análise transcricional das formas de micélio e levedura de *P. brasiliensis* (Felipe *et al.*, 2005; Andrade *et al.*, 2006) lançou as bases para os trabalhos seguintes, realizados em paralelo e em colaboração com esta tese de doutorado, que compreendeu a análise da modulação gênica de *P. brasiliensis* após co-cultivo e internalização por macrófagos (Tavares *et al.*, 2007; Derengowiski *et al.*, 2007; Fernandes, *et al.*, 2007). Neste sentido, foi avaliado, por microarranjos de cDNA, o perfil de expressão gênica de *P. brasiliensis* recuperado

diretamente do interior de macrófagos murinos não ativados, após 6 horas de infecção, sem nenhuma passagem posterior *in vitro* (Tavares *et al.*, 2007). Estes autores mostraram que o gene que codifica para a enzima regulatória chave fosfofrutoquinase (*pfka*) foi reprimido bem como as enzimas *pbgapdh, pgk, gpmA* e *eno*. O gene que codifica para a cistationina beta liase (*metG*), cujo produto é envolvido na produção de metionina, foi induzido o que evidencia uma importante resposta adaptativa deste patógeno para sobreviver dentro dos macrófagos, onde a concentração de aminoácidos e glicose é extremamente baixa. Além de genes relacionados ao estresse nutricional, a indução de genes envolvidos com estresse oxidativo (*sod3* e *hsp60*) também foi observada por Tavares *et al.* (2007). O gene *sod3* codifica para uma proteína envolvida na eliminação de ânios superóxido e corresponde a uma provável proteína de membrana GPI-ancorada (Castro *et al.,* 2005). Os autores sugerem que o aumento da expressão deste gene possivelmente favoreceria uma melhor acessibilidade aos derivados de ânion superóxido e subsequente detoxificação de intermediários reativos de oxigênio produzidos pelo hospedeiro. Adicionalmente o gene que codifica para a *hsp60* também foi induzido. A função protetora da HSP60 é relacionada com sua habilidade de bloquear a liberação de ferro ferroso reduzido proveniente da oxidação de proteínas mitocondriais (Cabiscol *et al.*, 2002). Neste sentido, os autores sugerem que a indução de *hsp60* por *P. brasiliensis* exposto a radicais oxidativos produzido pelos macrófagos pode ter uma função protetora similar. Este trabalho revelou a modulação de genes de *P. brasiliensis* envolvidos principalmente com estresse nutricional e oxidativo, sugerindo que o fungo responde ao ambiente inóspito encontrado no fagossomo.

Derengowski *et al.* (2007) utilizaram RNA de *P. brasiliensis* recuperado de leveduras internalisadas por 9 horas em macrófagos para análise de expressão de genes sabidamente envolvidos com estresse nutricional. Estes autores observaram a indução de genes que codificam para enzimas do ciclo do glioxalato (*icl* e *mls*), mostrando que o patógeno é capaz de utilizar compostos de 2 carbonos para a síntese *de novo* de glicose via gliconeogênese, bem como do gene que codifica para a enzima chave e regulada da gliconeogênese, a fosfoenolpiruvato carboxiquinase (*pck*). A co-regulação de genes do ciclo do glioxalato e da via da gliconeogênese observada por Derengowski *et al.* (2007) é consistente com a importância destas vias no fornecimento de energia a partir de compostos de 2 carbonos e metabolismo de ácidos graxos, como ocorre dentro do fagolisossomo (Lorenz *et al.*, 2002; Barelle *et al.*, 2006; Rude *et al.*, 2002). Posteriormente, Fernandes *et al.* (2007) observaram a

repressão dos genes *ras1* e *ras2* (componentes da via de sinalização) de *P. brasiliensis* também recuperado de macrófagos murinos após 9 horas de infecção. Provavelmente a repressão destes genes ocorre devido à limitação nutricional encontrada no interior do macrófago, já que os genes ras estão envolvidos na ativação das vias de sinalização que respondem ao choque térmico e controlam o estado nutricional da célula (Engelberg *et al.*, 1994, Breviario *et al.*, 1988, Wang *et al.*, 2004). Em contraste, em condições que mimetizam a via hematológica de disseminação deste patógeno, Bailão *et al.* (2006) observaram que uma indução de genes de *P. brasiliensis* envolvidos principalmente com estresse osmótico, não sendo verificada a repressão de genes da via glicolítica, já que o fungo se encontra em um ambiente rico em nutrientes. O perfil de expressão diferencial de *P. brasiliensis* em condições que mimificam a rota hematológica de disseminação do fungo revelou a indução de vários genes incluindo facilitador de transporte (*ctr3*), proteínas de resposta ao estresse (*hsp30, hsp70 and hsp90*) e remodelamento da parede celular (*sho1*, *pas-li*ke, *septin-1*).

Silva *et al.* (2007) em recente revisão (Figura 2) comparou o perfil de expressão gênica de leveduras de *P. brasiliensis* recuperadas do interior de macrófagos com a expressão gênica dessas leveduras após contato com sangue. No interior dos macrófagos o fungo expressa genes relacionados principalmente a estresse nutricional e oxidativo enquanto que em contato com o sangue, meio rico em nutrientes, é observado indução de genes principalmente relacionados a estresse osmótico (Silva *et al.*, 2007). As leveduras de *P. brasiliensis* fagocitadas pelos macrófagos são capazes de sensoriar e responder ao ambiente fagossomal. Os dados obtidos mostram um considerável grau de plasticidade transcricional do *P. brasiliensis* em resposta ao microambiente hostil do macrófago, o que possivelmente permite sua adaptação e conseqüente sobrevivência no interior destas células. A partir do sítio primário de infecção nos pulmões, a paracoccidioidomicose pode evoluir com a disseminação do fungo para outros órgãos ou tecidos por via hematológica e/ou sistema linfático (Franco, 1987) e nestas condições novamente ocorre uma resposta adaptativa revelando a versatilidade deste patógeno (Silva *et al.*, 2007).
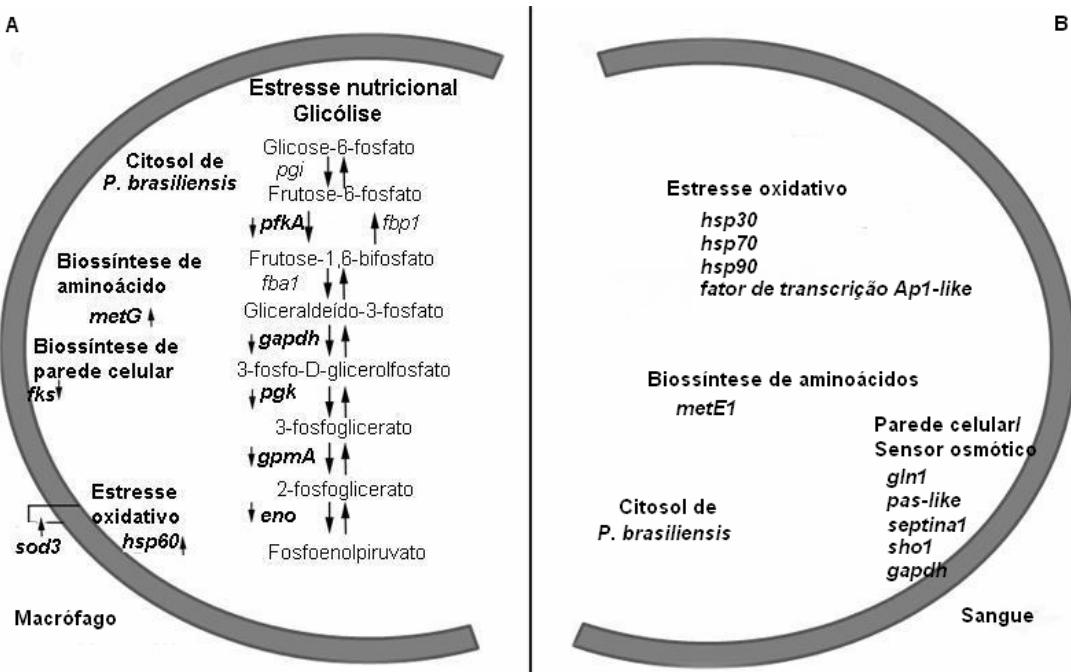
Figura 2. Modelo da adaptação de *P. brasileinsis* ao ambiente do hospedeiro (Silva *et al.*, 2007). As setas menores ↑ e ↓ indicam os genes induzidos e reprimidos, respectivamente. (**A**) Sobrevivência de *P. brasiliensis* no fagossoma do macrófago. Estresse oxidativo - *sod3* e *hsp60;* estresse nutricional na via de biossíntese de aminoácidos - *metG*; Glicólise - *pfkA*; *pbgapdh; pgk; gpma* e *eno*; Síntese de parede celular - *fks*. (**B**) Indução de genes de *P. brasiliensis* durante a disseminação via hematogênica. Estresse oxidativo - *hsp30, hsp70* e *hsp90, Ap1 -like transcription factor*; biossíntese de aminoácidos - *metE1*; parede celular e sensor de osmolaridade - *gln1, pas-like protein, septin, sho1* e *gapdh*.

- *H. capsulatum*

Os fatores de *H. capsulatum* potencialmente relacionados à interação com o hospedeiro têm sido analisados principalmente por meio das técnicas de ruptura gênica, interferência de RNA e microarranjos (Colonna-Romano *et al.*,1998; Hwang, *et al.*, 2003; Nittler *et al.*, 2005 Rappleye & Goldman, 2006). Estudos explorando o dimorfismo de *H. capsulatum* têm revelado genes potencialmente envolvidos no parasitismo intracelular tais como *yps-3*, α-(1,3)-glucana sintase e a *cbp1* proteína ligante de cálcio (Keath *et al.*,1989; Klimpel & Goldman, 1987; 1988; Sebghati *et al.*, 2000). O gene *yps-3* codifica para uma proteína de 20 kDa que pode ser tanto secretada quanto se localizar na superfície da parede celular de *H. capsulatum*. Mutantes para este gene possuem uma capacidade diminuída da colonização dos órgãos de camundongos na fase inicial de infecção (Bohse & Woods, 2007). Rappleye *et al.* (2004) confirmaram por meio da técnica de RNA interferência a importância da α-(1,3)-

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glucana para a virulência de *H. capsulatum*. Em trabalhos seguintes, Rappleye *et al* (2007) demostraram que a α-(1,3)-glucana impede o reconhecimento da β-glucana pelos receptores da célula hospedeira, evitando assim que estas se tornem ativadas e permitindo ao fungo escapar da resposta imune por diminuir a secreção de TNF-α pelas células hospedeiras. Marion *et al.* (2006) identificaram por meio de mutação insercional e interferência de RNA o produto do gene *amy1* que codifica para uma alfa-(1,4)-amilase. Os experimentos de mutação e silenciamento gênico indicaram que a produção de α-1,3-glucana requer a alfa-(1,4)-amilase e que a perda da função do gene *amy1* atenua a capacidade de *H. capsulatum* matar macrófagos e colonizar pulmão de camundongos. A *cbp1* também é específica da forma de levedura de *H. capsulatum* (Batanghari & Goldman, 1997a). Leveduras de *H. capsulatum* submetidas a estresse na presença de cálcio possui maior capacidade de incorporar cálcio com a adição de *cbp1* exógeno do que leveduras sem *cbp1* exógeno (Batanghari *et al.*, 1998). Esses resultados sugerem que esta proteína provavelmente aumenta a aquisição de cálcio pelas leveduras quando estas se encontram em um ambiente com baixas concentrações de cálcio, por exemplo, no interior do fagolisossomo dentro dos macrófagos (Batanghari & Goldman, 1997b; Batanghari *et al.*, 1998). Experimentos de ruptura do gene *CBP1* demonstraram a importância da proteína codificada por este gene na sobrevivência e virulência de *H. capsulatum*. As leveduras mutantes foram incapazes de destruir os macrófagos *in vitro* ou de proliferar em modelo murino de infecção pulmonar (Sebghati *et al.*, 2000). O Ca^{+2} também atua como mediador intracelular se ligando a calmodulina. O complexo Ca^{+2}/calmodulina pode ativar proteínas quinases dependentes de Ca^{+2}/calmodulina em importantes vias de transdução de sinal intracelulares. El-Rady & Shearer (1997) isolaram e caracterizaram o cDNA completo que codifica para a calmodulina de *H. capsulatum*. A análise da expressão deste gene, por *"northern-blot"*, mostrou que ele é expresso tanto na fase de micélio quanto na fase de levedura. Entretanto, na forma de levedura, a quantidade de RNA mensageiro é duas vezes maior. Os autores sugerem que esta expressão aumentada na forma parasítica do fungo pode ser importante para sua sobrevivência no interior das células fagocíticas. Experimentos de mutação do gene *URA5* de *H. capsulatum* que codifica para a orotidina-monofosfato pirofosforilase (OMPpase), uma enzima da via de biossíntese de pirimidina, mostrou ser este gene essencial para a virulência do fungo tanto em modelo de infecção murino quanto em linhagem de células murinas (Retallack *et al.* 1999). A *hsp60* de *H.*

capsulatum é a principal molécula de superfície reconhecida pelos macrófagos via receptores β_2 integrina (Long *et al*., 2003). A hsp60 tem sido descrita com sendo importante em ativar macrófagos via CD14 utilizando um mecanismo similar ao de LPS (Kol *et al*., 2000).

Com a finalidade de fazer uma descrição mais completa da regulação da expressão gênica global nas duas fases morfológicas de *H. capsulatum*, Hwang *et al.* (2003) analisaram por microarranjos de DNA os genes induzidos na fase de micélio e levedura. Neste estudo foi observada a indução de genes da fase de levedura relacionados principalmente com metabolismo e disponibilidade de nutrientes, tais como: lisina permease (envolvida na via de produção de glutamato), a 4-hidroxifenilpiruvato dioxigenase (metabolismo de tirosina), a dihidrolipoamida desidrogenase (metabolismo de carbono e aminoácido). Os autores sugerem que a expressão aumentada destes genes nas células de levedura de *H. capsulatum*, provavelmente reflete a habilidade deste patógeno em se adapatar à condições limitantes de nutriente durante a infecção.

A habilidade que os macrófagos ativados possuem de restringir a multiplicação do *H. capsulatum* é dependente da produção de NO (Lane *et al*., 1994; Nakamura *et al*., 1994; Newman, 1999). Entretanto, o efeito inibitório do NO sobre *H. capsulatum* é predominantemente fungistático, o que sugere que *H. capsulatum* é capaz de resistir à morte por NO em células do hospedeiro (Nakamura *et al*., 1994). Para investigar os possíveis mecanismos envolvidos na habilidade deste fungo em sobreviver ao estresse gerado por espécies reativas de nitrogênio, Nittler *et al*. (2005) analisaram por microarranjos genes de *H. capsulatum* que são induzidos em resposta ao tratamento com doadores de RNS (espécies reativas de nitrogênio). Foi identificada nesta análise, a indução de genes relacionados à aquisição de ferro, produção de energia, resposta ao estresse, enovelamento e degradação de proteínas. A restrição ao ferro imposta pelo hospedeiro limita a replicação de *H. capsulatum* (Lane *et al*., 1991; Newman *et al*., 1994). Segundo os autores, a indução de genes relacionados à aquisição de ferro em resposta a exposição à RNS pode facilitar o crescimento intracelular de *H. capsulatum* na célula hospedeira. Nittler *et al.* (2005) sugerem ainda que se o estresse induzido por RNS é interpretado pelo *H. capsulatum* como um sinal de que ele se encontra no interior do macrófago, a indução de genes relacionados a oxidação e metabolismo de lipídeos pode refletir a tentativa de aquisição de nutrientes pelo fungo.

1.5. Mecanismos imunoregulatórios das células fagocíticas durante a interação patógeno-hospedeiro

As células fagocíticas (macrófagos, neutrófilos e células dendríticas) são consideradas a primeira linha de defesa contra infecções por microrganismos. Essas células podem ingerir e matar o patógeno invasor bem como liberar citocinas e quimiocinas que tornam a fagocitose e a atividade microbicida mais efetiva (resposta imune inata). A morte do microrganismo ocorre no fagossomo por meio de vários mecanismos tais como mudanças no pH, ataque por enzimas hidrolíticas, ataque por radicais livres e metabólitos tóxicos. Em seguida, os antígenos microbianos são processados e apresentados aos linfócitos, levando a uma resposta imune altamente específica – resposta imune adquirida (Janeway, 1992). O reconhecimento do patógeno pelas células fagocíticas durante a infecção ocorre por meio de múltiplas interações e sítios e envolve vários receptores (Janeway & Medzhitov, 2002; Romani, 2004; Fernandes *et al*., 2007). Os fatores do hospedeiro relacionados a esta interação são principalmente moléculas de adesão/internalização presentes na membrana, componentes das vias de transdução de sinal e fatores de transcrição. Estes componentes celulares irão induzir a secreção de citocinas e quimiocinas bem como de metaloproteinases de matriz, importantes no processo inflamatório, além de outros genes de resposta do hospedeiro facilitando a permanência ou a eliminação do patógeno.

1.6. Adesão e internalização dos patógenos pelos fagócitos

A primeira interação entre a célula fagocítica e o patógeno ocorre por meio da ligação dos receptores de reconhecimento padrão (PRRs – "Patterns Recognizing Receptors") do fagócito às estruturas moleculares conservadas dos microrganismos conhecidos como padrões moleculares associados aos patógenos (PAMPs – "Pattern Associated Molecular Pathogens") (Janeway & Medzhitov, 2002). Os PRRs podem estar presentes no soro, fluídos dos tecidos, membranas ou citoplasma e podem ser subdividida dentro de famílias relacionadas de acordo com sua estrutura e/ou função. Geralmente cada receptor reconhece um grande número de microrganismos baseados em sua especificidade individual com o ligante. Após o reconhecimento, alguns PRRs promovem a internalização do microrganismo (Janeway & Medzhitov, 2002; Romani, 2004). As principais formas de internalização de fungos pelos fagócitos ocorrem por meio dos receptores toll-like (TLRs), receptores de manose e integrinas.

a) Receptores toll-like (TLRs)

Os TLRs constituem uma família de dez receptores de reconhecimento imunes inatos necessários para detecção de um amplo espectro de produtos microbianos incluindo LPS, flagelina e lipoproteínas bacterianas. Eles são distintos uns dos outros na especificidade do ligante, no padrão de expressão e vias de sinalização, mas todos atuam na iniciação e ativação da imunidade (Figura 3). Os TLRs constituem um dos mais poderosos meios de modulação gênica, sendo que muitos genes são induzidos/reprimidos pela sinalização desses receptores após serem ativados por ligantes de origem microbiana conservados evolutivamente (Janeway, 1992; Carpenter & O'Neill, 2007). Os TLR2 e TLR4 são relacionados principalmente com a imunidade anti-fúngica. Estudos recentes têm demonstrado o envolvimento desses receptores no reconhecimento de patógenos como *Candida albicans*, *Aspergillus fumigatus* e *Cryptococcus neoformans* (Netea *et al*., 2004a; Netea *et al*, 2006). A infecção fúngica em camundongos nocaute para TLRs ou moléculas associadas, indica que TLRs específicos tais como TLR2 and TLR4 realizam diferentes funções na ativação de vários ramos da resposta imune inata. Netea *et al*. (2004b) sugerem que TLRs oferecem mecanismos de escape para certos microrganismos patogênicos, especialmente através da indução dirigida por TLR2 de citocinas antiinflamatórias. O TLR2 é o receptor mais especializado e reconhece uma grande variedade de PAMPs de bactérias, leveduras, fungos, parasitas e viroses. As PRRs que reconhecem bactérias e fungos se localizam geralmente sobre a superfície de células e as que reconhecem componentes virais são localizadas intracelularmente. Embora a maioria dos TLRs pareça funcionar como homodímeros, os TLR2 formam heterodímeros com TLR1 ou TLR6, no entanto, cada dímero apresenta diferente especificidade ao ligante.
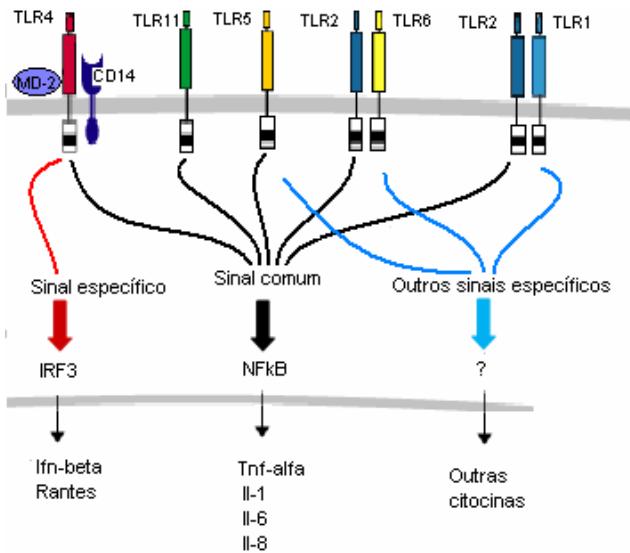
Figura 3. Representação esquemática dos principais receptores toll-like (TLR) envolvidos na modulação da resposta inflamatória induzida por patógenos. http://inet.uni2.dk/~iirrh/IIR/09inn/+TLRSig.htm. O receptor TLR4 interage com MD-2 e CD14 e transduz sinais específicos que irão ativar fatores de transcrição responsáveis por induzir a expressão do gene Ifn-β e Rantes. Todos os TLRs são capazes de ativar Nfkb. A expressão deste gene induz as principais citocinas (Tnf-α, Il-1, Il-6 e Il-8) envolvidas na resposta pró-inflamatória. TLR5 pode também transduzir sinais específicos que irão ativar outras citocinas por meio de um regulador transcricional não conhecido. TLR2 forma dímeros com TLR1 ou TLR6 levando a ativação de Nfkb ou de outro regulador transcricional com conseqüente indução de citocinas.

Os TLRs também podem atuar juntamente com outros co-receptores para aumentar a sensibilidade de interação com o ligante, como por exemplo, o reconhecimento de LPS por TLR4, requer o MD-2 e CD14 (Takeda *et al*., 2003). O TLR4, quando ativado, recruta moléculas adaptadoras dentro do citoplasma das células para propagar o sinal (Figuras 4). Quatro moléculas adaptadoras, conhecidas como MyD88, MAL, TRIF, e TRAM, são envolvidas na via de sinalização (Takeuchi *et al*., 2000a; Horng *et al*., 2002; Yamamoto *et al*.2002; Fitzgerald *et al*., 2003). Os adaptadores ativam outras moléculas dentro da célula, incluindo certas proteínas quinases (IRAK1, IRAK4, TBK1, e IKKi) que amplificam o sinal levando a indução ou repressão de genes que irão orquestrar a resposta inflamatória.O estudo de TLRs tem ampliado significativamente a compreensão das respostas imunes inatas e adaptativas oferecendo novas perspectivas relacinadas a agentes terapêuticos contra doenças infecciosas e inflamatórias (Takeda *et al*., 2003).
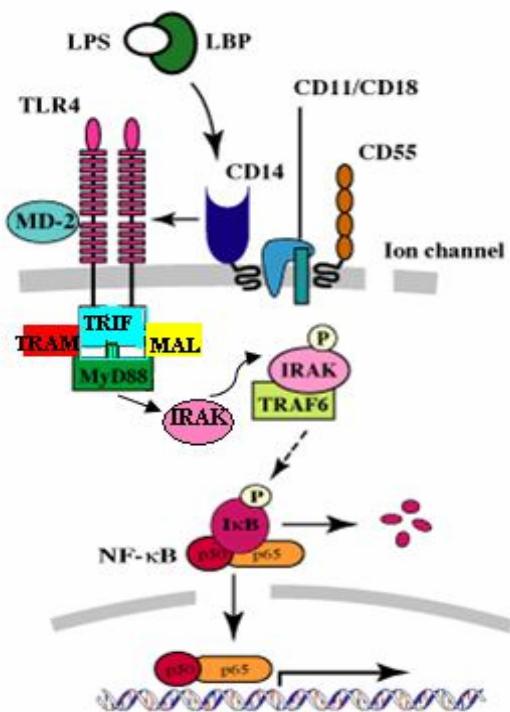
Figura 4. Representação esquemática da cascata de sinalização celular induzida via receptor TLR4. CD14 se liga a moléculas específicas presentes na superfície de patógenos e se liga ao TLR4 que por sua vez é co-ativado também por MD-2. TLR4, quando ativado, recruta as moléculas TRIF, MyD88, TRAM e MAL dentro do citoplasma das células que ativam IRAK para propagar o sinal. IRAK se liga a TRAF6 que por sua vez desfosforila IkB (o repressor de NFkB). A molécula de NFkB (composta pelas subunidades p50 e p65) é liberada e entra no núcleo indo ativar a transcrição de genes importantes no processo pró-inflamatório.

Adaptado de http://inet.uni2.dk/~iirrh/IIR/09inn/+TLRSig.htm.

b) Receptores de manose

Os receptores de manose (MR) estão entre os receptores pertencentes à família dos domínios CLEC (C-type lectin domain family) caracterizados por sua habilidade de se ligar a carboidratos de forma dependente de Ca^{2+}. Este receptor consiste de um domínio N-terminal rico em cisteína, um domínio de fibronectina tipo II e CRDs (Domínios de Reconhecimento do Carboidrato) (figura 5). Os MRs reconhecem preferencialmente oligomanoses α-ligadas que são preferencialmente ramificadas (Kanazawa, 2007) sendo capazes de reconhecer grande variedade de antígenos que vão desde moléculas próprias a moléculas presentes na superfície

de patógenos (Geijtenbeek *et al*., 2004). Os fagócitos expressam vários tipos de receptores lectinas tipo C sobre sua superfície para captura de antígenos (Kanazawa, 2007). O reconhecimento de *C. albicans* por macrófagos humanos e murinos é mediado por um receptor de manose que pode ligar a resíduos N-manosil e por TLR-4 ligados a resíduos O-manosil (Netea *et al*., 2006). A resposta protetora de células T à levedura patogênica *C. neoformans* é dependente do eficiente reconhecimento e internalização de manoproteínas por múltiplos MRs presentes na superfície de células dendríticas (DC) que por sua vez, fornecem uma interação crucial entre as respostas imune inata e adaptativa ao *C. neoformans* (Mansour *et al*., 2006). Jiménez *et al*. (2006) relataram que o aumento da expressão do receptor de manose é importante para a fagocitose de leveduras de *P. brasiliensis* pelos macrófagos.
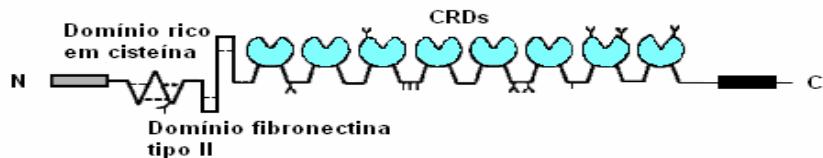
Figura 5. Representação esquemática do receptor de manose. O receptor de manose é constituído por uma cauda N-terminal que possui um domínio rico em fibronectina tipo II e um domínio rico em cisteína. Na sequência existe uma longa região composta de um domínio rico em carboidratos com ramificações finalizando na região C-terminal.

O reconhecimento de β-glucanas presentes especialmente na parede celular de fungos pode ocorrer por uma família de receptores não clássicos de lectina tipo C, denominados Dectina-1. Os receptores Dectina-1 não apresentam um resíduo de cisteína em sua região central e contém um motivo de ativação baseado no imunoreceptor com motivo de tirosina (ITAM)-like em sua cauda citoplasmática que é envolvida na ativação celular. Após a ativação da segunda tirosina ocorrem vários efeitos, tais como: indução de fagocitose, produção de espécies reativas de oxigênio (ROS) e produção de citocina resultante da ativação de NF-kB (Brown, 2006). Este receptor reconhece β-glucana de fungo sem necessidade de opsonização. Portanto, ele pode reconhecer várias espécies de fungos, incluindo *C. albicans* (Taylor *et al*., 2007), *Pneumocystis carinii* (Saijo *et al*., 2007), *Coccidioides posodaii* (Viriyakosoi *et al*., 2005) e *A. fumigatus* (Gersuk *et al*., 2006). Sua expressão por macrófagos é induzida por IL-4

e IL-13 e reprimida por LPS, IL-10 e dexametasona. A ausência deste receptor pode explicar porque alguns fungos apresentam a capacidade de evadir da resposta imune (Brown, 2006).

c) Integrinas

As integrinas são uma família de receptores de superfície que possuem função de adesão, migração, diferenciação e sobrevivência (Hynes, 2002). Essas moléculas são glicoproteínas heterodiméricas contendo subunidades α e β não covalentemente associadas, e são agrupadas em subfamílias de acordo com a identidade da subunidade β. Em humanos, oito diferentes subunidades β combinam com 18 diferentes subunidades α para formar 24 heterodímeros funcionalmente distintos. As integrinas têm um grande domínio extracelular responsável por interagir com ligantes extracelulares e um pequeno domínio intracelular que se liga ao citoesqueleto e proteínas de sinalização. Essas glicoproteínas assimilam informação do ambiente extra e intracelular atuando como um transdutor bidirecional de sinal através da membrana celular (DeMali *et al.*, 2003; Schwartz & Assoian, 2001). As integrinas CD18 e Mac-1 são altamente expressas em neutrófilos e macrófagos presentes no infiltrado de inflamação pulmonar de camundongos com paracoccidioidomicose experimental (Gonzalez *et al.*, 2005). Esses autores sugerem que durante o estágio inicial da infecção a indução dessas moléculas é importante para a patogênese desta micose. Durante a fase inicial de infecção por *H. capsultum*, macrófagos alveolares reconhecem leveduras deste fungo não opsonizados via a família de integrinas CD18 (LFA-1, CR3 e CR4) (Bullock & Wright, 1987; Newman *et al.*, 1990).

1.7. Quimiocinas liberadas pelos fagócitos durante a interação com microrganismos patogênicos

As quimiocinas são moléculas secretadas principalmente pelas células do sistema imune e formam gradiente de concentração dentro dos tecidos para atrair leucócitos. Estas moléculas são subdividas nos subgrupos C, CC, CXC, CX_3C, dependendo da posição dos dois resíduos de cisteínas da porção N-terminal na sequência (Ernst *et al.*, 1994; Bazan *et al.,* 1997; Zlotnik & Yoshie, 2000). A expressão de quimiocinas tem sido detectada em associação com a maioria das infecções por microrganismos patogênicos. Em um microambiente de infecção, desde uma virose até parasitas multicelulares, quimiocinas específicas podem ser requeridas para induzir uma resposta mais efetiva por meio da mobilização de uma subpopulação de

leucócitos apropriados com funções efetoras (Vadeboncoeur *et al.*, 2003; Nixon *et al.*, 2000; Dongari-Bagtzoglou *et al.*, 1999; Chiu & Chensue, 2002).

A interação de fagócitos com patógenos intracelulares ou com seus produtos presentes na parede celular induz a ativação e secreção de citocinas chaves no processo inflamatório tais como fator de necrose tumoral alfa (TNF-α), interleucina 1 (IL-1), IL-6, IL-8, IL-10, IL-12, e fator de crescimento tumoral-β (TGFβ) (De Waal Malefty, *et al.* 1991; Cano *et al.*, 1992). Todas essas citocinas apresentam atividade estimuladora ou supressora, exercendo seus efeitos tanto de forma autócrina como parácrina sobre os macrófagos, interagindo com os receptores específicos presentes nessas células e dessa forma modulando a sua função (Adams & Amilton, 1997; Moonis *et al.*, 1992). Citocinas como interferon-γ, (IFNγ), TNF-α, IL-1 e fator estimulador de colônia de granulócitos e macrófagos (GMC-CSF) são importantes no processo de ativação de macrófagos, enquanto que IL-6, IL-10 e TGFβ são considerados fatores de desativação destas células (Figueiredo *et al*, 1993; Nathan *et al.*, 1983). O desequilíbrio na produção de citocinas com atividade estimuladora ou supressora, após a infecção por fungos patogênicos pode determinar a evolução da doença ou a morte do microrganismo.

IFN-γ é a citocina que caracteriza o padrão Th1 de resposta imunológica e sua principal função é ativar macrófagos e células T citotóxicas que possuem função essencial no controle da infecção por microrganismos patogênicos (Karhawi *et al.*, 2000; Gonzalez *et al.*, 2000). Esta citocina modula a expressão de quimiocinas e receptores de quimiocinas e determina quais as células que irão infiltrar no pulmão de camundongos infectados com *P. brasiliensis* (Souto *et al.*, 2003). Mamoni *et al.* (2005) verificaram por RT-PCR o aumento na expressão dos genes que codificam para as quimiocinas CXCL10 e CXCL9 e este aumento foi relacionado com a produção de IFN-γ produzido por células de indivíduos com Paracoccidioidomicose (PCM) infecção. Além disso, em PCM experimental em camundongos foram detectados altos níveis de CCL5, CCL2, CXCL10 e CXCL9 concomitante com infiltração de células mononucleares no pulmão (Souto *et al.*, 2003). Macrófagos alveolares e fluídos de lavado bronco alveolares (BAL) de pacientes com PCM produzem e contem altos níveis de MIP-1α, IL-6 e TNF-α (Fornazim *et al.*, 2003). Além disso, foi observada um intenso infiltrado de neutrófilos no sítio da infecção, juntamente com aumento dos níveis de secreção das quimiocinas KC, MCP-1, MIP-1α, RANTES, Mig e IP-10 bem como de CCR5 e CXCR3 em pulmão de camundongos infectados com *P. brasiliensis* (Souto *et al.*, 2003).

Adicionalmente, Medeiros *et al*., (2004) em modelo de infecção experimental murino verificou que leveduras de *H. capsulatum* inoculados via intraperitoneal induzem a secreção de MIP-1α sugerindo que esta citocina está correlacionada com o influxo de células para o local da infecção.

TNF-α atua sinergisticamente com IFN-γ na ativação da resposta imune celular no controle da infecção por fungos patogênicos (Louie *et al*., 1994; Smith *et al*., 1990). O aumento dos níveis séricos desta citocina é considerado importante fator na defesa contra o *P. brasiliensis* (Mendes *et al*., 1991; Silva & Figueiredo, 1991). Parise-Fortes *et al*. (2000) verificaram que co-cultura de macrófagos peritoneais de hamsters inoculados com uma cepa virulenta de *P. brasiliensis* apresentava, em estágios iniciais da infecção, altos níveis de TNF-α, limitando a disseminação do fungo, através da ativação macrofágica e, conseqüentemente, do aumento da atividade fungicida. Macrófagos peritoneais de camundongos suscetíveis à infecção por *P. brasiliensis* secretam baixos níveis de TNF-α em comparação com animais resistentes (Calich e Kashino, 1998). Camundongos geneticamente deficientes do receptor para TNF-α são incapazes de controlar a infecção por *P. brasiliensis*, não desenvolvendo resposta granulomatosa ao agente agressor (Souto *et al*., 2000). Além disso, macrófagos ativados por TNF-α são capazes também de inibir a transição conídeo-levedura em *P. brasiliensis* (Gonzalez *et al*., 2004). Adicionalmente, Calich e Kashino (1998) relataram no estágio inicial de infecção com *P. brasiliensis* altos níveis de secreção de TNF-α, coincidindo com a produção de citocinas por células do linfonodo e contínua secreção de IL-12.

1.8. Metaloproteinase de matriz e sua função na interação patógeno-hospedeiro

Metaloproteinases de matriz (MMPs) constituem uma família de endopeptidases extracelulares dependentes de cálcio e zinco que degradam componentes da membrana basal e da matriz extracelular sendo, portanto, importantes moléculas efetoras para a migração de células do sistema imune para o local da infecção (Sternlicht *et al*., 2001). As MMPs também possuem função regulatória (Opdenakker *et al*., 2001), já que elas podem modular a atividade de citocinas e quimiocinas através de processamento proteolítico (Schonbeck *et al*., 1998; Van den Steen *et al*., 2000; McQuibban *et al*., 2000). Por outro lado, considerando sua função no remodelamento do colágeno e da matriz celular, as metaloproteinases de matriz também podem atuar durante a infecção por fungos, facilitando a invasão pelo patógeno (Claveau *et*

al., 2004). Neste sentido, Rodriguez *et al.* (1997) observaram a indução de genes que codificam para metaloproteases bem como a secreção destas proteínas no sobrenadante de macrófagos co-cultivados com *A. fumigatus* e sugerem que este evento provavelmente está correlacionado com o desenvolvimento de aspergilose invasiva. Adicionalmente a indução da expressão de genes que codificam para metaloproteinases de matriz (MMP1, MMP9, MMP10 e MMP14) tem sido observada em macrófagos infectados por diferentes patógenos tais como *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Salmonella typhi* e *A. fumigatus* (Nau *et al.*, 2002; Cortez *et al.*, 2006). Dessa forma a indução de genes relacionados ao remodelamento da matriz extracelular possivelmente é um mecanismo desenvolvido pelos patógenos para aumentar a eficiência de invasão celular e/ou tecidual.

1.9. Mecanismo de destruição do patógeno pelos fagócitos

A ativação e produção de citocinas pelos fagócitos e a internalização de microrganismos por células fagocitárias promovem a exposição do patógeno a um novo ambiente hostil com alterações no pH e enzimas degradativas, que estão presentes no fagossomo. Postula-se que o fagossomo, além de ser um ambiente de carência nutricional, apresenta condições extremas de estresse oxidativo devido às altas concentrações de espécies reativas de oxigênio (ROS) e nitrogênio (RNS) produzidas (Graham *et al.*, 1999; Schnappinger *et al.*, 2003). Estudos empregando modelos experimentais com macrófagos demonstraram que a atividade microbicida dessas células está relacionada fundamentalmente com a produção de NO e seus metabólitos. Essas espécies reativas são geradas a partir da interação de NO com outras moléculas, como o íon superóxido (O_2^-) que resulta na produção de um composto altamente reativo, o peroxinitrito ($ONOO^-$), que subseqüentemente decompõe-se em outras moléculas também altamente reativas. As espécies reativas causam uma variedade de alterações no DNA incluindo quebras de fitas e de-aminação, enquanto que em proteínas, causam modificações que envolvem nitrosilação de resíduos de cisteína e tirosina, além de inativação de enzimas com núcleo ferro-enxofre (Graham *et al.*, 1999; Schnappinger *et al.*, 2003; Nathan *et al.*, 2000). Essas modificações podem afetar enzimas metabólicas vitais, proteínas da cadeia respiratória da mitocôndria e síntese de DNA, causando efeitos citostáticos e citotóxicos *in vitro* e *in vivo* contra uma grande variedade de microrganismos, incluindo *Schistosoma mansoni* (James *et al.*, 1989), amastigotas de *Leishmania major* (Liew *et al.*, 1990), *Trypanosoma cruzi* (Norris *et al.*, 1995), *C. neoformans*

(Lee *et al.*, 1994), *H. capsulatum* (Lane *et al*., 1994), a forma de patogênica de *C. albicans* (Blasi *et al*., 1995), a forma leveduriforme de *Penicillium marneffei* (Kudeken *et al*., 1998), *M. tuberculosis* (Chan *et al*., 1995), entre outros. Bocca *et al.* (1998) mostraram um aumento na taxa de síntese de NO em camundongos infectados com o *P. brasiliensis,* corroborando assim os dados de que NO está envolvido na morfogênese e patogenicidade do fungo. Gonzalez *et al.* (2000) avaliaram a ação de NO produzidos por macrófagos peritoneais murinos ativados por IFN-γ em conídeos de *P. brasiliensis*, e observaram que o NO inibe o processo de transição de conídio para levedura.

A interação de *P. brasiliensis* e *H. capsulatum* com macrófagos é o evento chave na patogênese da PCM e da histoplasmose, respectivamente. Os macrófagos inicialmente fornecem um ambiente para a replicação do fungo e disseminação e então subsequentemente atuam como células efetoras finais para remover o patógeno do hospedeiro. As estratégias que estes patógenos utilizam para sobreviverem em macrófagos ainda é pouco conhecida. Newman *et al.* (1992) e Eissenberg *et al.* (1993) demonstraram que leveduras de *H. capsulatum* sobrevivem tanto em macrófagos humanos quanto em murinos por diminuir a fusão com o fagolisossomo e aumentar o pH, respectivamente. Em macrófagos murinos, a acidificação normal do fagossomo é prevenida pela inibição, por *H. capsulatum,* da inserção da ATPase vacuolar de macrófago (V-ATPase) na membrana fagossomal (Strasser *et al*., 1999). Esta estratégia provavelmene evita a ação das hidrolases ácidas sobre as leveduras e aumenta a capacidade de *H. capsulatum* adquir o ferro disponibilizado pelas transferrinas, cujo nível de saturação de ferro é reduzido pela metade em pH 6,5 (Princiotto *et al*., 1975). Entretanto, Newman *et al.* (2006) demonstraram que as hidrolases lisossomais de macrófago humanos não necessitam de um pH ácido para matar e degradar leveduras de *H. capsulatum,* sendo este mecanismo utilizado apenas pelos macrófagos murinos. A maior estratégia desenvolvida pelo *H. capsulatum* para aumentar sua sobrevivência em macrófagos humanos se encontra na habilidade das leveduras deste patógeno diminuírem a fusão do fagolisossomo (Eissenberg *et al*., 1993), assim como é observado na interação dos macrófagos com outros microrganismos tais como *M. tuberculosis* (Armstrong *et al*., 1971; Hart *et al*., 1987; Sturgill-Koszycki *et al*., 1994; Crowle *et al*., 1991; Clemens *et al*., 1995), *Mycobacterium avium* (Rastogi *et al*., 1992; Oh *et al*., 1996; Schaible *et al*., 1998), *Toxoplasma gondii* (Jones & Hirsch, 1972).

1.10. Perfil transcricional de macrófagos durante a interação com patógenos

Os macrófagos constituem a principal célula de defesa do sistema imune inato na maioria das infecções por microrganismos. Embora muitos trabalhos tenham sido realizados nos últimos anos buscando um melhor entendimento dos mecanismos ativados nestas células durante a interação com patógenos, os estudos se limitaram a um número reduzido de moléculas. Neste sentido a tecnologia de microarranjos abriu uma nova perspectiva ao ampliar o conhecimento em nível molecular das alterações sofridas por estas células durante a interação com o patógeno. Esta tecnologia tem sido aplicada extensivamente principalmente para macrófagos infectados com bactérias patogênicas. Assim, microarranjos de cDNA de macrófagos infectados com a bactéria intracelular *Brucella abortus* revelaram a indução de genes associados à inflamação de forma semelhante ao observado em experimentos de arranjos que utilizaram outras bactérias intracelulares gram-negativas tais como *Salmonella enterica* serovar Typhimurium e *Listeria monocytogenes* (Rosenberger *et al*., 2000; Cohen *et al*., 2000; Eskra *et al*., 2003). Recentemente, Weiss *et al*. (2004) analisaram por microarranjos o padrão sequencial da expressão gênica de macrófagos bovinos incubados com dois diferentes isolados micobacterianos e identificaram genes potencialmente envolvidos na eliminação destes microrganismos. Muitos genes associados a membranas endossomais ou lisossomais foram diferencialmente expressos, por exemplo, verificou-se a indução da expressão gênica de H$^+$ ATPases e a repressão da proteína 2 de macrófago associada ao lisossomo, Src tirosina quinase, proteína de tráfico de vesícula. Além disso, foi observada uma repressão de genes que pró-apoptóticos dos macrófagos induzido por um dos isolados bacterianos, reforçando os estudos funcionais que relataram uma diminuição da porcentagem de apoptose neste mesmo isolado (Weiss *et al*., 2004). A resposta de células dendríticas e macrófagos humanos a *M. tuberculosis* e aos protozoários filogeneticamente distintos (*Leishmania major, Leishamania donovani, Toxoplasma gondii*) e helminto (*Brugia malayi*) também foi analisada por microarranjos (Chaussabel *et al*., 2003). Nesta análise, esses autores observaram que na ausência de estímulos desses microrganismos, as células dendríticas e os macrófagos expressam constitutivamente 4.000 genes, sendo que 96% destes são comuns aos dois tipos celulares (macrófagos e células dendríticas). Dessa forma, esta análise revelou a existência de grupos funcionalmente similares de genes que são coordenadamente regulados em ambos os tipos celulares, tais como os relacionados à resposta inflamatória quando estas células são infectadas por *L. major* e *L. donovani*. A associação de características funcionais

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com cada agente infeccioso é consistente com o conceito de que células apresentadoras de antígenos possuem um padrão pré-determinado de sinalização para responder a diferentes patógenos (Chaussabel *et al.*, 2003).

Recentemente, trabalhos avaliando o perfil transcricional de macrófagos durante a infecção com microrganismos baterianos e micobacterianos, foram realizados (Rosenberger *et al.*, 2000; Cohen *et al.*, 2000; Eskra *et al.*, 2003; Chaussabel *et al.*, 2003; Weiss *et al.*, 2004). No entanto, o número de trabalhos relatando a análise do perfil transcricional de macrófagos infectados por fungos ainda é reduzido. Dentre tais trabalhos realizados recentemente destacam-se aqueles utilizando os fungos oportunistas *C. albicans* e *A. fumigatus* (Kim *et al.* 2005; Cortez *et al.* 2006). Neste contexto, Kim *et al.* (2005) analisaram a resposta transcricional de monócitos humanos normais infectados *in vitro* por *C. albicans* através de microarranjos de cDNA num período de 0 a 18h de infecção. Nesta análise foi observado aumento da expressão de genes que codificam citocinas proinflamatórias, incluindo TNF-α, IL-1, IL-6, durante as primeiras 6 horas de infecção, que coincidiu com o aumento da fagocitose. A expressão desses genes retornou próximo aos níveis basais em 18 horas de infecção. Genes codificando para quimiocinas, incluindo IL-8, proteínas inflamatórias de macrófagos 1, 3 e 4 e proteína de quimiotração de monócitos também foram fortemente induzidos no intervalo de 4 a 6 horas de infecção, assim como os receptores de quimiocinas CCR1, CCR5, CCR7 e CXCR5. A expressão de genes cujos produtos parecem proteger a viabilidade de monócitos, tais como os relacionados à proteína BCL2, metalotioneínas, CD71, e SOCS3, são induzidos em 4 a 6 horas e permanecem elevados até 18 horas de interação. Dessa forma, a expressão gênica diferencial de monócitos infectados por *C. albicans,* analisada por microarranjos de cDNA revela a indução de uma cascata dinâmica de expressão de genes cujos produtos são relacionados ao recrutamento, ativação e proteção de monócitos humanos no período inicial de infecção. Adicionalmente, com o objetivo de analisar a cinética inicial de expressão gênica de monócitos humanos infectados com conídios de *A. fumigatus,* Cortez *et al.* (2006) também utilizaram a tecnologia de microarranjos de cDNA. Nesta análise, os autores verificaram a indução de genes codificando citocinas e quimiocinas envolvidas na defesa do hospedeiro contra *A. fumigatus*, incluindo IL-1α, IL-8, CXCL-2, CCL-4, CCL-3 e CCL-20, de 2 a 6 horas de co-cultivo, que coincide com aumento na fagocitose. Além disso, observou-se que o gene que codifica para CD14 foi reprimido, e os genes codificando para IL-10 e metaloproteinase 1 de matriz foram induzidos.

Vale ressaltar que não existem na literatura até o momento dados de análise global e da cinética de interação relativos à expressão gênica diferencial do hospedeiro, por microarranjos de cDNA, em função dos processos de infecção pelos patógenos fúngicos *P.brasiliensis* e *H.capsulatum*, objeto deste estudo.

2. JUSTIFICATIVA

As células de defesa do hospedeiro, em especial os macrófagos desempenham funções muito importantes no estabelecimento e desenvolvimento da PCM e histoplasmose. Apesar disso, poucos estudos foram realizados para analisar as alterações de expressão gênica em resposta a infecção por *P. brasiliensis* e *H. capsulatum*. Os trabalhos publicados são, em sua maioria, relacionados aos genes específicos que codificam para moléculas de defesa relacionadas às citocinas e moléculas relacionadas à fagocitose. Diferentemente, vários estudos, utilizando modelos de infecção microbiana, vêm aplicando tecnologia genômica para verificar mudanças no perfil transcricional na interação hospedeiro-patógeno.

Este trabalho de doutorado propõe-se pela primeira vez analisar o perfil cinético global de expressão gênica coordenada das moléculas do macrófago em resposta aos fungos patogênicos *P. brasiliensis* e *H. capsulatum*. Adicionalmente, com base nos dados de expressão gênica do *P. brasiliensis* recuperado do interior do macrófago, conduzido em paralelo a este trabalho em nosso grupo (Tavares *et al.*, 2007), foi possível analisar as alterações em nível molecular, ocorridas no macrófago e no *P. brasiliensis*, em 6 horas da interação patógeno-hospedeiro. Os resultados obtidos no presente trabalho são pioneiros no que se refere à análise em larga escala da expressão gênica na interação patógeno-hospedeiro em fungos. Dessa forma, estes estudos irão ampliar o entendimento do processo infectivo destes dois importantes patógenos, *P. brasiliensis e H. capsulatum*, além de fornecer as bases moleculares de conhecimento para o desenvolvimento de novas estratégias no tratamento destas duas micoses sistêmicas.

3. OBJETIVO

Analisar a cinética da expressão gênica global de macrófagos infectados com *brasiliensis* ou *H. capsulatum.*

3.1. Metas

1- Analisar a expressão diferencial global e temporal dos genes de macrófagos durante a interação macrófago-*P. brasiliensis* por microarranjos de cDNA; quantificação das citocinas secretadas pelos macrófagos por ELISA e validação da expressão diferencial de genes dos macrófagos chaves por PCR em tempo real;

2- Analisar a expressão diferencial global e temporal dos genes de macrófagos durante a interação macrófago-*H. capsulatum* por microarranjos de cDNA e quantificação das citocinas secretadas pelos macrófagos por ELISA;

3- Realizar uma análise comparativa da resposta do hospedeiro frente à infecção causada pelos dois patógenos;

4- Propor um modelo de eventos que ocorrem em nível molecular durante a interação *P. brasiliensis*-macrófago, em decorrência dos dados obtidos neste trabalho de resposta dos macrófagos e dados do nosso grupo relativos à resposta de expressão gênica do patógeno após internalização pelos fagócitos.

4. METODOLOGIA

4.1. Fungos e condições de cultivo

P. brasiliensis: As células leveduriformes de *P. brasiliensis* isolado Pb01 (ATCC-MYA-826) foram crescidas em meio de cultura semi-sólido Fava Netto semi-sólido (0,3% protease peptona, 1% peptona, 0,5% extrato de carne, 4% glicose, 0,5% extrato de levedura, 0,5% NaCl, 1.7 % agar, w/v pH 7,2) a 37ºC por 7 dias antes da infecção dos macrófagos murinos.

H. capsulatum: O fungo *H. capsulatum* foi isolado de um paciente do Hospital das Clínicas de Ribeirão Preto-SP e é mantido em ágar-sangue com passagens periódicas em

camundongos da linhagem 129. As leveduras utilizadas nos experimentos de infecção foram previamente recuperadas de camundongo em meio ágar sangue por 20 dias a 37ºC.

4.2. Isolamento e cultivo dos macrófagos murinos

Macrófagos peritoneais: Macrófagos murinos peritoneais foram utilizados nos experimentos de infecção com o *P. brasiliensis*. Camundongos Balb/c (Brummer *et al*. 1989) foram injetados intraperitonealmente com 2 mL de tioglicolato 3% estéril, após 72 horas, os animais foram sacrificados e suas células recuperadas por lavagem peritoneal com 10 mL de meio de cultura RPMI-1640 gelado (Sigma), suplementado com 20 mM de HEPES, 1,5g/L de bicarbonato de sódio, 25 µg/ml de gentamicina e heparina (10 U/ml). As células peritoneais foram dispensadas em frascos de cultura de 150 cm^2 ($2x10^7$ células/frasco) e incubadas a 37ºC por 1 hora. Após a remoção das células não aderentes através de lavagem, as células aderentes foram incubadas em RPMI suplementado adicionado de 10% de soro fetal bovino (SFB) inativado a 37 ºC por 16 h.

Macrófagos J774: A linhagem de macrófagos J774.1 (ATCC TIB-67) foi utilizada nos experimentos de infecção com *H. capsulatum*. Essa linhagem J774.1 foi cultivada e mantida no mesmo meio e condições utilizadas para a manutenção dos macrófagos peritoneais descrito anteriormente.

4.3. Infecção dos macrófagos murinos com as leveduras de *P. brasiliensis* e *H. capsulatum* - Infecção com o *P. brasiliensis*:

a) **análise da cinética da internalização de leveduras de *P. brasiliensis* por macrófagos murinos**

Leveduras de *P. brasiliensis* foram ressuspendidas em meio RPMI suplementado com HEPES 20 mM e bicarbonato de sódio 1,5g/L contendo 20% de soro fresco de camundongos Balb/c. O protocolo de opsonização seguiu por meio da incubação da suspensão de leveduras a 37°C por 30 min. As culturas de macrófagos presentes nas microplacas foram infectadas com 1×10^5. As co-culturas foram então incubadas a 37°C em atmosfera de ar umidificada contendo 5% de CO_2. Após 1, 3, 6 e 12 h de infecção, as lamínulas foram removidas e lavadas com meio RPMI pré-aquecido a 37 ºC a fim de retirar as leveduras extracelulares ou fracamente aderidas aos macrófagos. As lamínulas contendo a co-cultura foram então fixadas com metanol e coradas com Wright-Giemsa. Utilizando microscopia óptica (magnitude de

1000×), cinqüenta a cem leveduras de *P. brasiliensis* foram contadas no intuito de determinar a porcentagem de leveduras aderidas internalizadas a cada ponto pós-infecção. Os experimentos foram realizados em triplicata e quatro a dez campos microscópicos foram analisados. Os resultados são apresentados com média ± SEM ("*standard error of the médium*").

b) Infecção do macrófago com *P. brasiliensis* para extração do RNA total dos macrófagos e dosagem de citocinas

Células leveduriformes de *P. brasiliensis* foram suspensas em 3 mL de meio RPMI contendo 20% de soro fresco de camundongo. A opsonização foi realizada incubando suspensões das células a 37ºC, por 30 minutos. As monocamadas de macrófagos dos frascos de cultura de 150 cm^2 (2×10^7 macrófagos/frasco) foram então infectadas com 4×10^6 células de levedura, na proporção levedura-macrófago de 1:5. As culturas foram incubadas a 37 ºC sob uma atmosfera de 5% de CO_2 por 6, 24 e 48 horas para as análises de modulação da expressão gênica durante a cinética de infecção dos macrófagos com *P. brasiliensis*. Para dosagem de citocinas 5×10^5 células peritoneais de macrófagos e 1×10^5 células fúngicas opsonizadas com soro foram preparadas em placas de cultura de 24 poços e incubadas como descrito anteriormente. Após 6, 24 e 48 horas, o sobrenadante dos macrófagos infectados e não infectados foi coletado e estocado a -20ºC. Os experimentos de dosagem de citocina foram feitos em triplicata para cada tempo.

- Infecção com o *H. capsulatum*: O procedimento para a infecção dos macrófagos J774.1 com *H. capsulatum* e a coleta do sobrenadante para dosagem de citocinas foi o mesmo utilizado para os macrófagos peritoneais com exceção de: a) as células J774.1 foram incubadas previamente por 18 h em meio contendo 2% de SFB; b) no momento da inoculação das leveduras foi utilizado um meio novo contendo 5% de SFB; c) as monocamadas de macrófagos foram infectadas com uma proporção de levedura-macrófago de 1:1 e os tempos de infecção foram 2, 24 e 48 horas.

4.4. Extração do RNA total dos macrófagos murinos

Após cada tempo de infecção (*P. brasiliensis* (6, 24 e 48h) e *H. capsulatum* (2, 24 e 48h)), os fungos não aderidos foram removidos por exaustiva lavagem com RPMI pré-aquecido a 37 ºC. Os macrófagos foram lisados com o reagente TRIzol® (Invitrogen, USA). O RNA total dos macrófagos infectados e controle foram extraídos com TRIzol®, conforme

protocolo do fabricante. Os experimentos foram realizados em triplicata para cada tempo de infecção (*P. brasiliensis* (6, 24 e 48h) e *H. capsulatum* (2, 24 e 48h)), o RNA dos macrófagos foi extraído em cada experimento individualmente e posteriormente reunidos para ser utilizado nos experimentos de microarranjos de cDNA, constituindo assim um *"pool"* de RNA de 3 experimentos independentes.

4.5. Experimentos de microarranjos em náilon

Os microarranjos de cDNA em náilon (contendo 624 clones) e em lâmina de vidro (contendo 4.500 clones) foram realizados para analisar a expressão diferencial dos genes dos macrófagos infectados com *P. brasiliensis* e *H. capsulatum*, respectivamente. Os clones de cDNA de sequências expressas de camundongo (ESTs) utilizados neste trabalho foram obtidas de uma livraria de timo normalizada, 2NbMT, disponível no consórcio I.M.A.G.E. (http://image.llnl.gov/image/html/iresources.shtml). Os insertos de cDNA foram clonados em três vetores (pT7T3D, pBluescript and Lafmid) e foram amplificados por PCR em placas de 384- ou 96-well poços com os seguintes primers, que reconhecem os três vetores, LBP 1S 5'-GTGGAATTGTGAGCGGATACC-3' forward e LBP 1AS 5'-GCAAGGCGATTAAGTTTGG-3' reverso. Água destilada e um segmento de DNA poli (A)$_{80}$ foram utilizados como controle negativo.

4.6. Microarranjos em náilon de macrófagos infectados com *P. brasiliensis*
a) Construção e hibridização dos microarranjos de cDNA e análise de dados

Os produtos de PCR constituídos pelos insertos de cDNA foram aplicados em duplicata em 2.5×7.5-cm Hybond® N$^+$ membranas de náilon (GE Health Care, USA) com um Array Spotter Geração III. Cada microarranjo assim construído contém 624 genes murinos relacionados a processos imunes, principalmente os envolvidos na resposta imune inata. As membranas foram primeiramente hibridizadas contra os oligonucleotídeos (hibridização do vetor) [γ-^{33}P] ATP-marcado. A quantidade de DNA depositada em cada ponto foi estimada pela quantificação dos sinais de hibridização. Em seguida, a sonda do vetor foi removida e as membranas foram utilizadas para a hibridização contra as sondas de cDNA complexas [α-^{33}P]dCTP- marcada (GE Health Care, USA), preparadas por transcrição reversa utilizando oligo(dT)$_{12-18}$, 10 µCi/µl (≥ 3000Ci/mM) de [α-^{33}P]dCTP (GE Healthcare, USA) e 10 µg do "pool" de RNA total isolado de macrófagos infectados e não infectados, em cada tempo da

cinética de infecção. A radioatividade total incorporada (30x 10^6 - 50 x 10^6 da sonda marcada em 100µL) foi avaliada pela leitura em aparelho de cintilação líquida (Tri-Carb 2100TR - UltimaGold, Packard). As sondas complexas marcadas com [α-^{33}P]cDNA foram hibridizadas contra os microarranjos por 48 horas a 65°C. As membranas foram lavadas e expostas em placas de imagem radiosensíveis por 48 horas, que posteriormente, foram digitalizadas por um phosphor imager (Cyclone, Packard Instruments) para captura dos sinais de hibridização. Depois da normalização, o programa SAM (Significance Analysis of Microarrays) foi utilizado para analisar as variações significativas da expressão gênica, entre as condições controle e experimental. Resumidamente, o programa SAM é baseado no test-t estatístico, especialmente modificado para este tipo de análise. Genes significativamente modulados foram identificados após serem avaliados por um critério de corte estatístico (q value < 5 %; FDR < 5 %) e variação do número de vezes que o gene foi induzido ou reprimido ($\geq$ 1,5- indução ou repressão). Os dados dos microarranjos foram depositados no ArrayExpress e possuem os seguintes números de acesso: A-MEXP-744 e E-MEXP-1093.

b) Delineamento experimental

Preparo das membranas de microarranjos de cDNA

624 clones da biblioteca de cDNA de timo de camundongo

⇩

Tratamento das membranas

⇩

Pré-hibridação das membranas

⇩

Marcação com oligo-vetor

⇩

Lavagem e exposição das membranas

⇩

Revelação das imagens e quantificação dos sinais

⇩

Lavagem das membranas para retirar o oligo-vetor

⇩

Membranas prontas para receber a sonda complexa
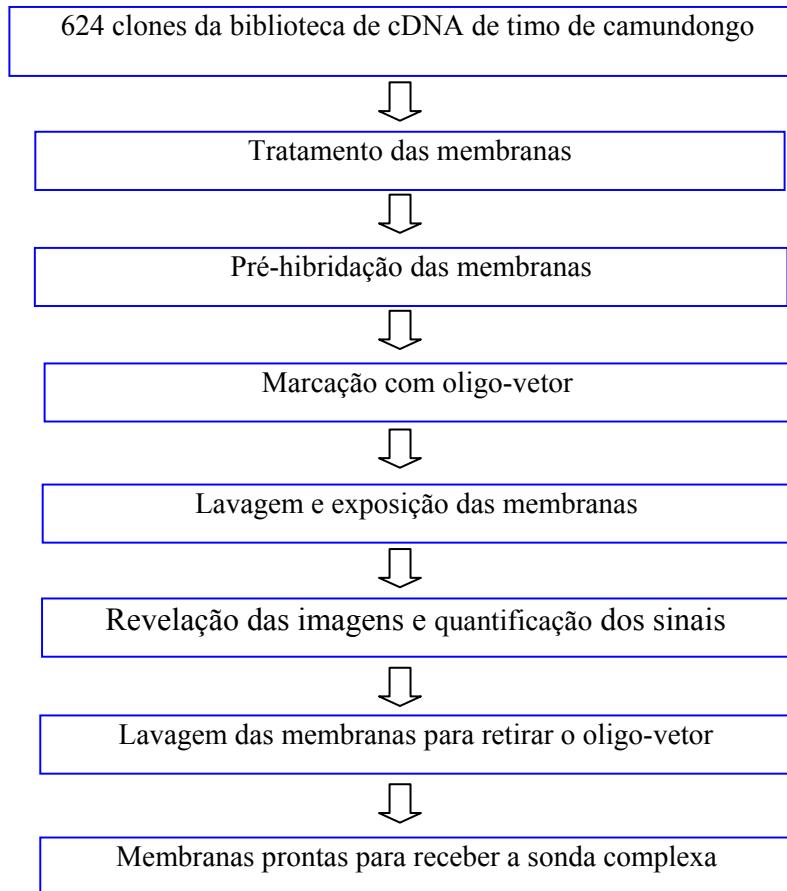
c) Validação por PCR em tempo real dos genes diferencialmente modulados

A mesma amostra de RNA analisada nos experimentos de microarranjos foi também utilizada para a confirmação da expressão diferencial por PCR em tempo real. Após o tratamento com DNAse I na presença de um inibidor de RNAse, quantidades iguais de RNA (0,5μg) foram submetidas a transcrição reversa (Superscript$^{®}$ III, Invitrogen, USA) usando um primer oligo (dT)$_{12-18}$ e submetidas a PCR em tempo real. As reações de amplificação foram realizadas com o aparelho 7900HT Sistema de Detecção de Sequência ABI PRISM (Applied Biosystems, USA) em uma reação de 12μl contendo: 0,4μM de cada primer, 6μl of SYBR Green PCR 2 × Master mix e 0,2 μl de molde de cDNA. Após a desnaturação inicial a 95°C por 10 min, as amplificações foram realizadas com 40 ciclos 95°C por 15 segundos, seguidos por 60°C por 1 minuto. Para confirmar a especificidade da amplificação, os produtos da PCR foram submetidos à análise da curva de anelamento. O método de comparação CT (Crossing Threshold), aplicando o gene constitutivo de macrófago ribossomal Rps9 foi usado para avaliar o valor de expressão de cada gene de interesse do macrófago infectado pelo fungo em comparação com o controle. Os experimentos de PCR em tempo real foram realizados duas vezes e em duplicata para todos os genes analisados. Todos os pares de oligonucleotídeos foram desenhados baseando-se nas sequências obtidas da base de dados do transcriptioma de camundongo (http://www.informatics.jax.org) utilizando o Primer Express software (Applied Biosystems). A sequência dos oligonucleotídeos utilizados para confirmar os dados dos microarranjos estão listados na tabela 1.

Tabela 1. Oligonucleotídeos usados no experimento de RT-PCR em tempo real para validação dos resultados do microarranjo

Clone ID	Gene	Senso (5´-3´)	Anti-senso (5´-3´)
	Rps9	CGCCAGAAGCTGGGTTTGT	CGAGACGCGACTTCTCGAA
574821	Clec1b	CTCTTCTTGGTGGCGTGTGA	AACAACCAGCCCCATGGA
575033	Nfkb	AGCCAGCTTCCGTGTTTGTT	AGGGTTTCGGTTCACTAGTTTCC
21961	Nfkrf	ACCTTTCAACCTACGATGGTCAGA	GAGCTCTCACATGGAATTTGGAA
575038	Tnf	GTACCTTGTCTACTCCCAGGTTCTCT	GTGGGTGAGGAGCACGTAGTC

d) Quantificação de TNF-α e IL-12

      Sobrenadantes da cultura de 6, 24 e 48 horas de infecção foram congelados, estocados a -20°C e descongelados imediatamente antes da reação de quantificação. As citocinas foram quantificadas utilizando kit comercial de ELISA (Pharmigen). Uma diluição do anticorpo de TNF-α (1:250 em 0.1 M tampão carbonato pH 9.5) anti-camundongo foi usado para cobrir microplacas de 96 poços (Nunc) por 12 horas a 4 °C. A seguir as placas foram lavadas e o diluente de ensaio foi adicionado (PBS e Soro Fetal Bovino 10%). Após 1 hora de incubação a temperatura ambiente as placas foram lavadas e as amostras foram adicionadas e incubadas por duas horas. Em seguida, a placa foi lavada e o conjugado estreptavidina-horseradish peroxidase (1:250) foi adicionado. A placa foi incubada por mais 1 hora a temperatura ambiente e lavada; posteriormente, foram adicionados 100µl do substrato de detecção (tetrametilbenzidine). A curva padrão foi preparada com citocina recombinante. A absorbância foi avaliada 30 minutos após incubação com o substrato de detecção a 405 nm, em uma leitora de placa de ELISA (TP-Reader, USA.). Os experimentos foram realizados em triplicata. O teste ANOVA foi realizado usando o GraphPad Prism version 4.00 para Windows, GraphPad Software, San Diego Califórnia, USA.

4.7. Microarranjos em lâminas de vidro dos macrófagos infectados com *H. capsulatum*
a) Obtenção do RNA total de referência (pool)

      Nos experimentos de microarranjos de cDNA em vidro foi utilizado um padrão de RNA denominado "RNA de referência" o qual foi preparado misturando-se amostras de RNA de células de diferentes tecidos de camundongos BALB/c (pool de referência). O RNA de referência não corresponde ao controle experimental, que por sua vez foi extraído de macrófagos não infectados.

b) Preparação dos microarranjos de cDNA em lâminas de vidro

      As amostras dos produtos de PCR obtidas conforme descrito no item 5 foram preparadas para deposição em lâminas de vidro adicionando-se mesmo volume (1:1) de reagente D (Amersham Biosciences) e transferidas para microplacas de 384 poços (Genetix). Através do robô Array Spotter Generation III (Amersham Biosciences) as amostras foram depositadas sobre as lâminas por um conjunto de 12 agulhas que depositam cada uma um volume de 0,9nL baseada na ação de capilaridade na superfície das lâminas de vidro. Após a deposição de cada conjunto de amostras, as agulhas foram lavadas automaticamente em uma

estação de lavagem que utiliza sucessivamente água purificada (18 megohm), etanol absoluto (Merck), solução de KOH 0,2M e água novamente. As agulhas foram secas com nitrogênio 5,0 analítico antes das amostras seguintes serem carregadas. A câmara de deposição das amostras em lâminas do Array Spotter III possui temperatura e umidade controladas., sendo a umidade relativa de deposição próxima de 55% e a temperatura em torno de 25°C. Após a deposição e secagem de todas as amostras nas lâminas, os pontos contendo as seqüências de cDNA foram fixados por "cross-linking" irradiando-se com 500 mJ de energia UV (Hoefer UV Crosslinker).

c) Marcação das sondas de cDNA com fluorocromos Cy3 e Cy5

As amostras de RNA (experimento e pool) foram marcadas utilizando o kit Cyscribe Post-Labelling (Amersham Biosciences) que permite a preparação do cDNA em dois passos. No primeiro passo ocorre a síntese da primeira fita de cDNA com a incorporação de nucleotídeos amino-alil dUTP modificados, e posterior degradação da cadeia de mRNA e purificação do cDNA para remoção de nucleotídeos livres e oligômeros. No segundo passo, o cDNA é marcado com formas reativas de ésteres NHS Cy3 (utilizado para marcar as amostras) e Cy5 (utilizado para marcar o pool de RNA) que se ligam aos nucleotídeos modificados. Após um processo de purificação para eliminação dos CyDye não incorporados a sonda está pronta para hibridação.

- Preparação da primeira fita de cDNA por incorporação de AAdUTP

Em um tubo tipo Eppendorf, em banho de gelo, foram adicionados 10µg de RNA total, 1µl de oligonucleotídeo randômico, 3µl de oligo (dT) e 0,5µl de controle denominado "spike mix" para o Universal ScoreCard em um volume total de 11µl (proporção de 2 µl de spike mix para cada 1µg de mRNA marcado). Todo esse procedimento é feito em duplicata A reação foi misturada cuidadosamente e incubada a 70ºC por 5 min, sendo posteriormente resfriada a 4ºC durante 5min. A extensão da cadeia de cDNA foi realizada utilizando 4µl de tampão 5X CyScript, 2µl de DDT 0,1M, 1µl de nucleotídeo "mix", 1µl de AA-dUTP, 1µl de transcriptase reversa CyScript, em um volume final de reação de 20µl. A reação foi incubada a 42ºC por 1,5 horas. A degradação do mRNA foi realizada adicionando 2µl de NaOH 2.5M com incubação a 37ºC durante 15min, e 20µl de HEPES 2M. A purificação do cDNA foi realizada usando colunas CyScribe GFX.

- Incorporação de Cy3 e Cy5

A amostra de cDNA marcada com amino-alil e purificada foi adicionada a um tubo contendo a alíquota de CyDye NHS éster e ressuspendida várias vezes. O material foi centrifugado a 13.800g por 1min e incubada no escuro durante 1 hora. Adicionou-se 15µl de hidroxylamina 4M seguida de incubação no escuro durante 15 min a temperatura ambiente. Os cDNAs marcados também foram purificados com as colunas GFX.

- Quantificação do CyDye incorporado no cDNA

Após a purificação dos cDNAs marcados foi realizada a monitoração de incorporação dos fluorocromos por meio de leitura em espectrofotômetro VIS (Ultrospec 2100, Amersham Biosciences) em comprimento de onda de 550nm para Cy3 e 650nm para o Cy5 com amostras diluídas 100 vezes. A quantidade de Cy3 ou Cy5 incorporada no cDNA pode ser calculada através do seu coeficiente de extinção molar 150 000 l mol $^{-1}$ cm^{-1} para Cy3 e 250 000 l mol $^{-1}$ cm^{-1} para Cy5. As proporções de Cy3 e Cy5 incorporados no cDNA foram calculadas através da fórmula: (A)/E x Z x fator de diluição x 10 ^{12}, onde:A= absorbância de Cy3 a 550nm ou Cy5 a 650nm, E= coeficiente de extinção molar para Cy3 ou Cy5 x 10^{-6} e Z= volume (**µ**l) da sonda após purificação.

Para hibridações com os dois fluorocromos foram adicionados os cDNA marcados com Cy3 e Cy5 em um tubo de microcentrífuga protegido da luz. A solução de cDNA foi evaporada em um aparelho "Speed Vaccum". A seguir, o cDNA foi dissolvido em 6 **µ**l de água livre de nuclease e desnaturado a 95°C por 2 min. A solução foi imediatamente resfriada no gelo por 30 segundos. À essa reação foram adicionados 1,5 **µ**l de A$_{80}$ (1mg/ml) e o sistema foi incubado a 75°C por 45min.

d) Hibridação das lâminas com os microarranjos

As lâminas de microarrajos foram hibridadas utilizando um processador automático de lâminas, "Lucidea Automated Slide Processor"–ASP (Amersham Biosciences) que permite a hibridação e lavagens de lâminas em câmaras independentemente controladas. Este aparelho inclui um programa que automatiza a injeção de amostras líquidas e soluções de lavagens ou ar dentro das câmaras e possui parâmetros de controle de temperatura, velocidade de injeção e circulação destas soluções no interior das mesmas.

As sondas de cDNA marcadas com Cy3 (amostras) e Cy5 (pool de RNA) foram feitas da mesma maneira que no item anterior, e as lâminas foram hibridadas por 15 horas a 42°C. As condições de lavagens foram: 1XSSC/0,2%SDS (2 vezes por 20 segundos à temperatura

ambiente); 0,1X SSC/0,2% SDS (2 vezes por 20 segundos à temperatura ambiente); 0,1X SSC (2 vezes por 20 segundos à temperatura ambiente). A última lavagem das lâminas foi feita com isopropanol, sendo em seguida aquecidas à 42°C, e novamente lavadas com isopropanol. Após as lavagens as lâminas foram aquecidas a 60°C para secagem e posteriormente foram colocadas no "Array Scanner" (Amersham Biosciences) para obtenção das imagens.

e) Aquisição das imagens dos microarranjos

As lâminas foram colocadas no aparelho "Array Scanner Gen III" (Amersham Biosciences) e as imagens foram obtidas com feixes de raios laser para as duas cores, o comprimento de onda de excitação do laser de 532nm para o verde e 633nm para o vermelho para cada um dos "spots" sobre o microarray. A leitura da lâmina gera dois arquivos com imagens separadas para os dois canais de cores (Cy3 e Cy5) e uma imagem colorida de sobreposição produzida com auxílio do programa ImageQuant (Amersham Biosciences).

f) Análise dos Dados

A quantificação das imagens dos microarranjos foi obtida com o programa Array Vision (Amersham Biosciences). A filtragem, normalização e análise dos dados foram realizadas no ambiente estatístico R (Ihaka e Gentleman, 1996), com o auxílio dos pacotes Limma (Smyth, 2004), Bioconductor (Ihaka e Gentleman, 1996; Bates *et al*., 2002), Aroma (Bergtsson, 2004) e KTH (Wirta, 2003). Foram aplicados métodos de normalização "Printtip" Lowes (Lowess por agulhas) e re-escalonamento dos valores M pelo valor de MAD ("Median Absolute Deviation") (Yang *et al*., 2002). O método estatístico Bayesiana Empírica (B-test) foi utilizado para a detecção dos genes diferencialmente expressos (Smyth, 2004).

5. RESULTADOS

5.1. Cinética do índice de fagocitose *de P. brasiliensis* durante a interação com macrófagos murinos

Com o objetivo de verificar a porcentagem de internalização de *P. brasiliensis* pelos macrófagos murinos foi realizado ensaios de fagocitose durante o co-cultivo nos tempos de 1, 3, 6 e 12 horas. Observou-se nesta análise de adesão/internalização que em 3 horas de co-cultivo a maioria das leveduras se encontra fortemente aderidas aos macrófagos e somente após 6 horas é que a quantidade de leveduras internalizadas é maior que a de aderidas (Figura 6A) com média de uma levedura por célula (figura 6B). Baseado nesta cinética de interação

foi escolhido o tempo de 6h como o estágio inicial para a análise da expressão diferencial por microarranjos dos genes de macrófagos murinos infectados pelo *P. brasiliensis*. A taxa de fagocitose para o *H. capsulatum* já é bem conhecida (Newman *et al*., 1990) e de acordo com os dados da literatura foi definido o tempo de 2 horas como o estágio mais inicial da infecção a ser analisado pelos microarranjos de cDNA. Em 2 horas de co-cultivo de *H. capsulatum* com os macrófagos a maioria das leveduras já se encontra aderidas e/ou internalizadas.
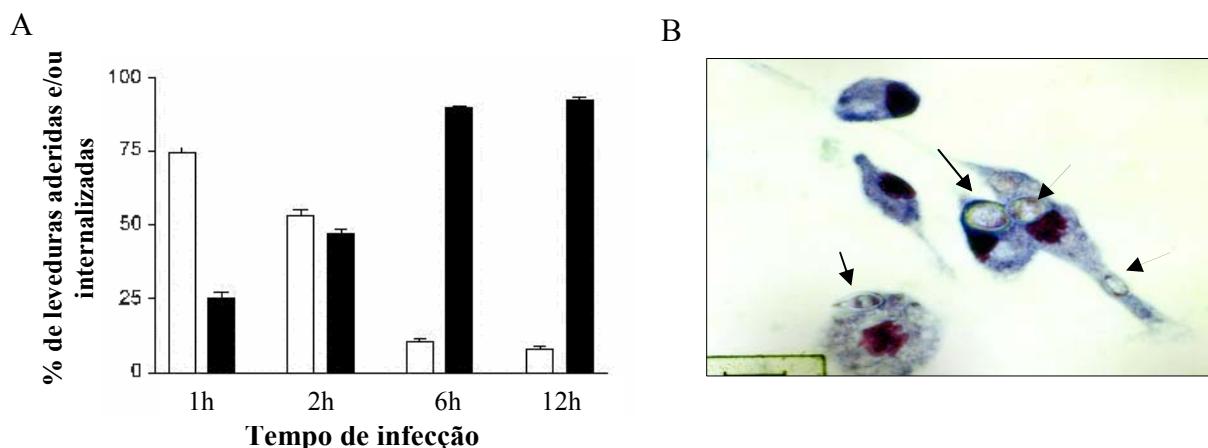
Figura 6. Cinética da infecção dos macrófagos murinos pelo *P. brasiliensis*. **(A)** Percentagem de leveduras de *P. brasiliensis* aderidas (barra branca) e internalizadas (barra preta) ao longo de 12 horas de infecção *ex vivo*. **(B)** Microfotografia (1000 x) representativa do ponto de 6 horas de co-cultivo. Setas maiores: leveduras internalizadas; Seta menor: levedura aderida lateralmente. Macrófagos e leveduras opsonizadas foram incubados obedecendo a uma razão de levedura/macrófago de 1:5 a 37 ºC em atmosfera de 5% de CO_2. Os dados mostrados são derivados de dois experimentos realizados em triplicata (média ± SEM).

5.2. Análise em gel de agarose do RNA total extraído dos macrófagos infectados ou não pelos fungos *P. brasiliensi* e *H. capsulatum*

A integridade dos RNAs dos macrófagos infectados ou não por *P. brasiliensis* foi avaliada pela técnica de eletroforese em gel de agarose 1% corado com brometo de etídeo. Na figura 7 é possível observar as bandas ribossomais maiores (26S - fungo e 28S - macrófago) e menores (18S – banda comum dos fungos e macrófago) bem definidas e ausência de degradação. A quantificação e confirmação da pureza das amostras foram realizadas pelo uso do aparelho Gene Quant (Amersham).
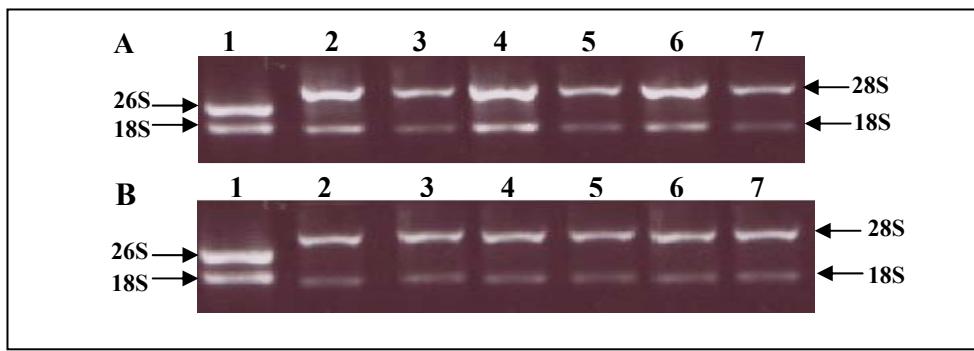
Figura 7. Pefil eletroforético do RNA total extraído dos macrófagos.

(A) Poço 1: RNA de *P. brasiliensis*; Poços 2, 4 e 6: RNA dos macrófagos murinos infectados com *P. brasiliensis* por 6, 24 e 48 horas, respectivamente; Poços 3, 5 e 7: RNA dos macrófagos murinos não infectados após 6, 24 e 48 horas de cultura, respectivamente.

(B) Poço 1: RNA de *H.capsulatum*; Poço 2, 4 e 6 RNA dos macrófagos murinos infectados com *H. capsulatum* por 2, 24 e 48 horas, respectivamente; Poços 3, 5 e 7: RNA dos macrófagos murinos não infectados após 2, 24 e 48 horas de cultura.

PARTE I

5.3. Análise da expressão gênica dos macrófagos peritoneais após infecção com *P. brasiliensis*.

A expressão gênica diferencial foi analisada em macrófagos peritoneais após 6, 24 e 48 horas de infecção com *P. brasiliensis* isolado Pb01. Três experimentos independentes de microarranjos para cada tempo foram realizados e os níveis de transcrição dos 624 genes envolvidos em processos inflamatórios, fagocitose, transdução de sinal, regulação da transcrição e apoptose, entre outros foram determinados.

A análise dos dados de expressão gênica dos macrófagos, pelo programa SAM, revelou 273 genes significativamente modulados após infecção com *P. brasiliensis*. No entanto, somente aqueles genes com uma indução/repressão ≥ 1,5 foram considerados para as futuras análises. Este critério de estringência reduziu o número de genes diferenciais para 118 (105 induzidos e 13 reprimidos), quando as células infectadas foram comparadas com aquelas não

infectadas, nos tempos mencionados anteriormente. A maioria dos genes modulados em macrófagos infectados com *P. brasiliensis* foi observada em 24 horas.

A tabela 1 mostra a lista dos genes induzidos em 6 horas de incubação, estágio relativamente inicial da infecção do macrófago. Um total de 28 genes de macrófagos peritoneais infectados com *P. brasiliensis* apresentaram mudanças na expressão gênica após 6h de interação. Os genes pró-inflamatórios relacionados à citocinas incluem: Irak2, Il7r, Ccl21, Ccl22 e Cxcl1; os genes associados à membrana: Cle1b, Cd8 e Mmp17. Os genes induzidos relacionados à transcrição foram representados por Ier5, Ccr4-not10 e Stat1. Proteínas STAT são fatores de transcrição que mediam a sinalização dirigida por citocinas. Os genes relacionados à transdução de sinal induzidos em 6h de infecção foram: Grb2, Cd37 e Rasa3. Além disso, foi observada a indução de genes relacionados à apoptose e estado redox tais como: Granzima A (Gzma) e tiorredoxina, respectivamente. Adicionalmente, três genes relacionados que não possuem função conhecida foram induzidos. Não foi observada a repressão de nenhum gene de macrófago contido no microarranjo, no tempo de 6h de infecção com *P. brasiliensis*.

Tabela 1. Genes de macrófagos induzidos após 6 h de infecção com o *P. brasiliensis*

Categoria	Descrição	Gene	Clone ID[a]	Indução
Inflamação				
	Similar ao receptor de interleucina-1 associado a quinase 2	Irak2	6474954	1,74
	Receptor de interleucina 7	Il7r	578171	2,19
	C-C ligantes de quimiocinas 21	Ccl21	576394	1,94
	C-C ligantes de quimiocinas 22	Ccl22	577486	2,20
	C-X-C ligantes de quimiocinas 1	Cxcl1	5321155	1,85
	Histocompatibilidade 2, antígeno E beta classe II	H2-Eb1	574155	2,51
	Antígeno CD28	Cd28	576501	2,20
Proteínas de membrana				
	C- receptor tipo lectina 1b	Clec1b	574821	4,63
	Antígeno CD8, cadeia alfa	Cd8	1247019	1,61
	Metaloproteinase de matriz 17	Mmp17	30850	1,84

Antígeno 6 de linfócito, locus A	Ly6a	581749	2,39
Regulação transcricional			
Resposta inicial imediata 5	Ier5	574793	1,83
Complexo de transcrição CCR4-NOT, subunidade 10	Cnot10	576406	2,33
Ativador da transcrição e da transdução de sinal 1	Stat1	583302	1,70
Transdução de sinal			
Fator do receptor de crescimento ligado a proteína 2	Grb2	575387	2,64
Antígeno CD37	Cd37	640080	1,56
Ras p21 ativador de proteína 3	Rasa3	582174	1,56
Proteína 1 do complexo T	Tcp1	640974	2,39
Apoptose			
Granzima A	Gzma	572830	1,56
Transferase			
Serina hidroximetil transferase 1 (solúvel)	Shmt1	573743	1,82
Proteína O-fucosiltransferase 2	Pofut2	575950	1,59
Outras funções			
Gama-glutamil carboxilase	Ggcx	582706	1,55
Repair de raio-X	Xrcc4	574242	1,59
Proteína com motivo de ligação a RNA 4B	Rbm4B	575510	1,80
Tiorredoxina	-	582490	1,82
Segmento de DNA, Chr2	D2Ertd	575429	2,37
Sequência transcrita	-	582106	1,91
Sequência transcrita	-	640795	1,99

[a] Identificação do gene na base de dados do SOURCE (http://source.stanford.edu/).

Em 24 horas de infecção, 59 genes foram diferencialmente expressos (54 induzidos e 5 reprimidos) (tabela 2). O gene codificando a citocina Ccl21 e outros genes relacionados à inflamação tais como Irak2, Pparbp e Ly86 foram induzidos. A indução de genes envolvidos na regulação da transcrição foi observada para o inibidor de Stat1, Ccr4-not 2, Tcf12, Foxo1, Nfkb e IkB. Alguns dos genes pró-inflamatórios regulados por NFkB incluem NOS2, TNF-α,

IL-1, COX-2 e várias quimiocinas, as quais podem aumentar a inflamação por atrair células inflamatórias adicionais para o sítio da infecção. A proteína NfkB também regula a transcrição de seu inibidor IkB (Karin & Ben, 2000) . Os genes relacionados à membrana Clec1b e Ddr1, que estão relacionados a uma fagocitose mais eficiente, foram induzidos, bem como componentes do complemento C2 e C3. Vários genes envolvidos no processo da ativação de macrófagos através de transdução de sinais (Mapk1, Mapk8ip3, Mapk8, Txk e Rab3d) foram induzidos. O gene que codifica Rab3d é um membro da família de proteínas Rab ligadoras de GTP, que são envolvidas na via de tráfico vesicular. Seis genes relacionados a apoptose foram induzidos na presente análise, incluindo aqueles que codificam as caspases pró-apoptóticas 2, 3 e 8 os inibidores Fas-i e Casp8r. Além disso, 5 genes tiveram sua expressão reprimida, sendo que dois são relacionados à inflamação (Ccl6 e Cxcl4) e os outros três genes reprimidos não apresentam função conhecida.

Tabela 2. Genes de macrófagos modulados após 24h de infecção com *P. brasiliensis*

Categoria	Descrição	Gene	Clone ID[a]	Indução/ Repressão
Genes induzidos				
Inflamação				
	C-C ligantes de quimiocinas 21	Ccl21a	576394	10,87
	Similar ao receptor de interleucina-1associado a quinase 2	Irak2	6474954	1,71
	Proteína ligante do receptor que ativação a proliferação do peroxissoma	Pparbp	582634	24,58
	Antígeno 86 de linfócito	Ly86	583305	5,88
	Antígeno CD37	Cd37	640080	2,00
	Relacionada à ativação de células T	Lat	582840	2,05
	Componente 2 do complemento (com H-2S)	C2	583642	3,47
	Componente 3 do complemento	C3	582886	1,80
Proteínas de membrana				

C- receptor tipo lectina 1b	Clec1b	574821	1,80
Família do receptor de domínio discóide, membro 1	Ddr1	575615	3,11
Metaloproteinase de matriz 17	Mmp17	30850	1,55
Selectina	Sell	621878	4,76
Proteína que interage com o adaptador transmembrana SHP2	Sit1	576098	1,65
Antígeno CD8, cadeia alfa	Cd8	1247019	2,06
Receptor gama de célula T, região constante	TcrgC	640129	1,58

Regulação transcricional

Proteína inibidora de STAT 1 ativado	Pias1	577047	1,89
Complexo de transcrição CCR4-NOT, subunidade 2	Cnot2	572999	3,46
Fator de transcrição 12	Tcf12	575368	2,62
Forkhead box O1	Foxo1	574862	19,41
Subunidade P50 de NFKappaB	Nfkb	575033	23,47
Factor de repressão de NFKappaB	Nkrf	21961	6,02
Fator de ligação ao enhancer linfóide 1	Lef1	575374	1,85

Transdução de sinal

Proteina quinase mitógeno ativada 1	Mapk1	574250	1,73
Proteina quinase mitógeno ativada 8 que interage com a proteína 3	Mapk8ip3	576539	1,79
Protein quinase mitógeno ativada 8	Mapk8	576631	3,06
Tirosina quinase TXK	Txk	583533	1,67
Membro RAS da família de oncogenes	Rab3d	575981	1,52

Apoptose

Caspase 2	Casp2	573760	1,62
Caspase 3, cisteína protease relacionada a apoptose	Casp3	581767	1,68
Caspase 8	Casp8	5099113	1,54
Molécula inibidora de Fas apoptótico 2	Faim2	37367	1,52
Regulador de CASP8 e FADD-like	Cflar	640265	1,54

Transferase

Metiltransferase 11 contendo o domínio 1	Mett11d1	576502	13,57
Metiltransferase-like 3	Mettl3	575022	3,85
Proteína O-fucosiltransferase 2	Pofut2	575950	1,64

Outras funções

Fator de splicing, rico em arginina/serina 1	Sfrs1	582744	1,54
Atividade arsenato redutase	-	582490	1,50
Ciclina D3	Ccnd3	575230	1,66
Gama-glutamil carboxilase	Ggcx	582706	1,66
Protein Kelch	Kbtbd8	582871	1,73
Enzima ativadora de SUMO 1 subunidade 2	Sae2	576467	2,20
Proteína I que se liga ao DNA, 127kDa	Ddb1	4823153	1,65
Differencialmente expressa em FDCP 6	Def6	573651	2,1
Domínio 27 repetido de ankirina	Ankrd27	575492	1,59
Locus transcrito	-	583763	7,30
Locus transcrito	-	641074	2,22
Sequência transcrita	-	581675	2,34
Sequência transcrita	-	575134	1,80
Sequência transcrita	-	576644	1,53
Sequência transcrita	-	582394	2,04
Sequência transcrita	-	576140	1,63
Sequência transcrita	-	576430	4,47
Sequência transcrita	-	640779	1,98
Em múltiplo cluster	-	574242	1,71

Genes reprimidos

Inflamação

C-X-C ligantes de quimiocinas 4	Cxcl4	573339	0,26
C-C ligantes de quimiocinas 6	Ccl6	576828	0,52

Outras funções

Caseína quinase nuclear 1	Nucks1	573338	0,19

	Fbox5	575998	0,46
F.box e protein rica em repetição de leucina 5			
Antígeno 6 de linfócito, locus D	Ly6d	581909	0,46

Em 48 horas de infecção, 31 genes foram diferencialmente expressos, sendo 23 induzidos e 8 reprimidos (tabela 3). Os genes pró-inflamatórios induzidos incluem Irak2, Cxcl14 and Cxcl4. Entre os genes induzidos relacionados à inflamação estão Clec1b, Cd14, H2eb, Ly6a (antígeno de linfócito do complexo 6, locus A) e Adam8 (Desintegrina e domínio de metaloprotease 8). Os genes induzidos relacionados à transdução de sinais foram: Mapkk11, Ptk9-like e SH3-domínio Grb2-like 1 (Sh3gl1). Neste tempo de infecção, foi observado um maior número de genes reprimidos das seguintes categorias funcionais: inflamação (Ccl21), transdução de sinal (Rab2 e Mark2) e regulação da transcrição (Pias1, S7a11lc e RIKEN).

Tabela 3. Genes de macrófago modulados após 48 h de infecção com o fungo *P. brasiliensis.*

Categoria Descrição	Gene	Clone ID[a]	Indução/ Repressão
Genes induzidos			
Inflamação			
Similar to interleukin-1 receptor-associated kinase 2	Irak2	6474954	1,64
C-X-C ligantes de quimiocinas 14	Cxcl14	583442	1,58
C-X-C ligantes de quimiocinas 4	Cxcl4	573339	1.52
Proteínas de membrana			
C- receptor tipo lectina 1 b	Clec1b	574821	2,66
Antígeno CD14	Cd14	5120996	1,91
Histocompatibilidade 2, classe II antígeno E beta	H2-Eb	574155	1,63
Antígeno 6 de linfócito, locus A	Ly6a	581749	1,67
Domínio 8 de desintegrina e metaloprotease	Adam8	582054	1,51
Beta-2 microglobulina	B2m	576472	2,73
Beta-2 microglobulina	B2m	576493	3,11
Transdução de sinal			
Proteína quinase quinase quinase mitógeno ativada 11	Mapkkk11	575211	3,00
Tirosina quinase TXK	Txk	583533	1,61

Domínio SH3, GRB2-like1	Sh3gl1	582991	1,69
Twin filina, homólogo 2 da proteína ligante de actina	Twf2	640339	1,80
Outras funções			
Inibidor de serina (ou cisteína) proteinase inhibitor 1	Serpinh1	582567	1,98
13 days embryo head cDNA	-	575324	1,73
Receptor do translocador nuclear hidrocarbono-like	Arntl	582931	1,93
Gene I de translocação de células B, anti-proliferativo	Btg1	574654	1,95
Atividade de tioredoxina	-	582490	2,75
Citocromo b-245, polipeptídeo beta	Cybb	583187	2,05
Locus transcrito similar a heat shock de 27kDa	-	582517	2,45
Sequência transcrita	-	582338	2,12
Sequência transcrita	-	575870	1,69

<u>Genes reprimidos</u>

Inflamação

C-X-C ligantes de quimiocinas 2	Ccl2	573898	0,33

Regulação transcricional

Proteína inibidora de STAT 1 ativado	Pias1	577047	0,47
Família carreadora de soluto, membro 11	S7a1lc	575418	0,50
RlKEN	-	576643	0,29

Transdução de sinal

RAB2, membro RAS da família de oncogenes	Rab2	573835	0,50
MAP quinase 2 que regula a afinidade a microtúbulo	Mark2	574316	0,36

Outras funções

Subunidade do complexooligosacariltransferase	Stt3b	573897	0,36
Enzima que degrada insulina	Ide	577628	0,37

[a] Identificação do gene na base de dados do SOURCE (http://source.stanford.edu/).

5.4. Validação dos genes envolvidos na resposta imune

Com o objetivo de validar os dados dos microarranjos foi realizada a RT-PCR em tempo real com as mesmas amostras de RNA utilizadas nos experimentos de microarranjos. Foram escolhidos para a validação três genes chaves envolvidos na resposta imune (Nfkb,

Nkrf, and Clec1b) que se apresentaram diferencialmente expressos durante a infecção com *P. brasiliensis*. Nfkb e seu repressor Nkrf são reguladores do processo inflamatório e Clec1b é importante para uma fagocitose mais efetiva pelos macrófagos. A **tabela 4** mostra que o perfil de expressão de todos esses genes quando validados pela RT-PCR em tempo real, corrobora-se com os dados obtidos pelas análises de microarranjos. A magnitude de indução difere entre as metodologias, no entanto, isto é um resultado esperado devido às diferenças técnicas em relação ao protocolo de normalização, cinética e sensibilidade dos dois métodos empregados.

Tabela 4. Validação dos dados de microarranjo por RT-PCR em tempo real.

Clone ID	Gene	Indução[a]	
		Microarranjo	[b]RT-PCR em tempo real
574821	Clec2[c]	4,63	4,5
574821	Clec2[e]	2,65	1,7
575033	Nfk-b[d]	23,47	1,4
21961	Nkrf[d]	6,02	3,1

[a] O valor de indução gênica foi determinado após a normalização de cada gene pelo Rps9.

[b] Média de indução de dois independentes experimentos de RT-PCR em tempo real feitos em duplicate em [c]6 horas, [d]24 horas e [e]48 horas.

5.5. Análise do perfil de expressão de Tnf-α por RT-PCR em tempo real e dosagem de citocinas dos macrófagos infectados com *P. brasiliensis*

O TNF-α está associado com a atividade antimicrobiana dos macrófagos sendo uma importante citocina no controle de micoses (Louie, *et al*. 1994; Smith *et al*. 1990; Souto *et al*., 2000). No presente trabalho, o perfil de expressão de Tnf-α foi analisado por RT-PCR em tempo real associado à dosagem por ELISA, já que o gene que codifica para essa citocina não tinha sido incluído na membrana dos microarranjos de cDNA. Entretanto, esta limitação não eliminou a possibilidade de se verificar o perfil de expressão desta citocina extremamente relevante para a resposta inflamatória. Utilizando PCR em tempo real observou-se uma

indução de três vezes na expressão gênica de Tnf-α em 24 horas de infecção. Além disso, foi confirmado por ELISA que a indução do mRNA também resulta em um aumento na secreção da proteína. Dessa forma os experimentos de dosagem de TNF-α por ELISA, revelaram um discreto aumento de TNF-α em 6 horas de infecção, enquanto que em 24 e 48 horas, esta citocina foi fortemente secretada por macrófagos infectados quando comparado aos não infectados (**Figura 8**). Outra citocina importante na indução de uma resposta inflamatória é a IL12 que também foi avaliada a sua expressão. Entretanto nenhum aumento da produção desta citocina pelos macrófagos foi observado nos tempos de infecção analisados.
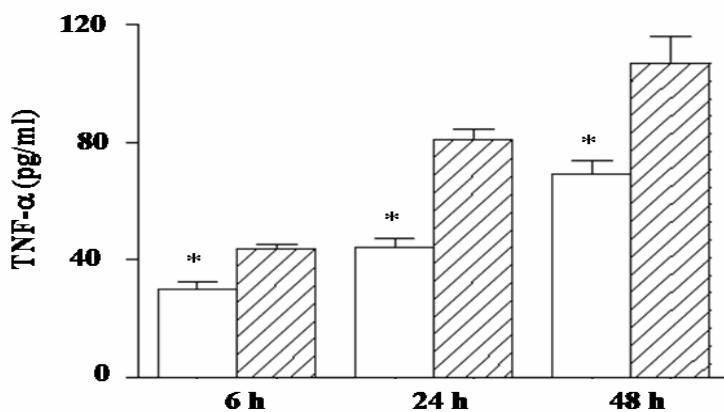
Figura 8. TNF-α liberado por macrófagos peritoneais em cultura. Macrófagos foram infectados com *P. brasiliensis* isolado 01. O sobrenadante foi coletado após 6, 24 e 48 horas. As concentrações da citocina foram determinadas por ELISA. One-way ANOVA com o teste de Dunnett's foram realizados para três experimentos independentes. As barras abertas representam as células não infectadas e as barras hachuradas representam as células infectadas. * $p < 0.01$ quando comparado a macrófagos não infectados.

PARTE II

5.6. Microarranjos de cDNA dos macrófagos infectados com *H. capsulatum*

Na segunda parte deste trabalho são apresentados os resultados obtidos a partir da análise de expressão gênica diferencial dos macrófagos durante a cinética de infecção pelo *H. capsulatum* (2, 24 e 48 horas), utilizando microarranjos em vidro contendo 4.500 clones de cDNA de macrófagos murinos. A utilização da plataforma de microarranjos em vidro para esta parte do trabalho foi possível uma vez que o grupo da USP/RP que colabora neste trabalho conseguiu dominar a tecnologia dos microarranjos em vidro, o que não ocorreu durante os

experimentos realizados com o outro patógeno *P.brasiliensis*. Certamente, estendendo a análise para 4.500 clones a avaliação global de expressão gênica na interação patógeno-hospedeiro ficará muito mais completa. Para cada tempo analisado foram realizados dois experimentos independentes de microarranjos (cada lâmina de microarranjo contendo os clones depositados em duplicata) e os níveis dos transcritos foram quantificados. A mudança de expressão gênica na cinética de infecção foi observada para um total de 320 genes, quando comparados os macrófagos infectados com aqueles não infectados. Os genes modulados foram agrupados em 12 principais categorias funcionais (inflamação, transcrição, transdução de sinais, apoptose, transporte, proteínas relacionadas à membrana, histonas, processamento de RNA, potencial redox, proteasoma, fosfatases e transferases). A maioria dos genes foi modulada no tempo de 48 horas, sendo que em 2 horas de infecção observou-se uma menor modulação gênica. O número de genes induzidos em relação aos reprimidos não foi expressivamente diferente durante a cinética de infeção.

A tabela 5 lista os genes de macrófagos infectados por *H. capsulatum* e modulados em 2 horas de infecção. O número de genes induzidos e reprimidos foram 54 e 16, respectivamente. Os genes induzidos foram classificados nas seguintes categorias funcionais: inflamação (Ccl2), transcrição (Mark2, Sox4 Irf8 e E2f2), transdução de sinais (Cd37, Lck, Jak3 e Arhgap4), transporte (Stx18, Slc12a7 e Nxf1), proteínas relacionas à membrana (Tmem115, Col27a1 e Itga5) e processamento de RNA (Prpf38b, Rpl29, Rpl15). Adicionalmente, um total de 18 genes de macrófagos relacionados a outras funções teve sua expressão induzida. Neste perído de 2 horas a infecção pelo *H. capsulatum* modulou negativamente os genes de macrófagos envolvidos principalmente com a transcrição.

Tabela 5. Genes de macrófagos induzidos após 2 horas de infecção com *H. capsulatum*.

Categoria	Descrição do gene	Gene	[a]Clone ID	Indução/ Repressão
Genes induzidos				
Inflamação				
	Ligante 2 de quimiocina (motivo C-C)	Ccl2	573898	1,3
Proteínas de membrana				
	Proteína transmenbrana 115	Tmem115	582843	1,2
	Procolágeno, tipo XXVII, alfa 1	Col27a1	1378843	1,2

Integrina, alfa 5	Itga5	135671	1,2

Regulação transcricional

MAP/quinase regulando afinidade ao microtúbulo 2	Mark2	574316	1,2
Gene contendo o box SRY 4	Sox4	639914	1,3
Fator regulador de interferon 8	Irf8	640774	1,3
Fator 2 de transcrição E2F	E2f2	640754	1,4

Transdução de sinal

Antígeno CD37	Cd37	640080	1,2
Proteína tirosina quinase de linfócito	Lck	640179	1,2
Janus quinase 3	Jak3	576670	1,2
Proteína ativadora de Ro GTPase	Arhgap4	582914	1,2
Região não catalítica da proteína adaptadora tirosina	Nck2	640608	1,2
Substrato 2 relacionado a RAS C3 botulinium	Rac2	640180	1,3

Transporte

Sintaxina 18	Stx18	640560	1,3
Família carreadora de soluto 12, membro 7	Slc12a7	639998	1,2
Homólogo 1 do fator de exportação do RNA nuclear	Nxf1	574499	1,2

Processamento de RNA

PRP38 fator de processamento de pre RNA	Prpf38b	639769	1,3
Proteína ribosomal L29	Rpl29	583177	1,2
Proteína ribosomal L15	Rpl15	583166	1,2

Outros

Aspartil aminopeptidase	Dnpep	641031	1,2
Contendo o domínio de homologia a espectrina, família A membro 1 específico para ligação de	Plekha1	640919	1,2
RIKEN cDNA 0610009K11 gene	-	582885	1,2
Predicted gene, EG668468	EG668468	1225946	1,2
Acil coenzima A desidrogenase	Acadsb	640536	1,2
RIKEN cDNA C330006K01 gene		640249	1,2
RIKEN cDNA 1110002B05 gene		639950	1,2
Proteína de ligação a DNA simples fita 2	Ssbp2	575853	1,2
Hexosaminidase B	Hexb	575852	1,2
RIKEN cDNA C230096C10 gene		583779	1,2
Similar ao estágio celular 2, grupo variável, membro	LOC62536	575653	1,2
Fator de crescimento derivado de hepatoma	Hdgf	639890	1,2

Contendo o domínio de sobrevivência do neurônio	Smndc1	639687	1,2
Ciclo 26 de divisão celular	Cdc26	640321	1,2
Hipóxia 1 induzido	Hyou1	639627	1,2
Gene de proliferação de melanócito 1	Myg1	583514	1,3
Gene de translocação de célula B 1, anti-	Btg1	574654	1,3
Zinc finger, contendo o domínio CCHC 7	Zcchc7	575396	1,5

Genes reprimidos

Regulação transcricional

Proteína zinc finger e homeobox 1	Zhx1	640278	0,7
Fator de transcrição de trans-atuação 1	Sp1	583862	0,8
Fator de splicing, rico em arginina/serina	Sfrs1	583110	0,7
Fator de splicing, rico em arginina/serina	Sfrs1	583038	0,7
Histona cluster 1	Hist1h2bc	640662	0,7
Fator de splicing, rico em arginina/serina	Sfrs1	583015	0,7
Fator de splicing, rico em arginina/serina	Sfrs1	582848	0,8
Família tubulina tirosina ligase-like, membro 4	Ttll4	582821	0,8

Outros

Proteína do complexo T 1	Tcp1	640974	0,8
DNA segment, Chr 10, ERATO Doi 610, expressed	D10Ertd	576334	0,8
Homólogo a MIS12(yeast)	Mis12	583716	0,7
Anemia de Fanconi, grupo de complementação	Fancc	583016	0,8
Lung RCB-0558 LLC cDNA,	-	583525	0,8
Metilmalonil-coenzima A mutase	Mut	583549	0,8
E26 oncogene de leucemia de ave 1, domínio 5´	Ets1	583649	0,8

[a] Identificação do gene na base de dados do SOURCE (http://source.stanford.edu/).

Em 24 horas de infecção do macrófago por *H. capsulatum,* 34 genes foram modulados (21 induzidos e 13 reprimidos; Tabela 6). Os genes induzidos são relacionados a inflamação (Ccl2, Cd28 e B2m), transcrição (Crsp2 e Nrip1), transdução de sinais (Tollip, Gimap3, Ywhah, Mapk8ip3 e Ncaph2), proteínas relacionadas a membrana (Col27a1) e outras categorias (Napl11, Bms11, Msn, Adfp, Gdap2 e Tdrd5). Neste tempo de infecção observou-se a repressão de genes que foram classificados nas seguintes categorias funcionais:

inflamação (Traf6 e Cd14), transcrição (Zhx1), proteínas relacionadas à membrana (Col6a1), citoesqueleto (Tuba2 e Ttl14), processamento de RNA (Rpl18a), histonas (Hist1h2bc) e relacionadas a outras funções (Rce1, Ankrd28, Psmc3 e Atad2).

Tabela 6. Genes de Macrófagos induzidos após 24 horas de infecção com *H. capsulatum.*

Categoria	Descrição do gene	Gene	[a]Clone ID	Indução/ Repressão
Genes induzidos				
Inflamação				
	Ligante 2 de quimiocina (motivo C-C)	Ccl2	573898	1,2
	Antígeno CD28	Cd28	576501	1,2
	Beta-2 microglobulina	B2m	576472	1,4
	Beta-2 microglobulina	B2m	576493	1,4
Relacionado à membrana				
	Procolágeno, tipo XXVII, alfa 1	Col27a1	1378843	1,3
Regulação Transcricional				
	Cofator requerido para ativação transcricional Sp1	Crsp2	640999	1,2
	Proteína que interage com o receptor nuclear 1	Nrip1	582841	1,2
Transdução de sinal				
	Proteína que interage com toll	Tollip	4951445	1,2
	GTPase, membro 3 da família IMAP	Gimap3	574526	1,2
	Proteína de ativação de 3monooxigenase/triptofano 5-monoxigenase, polipeptídeo eta	Ywhah	640494	1,2
	Proteína 3 que interage com proteína quinase mitógeno	Mapk8ip3	576539	1,2
	Complexo não-SMC condensina II, subunidade H2	Ncaph2	583463	1,2
Outros				
	Proteína de assembléia do nucleossomo 1-like 1	Nap1l1	583645	1,2
	BMS1-like, proteína de assembléia do ribossomo	Bms1l	639828	1,2
	Em múltiplos clusters		640263	1,2
	Moesina	Msn	583860	1,2

Proteína relacionada à diferenciação do adipócito	Adfp	640464	1,2
RIKEN cDNA 2610209M04 gene	-	640363	1,2
Proteína associada à diferenciação induzida por	Gdap2	641674	1,4
Contendo o domínio 5	Tdrd5	1445729	1,5
RIKEN cDNA 0610009K11 gene		582885	1,2

Genes reprimidos

Inflamação

Fator 6 associado ao receptor de TNF	Traf6	1330069	0,8
Antígeno CD14	Cd14	5120996	0,8

Relacionado à membrana

Procolágeno, tipo VI, alfa 1	Col6a1	1281632	0,6

Regulação Transcricional

Proteína zinc fingers e homeoboxes 1	Zhx1	640278	0,6

Citoesqueleto

Tubulina, alfa 2	Tuba2	582927	0,7
Tubulina, alfa 2	Tuba2	639633	0,8
Família tubulina tirosina ligase-like, membro 4	Ttll4	582821	0,8

Processameto de RNA

Proteína ribosomal L18A	Rpl18a	583588	0,6

Histonas

Cluster 1 de histona, H2bc	Hist1h2bc	640662	0,6

Outros

Homólogo a RCE1, proteína prenil peptidase (*S. Cerevisiae*)	Rce1	583235	0,8
Domínio repetido de anquirina 28	Ankrd28	1345145	0,8
Proteasomo (prosomo, macropaina) subunidade 26S, ATPase 3	Psmc3	583912	0,7
Família ATPase, contendo o domínio AAA 2	Atad2	1345449	0,8

[a] Identificação do gene na base de dados do SOURCE (http://source.stanford.edu/).

A tabela 7 descreve os genes induzidos encontrados na categoria inflamação, os quais codificam para citocinas e quimiocinas (Il15, Ccl3, Ccr7, Cxcl4, Ccl17 e Ccl3) e os genes Prss16, Lrp12, Traf6, Hnrpdl, Igsf3 e Cd28. Neste período de infecção, os genes relacionados ao estresse celular tais como Hsp110 e Cct6a foram regulados positivamente, bem como os genes envolvidos com transcrição (Brpf1, Lef1, Sox4, Btf3, Gfi1, Nab2, E2f2 e Zzz3), transdução de sinais (Txk, Tbl1xr1, Rab27a e Gngt2), transporte (Cdc21, Atp6ap1, Kpna2, Spna2 e Tomm20) e proteínas de membrana (Tme48, 5-Mar e Col27a1). A indução da expressão gênica do macrófago neste período de 48 horas de infecção foi observada principalmente para os genes que são relacionados ao processamento de RNA (Rpl27a, Rps3a, Rps13, Rpl29, Rpp30, Rps25, Rbm4, Rpl6, Rps27l, Rbm39, Rpl22 e Rpl24). Os outros genes modulados positivamente pelo *H. capsulatum* foram aqueles que codificam proteínas relacionadas ao potencial redox (Glrx5 e Fdx1), proteassoma (Ube1x e Psma5), fosfatases (Hisppd1 e Ptp4a3) e transferases (Gnpnat1 e Gnptg).

Tabela 7. Genes de macrófagos induzidos após 48 horas de infecção com *H. capsulatum*.

Categoria	Descrição do gene	Gene	[a]Clone ID	Indução
Inflamação				
	Interleucina 15	Il15	1225014	1,2
	Ligante 4 de quimiocina (motivo C-X-C)	Cxcl4	573339	1,2
	Ligante 4 de quimiocina (motivo C-X-C)	Cxcl4	582960	1,2
	Ligante 17 de quimiocina (motive C-C)	Ccl17	1263461	1,2
	Ligante 3 de quimiocina (motivo C-C)	Ccl3	5228819	1,4
	Ligante 3 de quimiocina (motivo C-C)	Ccl3	1330016	1,2
	Receptor 7 de quimiocina (motivo C-C)	Ccr7	5180758	1,2
	Proteasoma, serina, 16 (timo)	Prss16	640501	1,2
	ALS2 C-terminal like	Als2cl	639955	1,2
	Proteína 12 relacionada a lipoproteína de baixa	Lrp12	640726	1,4
	Fator 6 associado a receptor de TNF	Traf6	1330069	1,2
	Ribonucleoproteína nuclear heterogêneo D-like	Hnrpdl	640936	1,2
	Superfamília de imunoglobulina, membro 3	Igsf3	640435	1,2
	Antígeno CD28	Cd28	576501	1,2
Relacionado à membrana				

Proteína transmembrana 48	Tmem48	640512	1,2
Ring finger associada à membrana (C3HC4)5	5-Mar	639904	1,2
Procolágeno, tipo XXVII, alfa 1	Col27a1	1378843	1,3

Regulação transcricional

Contendo bromodomínio 1	Brpf1	640236	1,2
Fator de ligação ao enhancer linfóide 1	Lef1	575374	1,2
Gene 4 contendo SRY-box	Sox4	583398	1,2
Gene 4 contendo SRY-box	Sox4	639914	1,3
Fator de transcrição básico 3	Btf3	583772	1,3
Fator de crescimento independente 1	Gfi1	640751	1,3
Proteína 2 de ligação a Ngfi- A	Nab2	640749	1,3
Fator 2 de transcrição E2F	E2f2	640754	1,3
Zinc finger, contendo o domínio ZZ 3	Zzz3	576612	1,2

Transdução de sinal

Tirosina quinase	Txk	583533	1,2
Transducina (beta)-like 1X-ligada ao receptor 1	Tbl1xr1	583935	1,2
RAB27A, membro RAS da família de oncogenes	Rab27a	640750	1,2
Proteína G, polipeptídeo 2	Gngt2	583496	1,2

Estresse

Proteína Heat shock 110	Hsp110	582878	1,2
Subunidade da chaperonina 6a (zeta)	Cct6a	582061	1,2

Transporte

Contendo o domínio coiled-coil 21	Ccdc21	583450	1,2
ATPase, transportadora de H+, proteína acessória	Atp6ap1	583065	1,3
Carioferrina alfa 2 (importina)	Kpna2	640444	1,2
Espectrina alfa 2	Spna2	583512	1,3
Homólogo da translocase da membrana	Tomm20	640575	1,4

Processamento de RNA

Proteína ribosomal L27a	Rpl27a	640775	1,2
Proteína ribosomal L27a	Rpl27a	640668	1,2
Proteína ribosomal S3a	Rps3a	639738	1,2
Proteína ribosomal S13a	Rps13	640463	1,2

Proteína ribosomal L29a	Rpl29	583177	1,2
Ribonuclease P/MRP subunidade 30 (humano)	Rpp30	640459	1,2
Proteína ribosomal S25	Rps25	583911	1,2
Proteína ribosomal L27a	Rpl27a	640214	1,2
Proteína 4 de ligação ao motivo de RNA	Rbm4	640067	1,2
Proteína ribosomal L6	Rpl6	583113	1,2
Proteína ribosomal S27-like	Rps27l	640461	1,2
Proteína 4 de ligação ao motivo de RNA	Rbm39	583446	1,2
Proteína ribosomal L22	Rpl22	640141	1,3
Proteína ribosomal L24	Rpl24	641145	1,2

Potencial redox

Homólogo a glutarredoxina 5 (*S. Cerevisiae*)	Glrx5	583838	1,2
Ferrodoxina 1	Fdx1	583500	1,2

Proteassoma

Enzima E1 ativadora de ubiquinina, ChrX	Ube1x	575553	1,2
Proteasoma, subunidade alfa tipo 5	Psma5	639989	1,2

Fosfatase

Contendo o domínio histidina ácido fosfatase 1	Hisppd1	583887	1,2
Proteína tirosina fosfatase 4a3	Ptp4a3	640485	1,3

Transferase

N-acetiltransferase glicosamina-fosfatase 1	Gnpnat1	642037	1,2
N-acetilglicosamina-1-fosfotransferase,	Gnptg	583444	1,3

Outros

Proteína de ligação ao RNA 1, CUG três vezes	Cugbp1	639926	1,2
RIKEN cDNA 1110002B05 gene	-	639950	1,2
Calpaína, pequena subunidade 1	Capns1	641638	1,2
Proteína nucleolar 5A	Nol5a	640557	1,2
Anexina 1	Anxa1	640284	1,2
Homólogo 1 do gene de distribuição nuclear (A	Nde1	640079	1,2
Proteína F-box 46	Fbxo46	1330038	1,2
Histocompatibilidade 13	H13	640912	1,2
RIKEN cDNA B230217O12 gene	-	639986	1,2
RIKEN cDNA A830010M20 gene	-	641649	1,2

Uroporfirinogênio III sintase	Uros	583476	1,3
RIKEN cDNA 2310066E14 gene	-	583443	1,2
RIKEN cDNA 5730403B10 gene	-	640888	1,2
Cisteína sulfínico ácido decarboxilase	Csad	640308	1,2
Contendo o domínio coiled-coil 58	Ccdc58	582929	1,2
RIKEN cDNA 2410131K14 gene	-	583765	1,2
Locus transcrito	-	1263520	1,2
Transcrito derivado de testis	Tes	582953	1,2
Homólogo 2 de angel (Drosófila)	Angel2	640692	1,2
Locus transcrito	-	640755	1,2
RIKEN cDNA 2010001A14 gene	-	640493	1,2
Fator 1 de ligação ao DNA do receptor de	Grlf1	640573	1,2
Homólogo 1 do enhancer de policombo (Drosófila)	Epc1	583089	1,2
Fosforribosil pirofosfatase sentetase 1	Prps1	640509	1,2
Anexina A5	Anxa5	576596	1,2
RIKEN cDNA 2810001G20 gene	-	640192	1,2
Glutamato oxaloacetato transaminase 1, solúvel	Got1	640640	1,2
Hect (homólogo ao UBE3A)	Herc1	583423	1,2
Proteína 11 relacionada ao miotubularina	Mtmr11	1265247	1,3
Receptor colinérgico, nicotínico, polipeptídeo	Chrne	1380475	1,2
RIKEN cDNA 4921537D05 gene	-	639871	1,2
RIKEN cDNA 4932414K18 gene	-	576636	1,2
Contendo o domínio de reparo G 2	Gpatch2	639834	1,2
Locus transcrito	-	640371	1,2
Locus transcrito	-	641074	1,4
Nucleotidase 1 ativada por cálcio	Cant1	1379942	1,3
RIKEN cDNA 9530077C05 gene	-	1265419	1,3
Timina DNA glicosilase	Tdg	642198	1,4
Proteína sinaptofisina lide	Sypl	640468	1,2
Metaderina	Mtdh	640549	1,5
Contendo o domínio 1 de sobrevivência do	Smndc1	639687	1,4
Proteína do receptor 1 do retículo endoplasmático	Kdelr1	640576	1,6
Contendo o domínio 5 tudor	Tdrd5	1445729	2,1

[a] Identificação do gene na base de dados do SOURCE (http://source.stanford.edu/).

Os genes de macrófagos que tiveram sua expressão modulada negativamente pela infecção com *H. capsulatum* estão listados na Tabela 8. A modulação negativa foi observada para 10 genes relacionados à inflamação (Nkrf, H2-k1, Sema7a, Il10, Il1b, Ccr5, Cd83, Cd8a, Cd27 e Laptm5), 7 genes envolvidos com a transcrição (Bag2, Uqcrfs1, Abt1, Btg2, Sfrs1, Rbm39, e Taf6) e 8 genes relacionados a transdução de sinais (Slk, Nck2, Grb2, Rragc, Prkar2b, Lck, Ahrgap4 e Cdk4). É importante ressaltar que nos tempos de 2 e 24 horas de infecção dos macrófagos pelo *H. capsulatum* não foi observada nenhuma modulação de genes envolvidos com apoptose. Entretanto, em 48 horas os genes pró-apoptóticos Casp8 e 2 foram modulados negativamente. Genes relacionados ao transporte (Slc35a4, Ywhah, Slc25a36, Slc22a7, Eif4ebp2 e Vamp1) e genes que codificam para proteínas relacionadas a membrana (Vcam1, Tlr2, Cib1 e Col6a1) também foram observados responder negativamente a infecção por *H. capsulatum*. Os demais genes modulados negativamente foram classificados nas seguintes categorias funcionais: desidrogenases (Adhfe1 e Aldh9a1), proteínas relacionadas ao citoesqueleto (Ttl14, Actr1a e Csnk1d), histonas (Hist1h2bc) e proteínas relacionadas ao processamento de RNA (Rpl7a, Rpl18a, Rbm39, Mrpl28, Sfrs1 e Nol7).

Tabela 8. Genes de Macrófagos reprimidos após 48 horas de infecção com *H. capsulatum*.

Categoria	Descrição do Gene	Gene	[a]Clone ID	Repressão
Inflamação				
	Fator de repressão de NF-kappaB	Nkrf	21961	0,6
	Histocompatibilidade 2, K1, região K	H2-K1	583608	0,7
	Histocompatibilidade 2, K1, região K	H2-K1	640137	0,8
	Histocompatibilidade 2, K1, região K	H2-K1	583704	0,8
	Domínio sema, domínio de imunoglobulina (Ig)	Sema7a	583573	0,8
	Domínio sema, domínio de imunoglobulina (Ig)	Sema7a	583574	0,8
	Interleucina 10	Il10	1002777	0,8
	Interleucina 1 beta	Il1b	3989461	0,8
	Receptor 5 de quimiocina	CCR5	2119876	0,5
	Antígeno CD83	Cd83	574651	0,8
	Antígeno CD8, cadeia alfa	Cd8a	1247019	0,6
	Antígeno CD27	Cd27	640155	0,7
	Proteína transmembrana 5 associada ao lisossomo	Laptm5	639946	0,8

Proteínas de membrana

Molécula 1 de adesão celular vascular	Vcam1	576563	0,8
Receptor Toll-like 2	TLR2	4753177	0,8
Ligante 1 de cálcio e integrina	Cib1	583939	0,7
Procolágeno, tipo VI, alfa 1	Col6a1	1281632	0,7

Regulação transcricional

Atanogene 2 associado a Bcl2	Bag2	573075	0,8
Ubiquinol-citocromo c redutase	Uqcrfs1	640752	0,8
Ativador da transcrição basal	Abt1	583686	0,7
Gene 2 de translocação de célula B	Btg2	574837	0,7
Gene 2 de translocação de célula B	Btg2	583751	0,7
Fator de splicing, rico em arginina/serina 1	Sfrs1	583015	0,8
Proteína 39 com motivo de ligação ao RNA	Rbm39	640923	0,8
TAF6 RNA polimeraseII,	Taf6	640288	0,8

Transdução de sinal

STE20-like quinase (levedura)	Slk	583214	0,8
Região não catalítica da proteína adaptadora	Nck2	640608	0,8
Receptor do fator de crescimento	Grb2	575387	0,7
Relacionado ao ligante C de Ras GTP	Rragc	583915	0,8
Proteína quinase, dependente de AMPc	Prkar2b	640704	0,6
Proteína tirosina quinase de linfócito	Lck	577700	0,8
Rho GTPase ativadora da proteína 4	Arhgap4	582914	0,7
Quinase 4 dependente de ciclina	Cdk4	640338	0,6

Apoptose

Caspase 8	Casp8	5099113	0,7
Caspase2	Casp2	573760	0,7

Transporte

Família carreadora de soluto 35, membro A4	Slc35a4	583816	0,8
Proteína de ativação da Tirosina 3-	Ywhah	640494	0,5
Família carreadora de soluto 25, membro36	Slc25a36	582799	0,8
Família carreadora de soluto 22 (transporta ânion	Slc22a7	574190	0,8
Fator eucariótico de iniciação da tradução 4E	Eif4ebp2	582913	0,8
Proteína 1 de membrana associada a vesícula	Vamp1	573892	0,8

Desidrogenase

Álcool desidrogenase contendo ferro, 1	Adhfe1	640899	0,8
Álcool desidrogenase 9, subfamília A1	Aldh9a1	641104	0,8

Citoesqueleto

Família tubulina tirosina ligase-like, membro 4	Ttll4	582821	0,8
Proteína 1 relacionada a actina (ARP) homólogo A	Actr1a	583567	0,7
Caseína quinase 1, delta	Csnk1d	573689	0,8

Histonas

Histona cluster 1, H2bc	Hist1h2bc	640662	0,6
Histona cluster 1, H2bc	Hist1h2bc	640662	0,5

Processamento de RNA

Proteína ribosomal L7a	Rpl7a	582916	0,6
Proteína ribosomal L18A	Rpl18a	583588	0,5
Proteína 39 com motivo de ligação ao RNA	Rbm39	583614	0,7
Proteína ribosomal mitocondrial L28	Mrpl28	640538	0,7
Fator de splicing, rico em arginina/serina 1	Sfrs1	583110	0,7
Fator de splicing, rico em arginina/serina 1	Sfrs1	582848	0,7
Fator de splicing, rico em arginina/serina 1	Sfrs1	583038	0,7
Proteína nucleolar 7	Nol7	640296	0,8

Outros

Proteína zinc fingers e homeoboxe 1	Zhx1	640278	0,5
Proteína zinc fingers 207	Zfp207	574462	0,8
Zinc finger, CCHC contendo o domínio 7	Zcchc7	575396	0,7
Fosfoproteína estimulada por Ena-vasodilatador	Evl	582896	0,6
Proteína sinaptofisina-like	Sypl	640468	0,5
Contendo o domínio PR 10	Prdm10	640355	0,7
Contendo o domínio coiled-coil-hélice-coiled-	Chchd8	574276	0,7
Homólogo B de unc-84 (C. Elegans)	Unc84b	583592	0,7
Âncora bige-like responsivo a LPS	Lrba	573828	0,7
Proteína preimplantação 3	Prei3	640488	0,7
Quitinase, ácida	Chia	583285	0,8
Família da poli (ADP-ribose) polimerase,membro	Parp3	640154	0,6

Adenosina deaminase	Ada	577879	0,8
Homólogo a MIS 12	Mis12	583930	0,8
Similar ao estéril motivo alfa contendo o domínio	LOC66670	583618	0,8
Membro 11 da família das quinesinas	Kif11	583152	0,6
Segmento de DNA, Chr4, Universidade do Estado	D4Wsu53e	582868	0,8
Contendo o domínio coiled-coil 109A	Ccdc109a	582796	0,8
Sintaxina 18	Stx18	640560	0,7
ELK4, membro da família oncogene ETS	Elk4	583566	0,7
Segmento de DNA, Chr4	D4Wsu53e	583478	0,8
Calpaína 2	Capn2	583248	0,7
Enzima que degrada insulina	Ide	574241	0,8
Enzima que degrada insulina	Ide	577628	0,8
Dinamina 1-like	Dnm1l	583791	0,8
Tuinfilina, proteína ligante de actina, homólogo 2	Twf2	640339	0,8
Paraplegia espática 20	Spg20	582846	0,8
Contendo o fold de ligação	Obfc2a	582845	0,8
Reparo de excisão	ERCC5	1308118	0,8
Similar ao estágio 2 celular, grupo variável,	LOC62536	575653	0,8
Sincolina	Sync	583601	0,8
Segmento de DNA, Chr4, Universidade do Estado	D4Wsu53e	583830	0,8
Dinactina 4	Dctn4	640452	0,8
Proteína 2 ligante de DNA simples fita	Ssbp2	575853	0,8
Domínio repetido WD 61	Wdr61	640880	0,8
Domínio repetido WD 61, contendo o domínio	Wdsub1	640769	0,8
Ácido sintase amino levulínico 1	Alas1	583710	0,7
Histidina decarboxilase	Hdc	576094	0,8
Anemia de Fanconi, grupo de complementação C	Fancc	583016	0,8
Gene 2 de translocação de célula B, anti-	Btg2	583751	0,7
UDP-GlcNAc:betaGal beta-1,3-N-	B3gnt2	574207	0,8
Segmento de DNA, Chr2, ERATO Doi 435,	D2Ertd435	583551	0,7
Célula B CLL/linfoma 7A	Bcl7a	582794	0,7
RIKEN cDNA 1500032D16 gene		583789	0,5
RIKEN cDNA 1500032D16 gene		583350	0,8

RIKEN cDNA 1500032D16 gene	576310	0,8
RIKEN cDNA 1500032D16 gene	583790	0,8
RIKEN cDNA 1500032D16 gene	640992	0,7
RIKEN cDNA 1500032D16 gene	640104	0,7
RIKEN cDNA 1500032D16 gene	641854	0,7
Em múltiplo clusters	640263	0,5
Em múltiplo clusters	640110	0,8
Em múltiplo clusters	640331	0,8
Locus transcrito	573863	0,7
Locus transcrito	583575	0,8
Locus transcrito	640875	0,8
Locus transcrito	583657	0,7

[a] Identificação do gene na base de dados do SOURCE (http://source.stanford.edu/).

5.7. Resposta comum do hospedeiro frente à infecção causada por *P. brasiliensis* ou *H. capsulatum*

Os dados de expressão diferencial dos macrófagos durante a interação tanto com *P. brasiliensis* quanto com *H. capsulatum* foram comparados no presente trabalho e observou-se que o padrão de modulação gênica dos macrófagos induzidos por estes dois fungos é semelhante no que se refere às categorias funcionas dos genes que são modulados. Neste sentido, verificou-se no presente trabalho a modulação, nas cinéticas de interação dos macrófagos com esses patógenos, de genes relacionados a inflamação, proteínas de membrana, regulação transcricional e transdução de sinal. Em todos os tempos de interação foram observados genes induzidos em todas essas categorias. Por outro lado, o mesmo não foi observado para os genes reprimidos. Os genes diferencialmente expressos que, de acordo com os dados da literatura, desempenham funções extremamente relevantes na interação patógeno-hospedeiro estão destacados em azul e aqueles que foram diferenciais nos macrófagos infectados tanto por *P. brasiliensis* quanto por *H. capsulatum* em pelo menos um dos tempos estão sublinhados (Tabela 9).

Os genes induzidos observados neste trabalho que são envolvidos na inflamação foram principalmente os relacionados à quimiotaxia e fagocitose, sendo que a maioria deles foi positivamente regulado pelo macrófago em 6 e 24 horas de interação com *P. brasiliensis* e em

48 horas de interação com *H. capsulatum*. A indução de genes envolvidos na fagocitose e na transdução de sinal foi relativamente igual na cinética de infecção por estes patógenos. Na categoria da regulação transcricional a maior indução de expressão gênica foi observada em 24 e 48 horas de interação dos macrófagos com *P. brasiliensis* e *H. capsulatum*, respectivamente, não sendo verificada a indução de genes em 48 de interação com *P. brasiliensis*.

A repressão de genes dos macrófagos relacionados a inflamação durante a interação tanto com *P. brasiliensis* quanto com *H. capsulatum*, não foi observado em 6 e 2 horas, respectivamente. Na categoria transdução de sinal foi observada repressão somente em 48 horas de interação dos macrófagos com esses dois fungos. A repressão de genes relacionados a regulação transcricional foi observado em quase todos os tempos das cinéticas de infecção, com exceção do tempo de 6 horas de infecção do macrófago com *P. brasiliensis*. Na categoria de proteínas de membrana não houve repressão de genes de macrófagos durante a interação com *P. brasiliensis*. Em contrapartida, em 48 horas de interação com *H. capsulatum* foi observada repressão de 4 genes, entre eles o que codifica para TLR2, envolvido na ativação celular.

Tabela 9. Genes de macrófagos modulados durante a infecção com *P. brasiliensis* e *H. capsulatum*.

Categoria	Macrófagos infectados com *P.*			Macrófagos infectados com *H.*		
Genes induzidos						
Inflamação	**6h**	**24h**	**48h**	**2h**	**24h**	**48h**
	Irak2	**Irak2**	**Irak2**	**Ccl2**	**Ccl2**	**Cxcl4**
	Ccl21	**Ccl21a**	**Cxcl4**	-	Cd28	**Ccl3**
	Ccl22	Pparbp	**Cxcl14**	-	-	**Ccr7**
	Cxcl1	Ly86	-	-	-	Il15
	Il7r	Lat	-	-	-	Prss16
	H2-Eb1	**C2**	-	-	-	Als2cl
	Cd28	**C3**	-	-	-	Cd28
	-	-	-	-	-	Lrp12
	-	-	-	-	-	Hnrpdl
	-	-	-	-	-	Igsf3
	-	-	-	-	-	Ccl17
Proteínas de						
	Clec1b	**Clec1b**	**Clec1b**	**Col27a1**	**Col27a**	**Col27a1**
	Mmp17	**Mmp17**	**Cd14**	**Itga5**	B2m	5-Mar
	Cd8	Cd8	H2-Eb	Tmem115	-	Tmem48

	Ly6a	Ddr1	B2m	-	-	-
	-	Sit1	Adam8	-	-	-
	-	Sell	Ly6a	-	-	-
	-	TcrgC	-	-	-	-
Transdução de sinal						
	Grb2	**Txk**	**Txk**	Jak3	Tollip	**Txk**
	Cd37	Cd37	Mapkkk11	Cd37	Gimap3	**Traf6**
	Rasa3	Mapk1	Sh3gl1	Lck	Ywhah	Tbl1xr1
	Tcp1	Mapk8ip3	Twf2	Arhgap4	Mapk8i	Rab27a
	-	Mapk8	-	Nck2	Ncaph2	Gngt2
	-	Rab3d	-	Rac2	-	
Regulação						
	Stat1	**Nfkb**		Mark2	Crsp2	Brpf1
	Ier5	**Nkrf**		Irf8	-	Lef1
	Cnot10	Cnot2	-	Sox4	-	Sox4
	-	Lef1	-	E2f2	-	E2f2
	-	Tcf12	-	-	-	Btf3
	-	Foxo1	-	-	-	Gfi1
	-	Pias1	-	-	-	Nab2
	-	-	-	-	-	Zzz3
Apoptose						
	-	**Casp2**	-	-	-	-
	-	**Casp8**	-	-	-	-
Genes reprimidos						
Inflamação						
	-	**Cxcl4**	**Ccl2**	-	Traf6	**Nkrf**
	-	**Ccl6**	-	-	**Cd14**	**Il10**
	-	-	-	-	-	**Il-1β**
	-	-	-	-	-	**Ccr5**
	-	-	-	-	-	Cd83
	-	-	-	-	-	Cd27
	-	-	-	-	-	H2-K1
	-	-	-	-	-	Sema7a
	-	-	-	-	-	Laptm5
	-	-	-	-	Col6a1	Col6a1
Proteínas de	-	-	-	-	-	Vcam1
membrana	-	-	-	-	-	**Tlr2**
	-	-	-	-	-	Cib1
	-	-	-	-	-	Slk
	-	-	-	-	-	Nck2
	-	-	-	-	-	Grb2
	-	-	-	-	-	Rragc
	-	-	-	-	-	Prkar2b
	-	-	-	-	-	Lck
	-	-	-	-	-	Arhgap4

	-	-	-	-	-	Cdk4
Regulação	-	Pias1	Rab2	Zhx1	Zhx1	Bag2
transcricional	-	S7a11lc	Mark2	Sp1	-	Uqcrfs1
	-	-	-	Hist1h2bc	-	Abt1
	-	-	-	Sfrs1	-	Btg2
	-	-	-	Ttll4	-	Sfrs1
	-	-	-	-	-	Rbm39
	-	-	-	-	-	Taf6
Apoptose						
	-		-	-	-	**Casp2**
	-		-	-	-	**Casp8**

6. DISCUSSÃO

6.1. Regulação transcricional de macrófagos infectados com *P. brasiliensis*

Com a finalidade de compreender a complexa interação entre as células hospedeiras e leveduras de *P. brasiliensis* foi analisada no presente trabalho a modulação de genes imunorregulatórios após infecção de macrófagos peritoneais por este patógeno. Este é o primeiro estudo que analisa o perfil cinético da expressão gênica coordenada de moléculas de macrófagos peritoneais em resposta a esse fungo. A caracterização da modulação da expressão gênica durante a cinética de infecção permite uma melhor compreensão da resposta imune inata do que a análise de apenas um único dado temporal. Os dados de microarranjos deste trabalho mostraram a expressão diferencial de genes extremamente relevantes durante a interação patógeno-hospedeiro, especialmente aqueles que são relacionados à resposta pró-inflamatória, fagocitose, apoptose e transdução de sinais.

A inflamação é um poderoso mecanismo protetor coordenado e controlado por citocinas e quimiocinas. Neste sentido, o aumento da expressão de quimiocinas tem sido detectado por análises de microarranjos em macrófagos infectados com *M. tuberculosis*, *C. albicans* e *A. fumigatus* (Wang *et al*., 2003; Kim *et al*., 2005; Cortez *et al*., 2006). Corroborando com esses trabalhos, os resultados apresentados nesta tese mostram que os genes que codificam quimiocinas associadas a migração de células de defesa para o sítio da infecção foram significativamente aumentados em macrófagos peritoneais em resposta ao *P. brasiliensis* (Ccl22, Cxcl1, Cxcl4 e Ccl21) em 6, 24 e 48 horas sendo mais expressiva no estágio inicial da interação (6h). As quimiocinas CXCL1 e CXCL4 são quimio-atraentes para

neutrófilos enquanto que CCL22 é quimiotática para monócitos. Recentemente, foi observado no pulmão de camundongos infectados com *P. brasiliensis* a indução de genes que codificam para outras quimiocinas que também atraem neutrófilos (KC e CCL3) e monócitos (CCL5, CCL2, CXCL10 e CXCL9); esta observação foi acompanhada por um intenso infiltrado dessas células de defesa no sítio da infecção importantes no estabelecimento de uma resposta inflamatória eficiente e de um granuloma bem formado para conter a disseminação do fungo para outros órgãos e/ou tecidos (Souto *et al.* 2003). A quimiocina Ccl21, em contraste a outras quimiocinas CC, que normalmente atraem monócitos, é quimiotática para linfócitos e se liga principalmente a receptores CCR7 estimulando a migração de células T e dendríticas para o local da infecção (Nagira *et al.* 1997; Yoshida *et al*., 1998). Em um modelo experimental de infecção de camundongos com formas amastigotas de *Leishmania donovani* deficientes para a produção de Ccl21 é observado uma maior susceptibilidade à infecção do que camundongos normais. Camundongos que não expessam Ccl21 possuem uma capacidade diminuída de migração e ativação de células dendríticas que normalmente são estimuladas por esta citocina auxiliando na eliminação de *L. donovani*. Além disso, observa-se uma formação tardia de granuloma o que diminui ainda mais a capacidade de contenção e eliminação deste patógeno pelo sistema imune (Ato *et al*., 2006). Dessa forma, o aumento da expressão de Ccl21 possivelmente é um mecanismo utilizado pelos macrófagos na tentativa de eliminar este patógeno por meio da indução da ativação e migração de células efetoras para o local da infecção aumentando a eficiência da resposta imune.

A expressão de genes codificando proteínas associadas a receptores de quimiocinas também foi avaliada neste trabalho, dessa forma, foi detectada a indução do gene que codifica para o receptor de IL-7 (IL-7r) na fase inicial de infecção (6 h) dos macrófagos infectados com o *P. brasiliensis*. Este receptor participa na indução da secreção de citocinas pró-inflamatórias por macrófagos e monócitos (Alderson *et al.,* 1991). A indução de IL-7r provavelmente está relacionada à ativação dos macrófagos em resposta à interação inicial com o *P. brasiliensis* possibilitando com isto uma ativação das propriedades fagocíticas e fungicidas desta célula. Além disso, foi observada no presente trabalho a indução gênica de Irak2 nos macrófagos durante a interação com *P. brasiliensis* em todos os tempos de infecção analisados. Recentemente, também foi detectada uma indução deste gene em monócitos humanos em 4 horas de infecção com *A. fumigatus* através de análises por microarranjos (Cortez *et al*., 2006). A proteína IRAK2 se liga ao receptor de IL-1 tipo I após a ligação de IL-1 promovendo a

ativação de uma cascata de sinalização intracelular que leva a indução transcricional do gene Nf-kb (Muzio *et al*., 1997). A expressão de Nf-kb leva a transcrição de genes da resposta imune, incluindo citocinas pró-inflamatórias tais como Tnf-α (fator de necrose tumoral-α), que atua na ativação de componentes da resposta imune inata, recrutando células inflamatórias para o sítio inicial da infecção (Karin & Ben, 2000). Neste contexto, vale ressaltar que também foi observado durante a interação dos macrófagos com *P. brasiliensis* a indução do gene Nf-kb, o que reforça os dados de indução da proteína IRAK2. Consistente com estes resultados, observações similares foram feitas por análises de microarranjos de macrófagos infectados com *Brucella abortus, M. tuberculosis, M. bovis* and *S. typhi* (Eskra *et al*., 2003; Wang *et al*., 2003; Nau *et al*., 2002). Provavelmente a modulação deste gene por *P. brasiliensis* irá induzir a expressão de genes que irão orquestrar a secreção de citocinas e migração de células envolvidas na resposta imune para o local da infecção (Figura 9).

A citocina pró-inflamatória TNF-α além de atuar como um indutor autócrino fornece um segundo sinal que induz uma atividade citotóxica em macrófagos ativados com IFN-γ por estimular a produção de NO (Green *et al*., 1990). No presente trabalho, a proteína TNF-α foi aumentada em todos os tempos após infecção do macrófago com *P. brasiliensis*, entretanto, a indução do gene que codifica esta proteína foi observada somente em 24 horas. Provavelmente, o aumento da proteína em seis horas de infecção é resultado da regulação pós-transcricional desta molécula. Esta proteína realiza uma importante função na prevenção de infecções fúngicas, no sentido de induzir uma eficiente resposta pró-inflamatória para a eliminação do patógeno do local da infecção (Louie, *et al*. 1994; Smith *et al*. 1990). Macrófagos peritoneais de hamster co-cultivados com *P. brasiliensis* (Parise-Fortes *et al*., 2000) secretam altos níveis de TNF-α, sendo que esta secreção é correlacionada com um aumento na ativação e atividade fungicida dos macrófagos impedindo a disseminação do fungo para outros órgãos e tecidos. Além disso, camundongos deficientes em produzir TNF-α são incapazes de gerar granulomas bem formados em resposta ao *P. brasiliensis* e de controlar a infecção por este fungo (Smith *et al*., 1990).

Apesar de ter sido incluído nas membranas dos microarranjos o gene que codifica para IL-12, não se mostrou diferencialmente expresso pelos macrófagos infectados, com *P. brasiliensis,* em nenhum dos tempos de infecção analisados no presente trabalho. Este resultado corrobora com o que foi observado neste trabalho pelo experimento de ELISA, em que não foi detectada a secreção de IL-12 pelos macrófagos durante a interação com o *P.*

brasiliensis. Além disso, este dado está de acordo com análises similares realizadas em macrófagos infectados com *M. tuberculosis* (Nau *et al*., 2002) e *H. capsulatum* (Marth & Kelsall, 1997), em que também foi observada uma supressão da secreção de IL-12. Provavelmente estes patógenos inibem a produção desta citocina no estágio inicial da infecção como um mecanismo de evasão da resposta imune inata do hospedeiro, sendo, portanto, esta uma estratégia evolucionária convergente destes microrganismos para sobreviverem no interior de macrófagos não ativados.
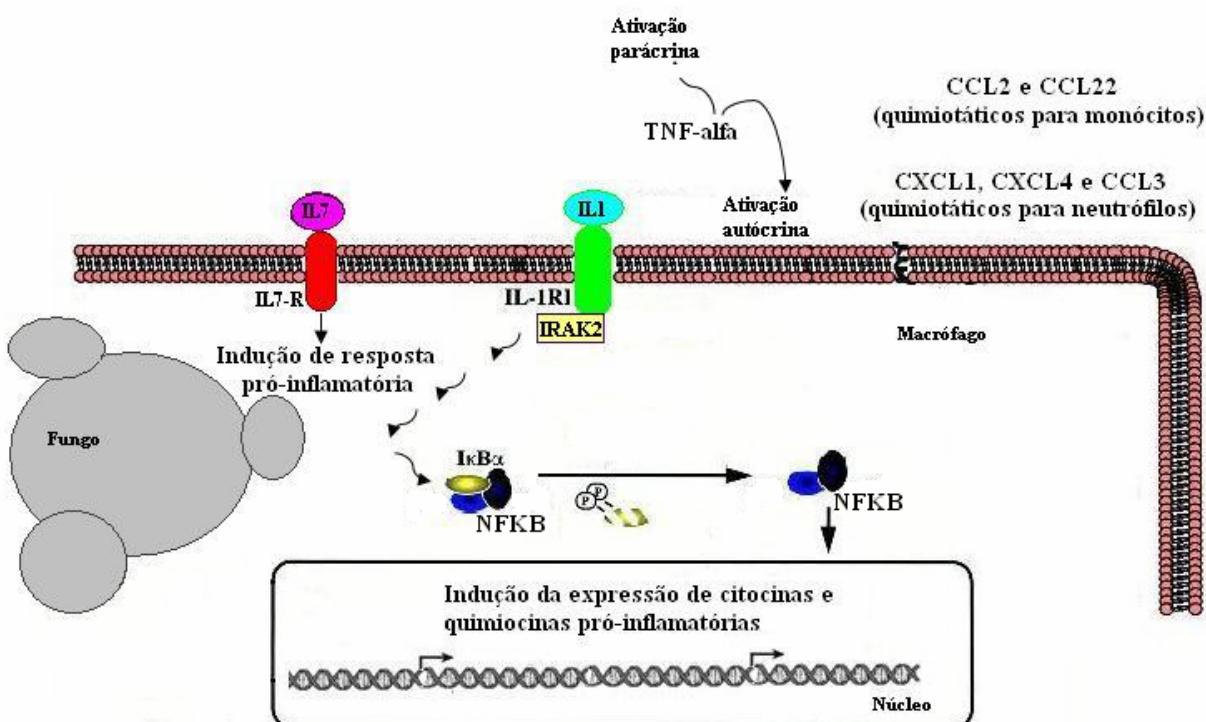
Figura 9. Proposta de modelo da modulação de genes relacionados ao processo pró-inflamatório de macrófagos murinos durante a interação com *P. brasiliensis*. Está representada na figura a indução dos genes que codificam para as seguintes proteínas: **Quimiocinas: CXCL1**, **CXCL4** e **CCL3** (C-X-C ligante 1, e 4 e C-C ligante 3 -quimiotáticos para neutrófilos); **CCL2** e **CCL22** (C-C ligante 2 e 22 -quimiotáticos para monócitos); **TNF**-alfa induz a migração de células fagocíticas para o local da infecção e induz a ativação desta células (ativação parácrina) e da própria célula que está secretando esta citocina (ativação autócrina); **Receptores de quimiocinas**: **IL7-R**: este receptor se liga a IL7 e induz uma resposta pró-inflamatória; **IRAK2**: esta molécula se liga ao receptor IL1-R, no qual a IL1 esta ligada, induzindo a ativação de Nfkb. **Fator de transcrição: NFKB**. A molécula de Nfkb quando no seu estado inativado se encontra no citoplasma ligada ao seu inibidor IkB. A fosforilação de IkB libera **NFKB** que entra no núcleo e atua como fator de transcrição de genes que codificam para quimiocinas e citocinas responsáveis por induzir uma resposta pró-inflamatória.

A expressão de genes codificando proteínas de membrana envolvidas em adesão e fagocitose também foram diferencialmente expressos por macrófagos durante a interação com *P. brasiliensis* tais como Cd14 e Clec1b. CD14 é uma glicoproteína glicosilfosfatidilinositol-ancorada expressa em leucócitos e é o maior receptor responsável pelos efeitos de LPS e vários produtos bacterianos, tais como peptideoglicanos e lipoarabinomanana sobre macrófagos, monócitos e neutrófilos. Além disso, estudos prévios demonstraram o envolvimento da proteína Cd14 no reconhecimento de fungos pelos macrófagos. Weiss *et al.* (2004) observaram a indução do gene Cd14 em macrófagos durante a fagocitose de diferentes isolados de *M. avium* em 6 e 24 horas. Dessa forma, pode-se postular que este padrão de expressão diferencial induzido pelo *P. brasiliensis* resultaria em uma invasão mais efetiva e/ou ativação da célula hospedeira.

O gene que codifica para Clec1b, também relacionado à adesão e fagocitose, foi induzido em todos os tempos de infecção do macrófago com *P. brasiliensis*, o que foi confirmado por PCR em tempo real em 6 e 48 horas de interação. A proteína de membrana, CLEC, é capaz de reconhecer resíduos de manose presentes sobre a superfície de um amplo espectro de microrganismos, incluindo *P. brasiliensis* e *H. capsulatum* (Linehan *et al.*, 2000; Azuma *et al.*, 1974; Kanetsuna *et al.*, 1969). Recentemente, evidências adicionais de sua importância para a fagocitose de *P. brasiliensis* foi obtida pelo tratamento de macrófagos com um análogo metil-manosídeo que reduz a frequência da fagocitose deste fungo (Jiménez *et al.*, 2006). Dessa forma, os resultados encontrados neste trabalho corroboram esses dados, visto que a indução de Clec1b é importante para *P. brasiliensis* resultando, provavelmente, em uma internalização mais efetiva do fungo. Gonzalez *et al.* (2005c) observaram a indução de outras moléculas (ICAM-1, VCAM-1, e Mac-1), que não estavam nos microarranjos do presente trabalho, também relacionadas à adesão celular. A expressão dessas moléculas foi aumentada nos pulmões de camundongos infectados com *P. brasiliensis* sugerindo sua participação no processo inflamatório e conseqüentemente, na patogênese da paracoccidioidomicose. Dessa forma, pode-se postular que o aumento da expressão de moléculas relacionadas à adesão e/ou fagocitose, observado no presente trabalho, provavelmente é um mecanismo induzido pelo fungo para facilitar sua invasão às células hospedeiras.

Os genes que codificam as metaloproteinases Mmp17 e Adam8 foram significativamente induzidos em macrófagos peritoneais em resposta ao *P. brasiliensis*. Metaloproteinases de matriz geralmente estão envolvidas na remodelação de tecidos, clivagem

de receptores de superfície e ativação de quimiocinas (Chang & Werb, 2001). Corroborando com estes resultados, análises de microarranjos de macrófagos infectados por diferentes patógenos tais como *M. tuberculosis*, *M. bovis, Salmonella typhi* e *A. fumigatus* também revelaram a indução das metaloproteinases MMP1, MMP9, MMP10 e MMP14 (Nau *et al.*, 2002; Cortez *et al.*, 2006). Considerando sua função no remodelamento do colágeno e da matriz celular, esses autores sugerem que a indução destas enzimas pode estar correlacionada com mecanismo de facilitação da invasão do fungo às células e tecidos do hospedeiro. Assim, pode-se sugerir que o aumento da regulação da expressão de Mmp17 e Adam8 de macrófagos, observadas no presente trabalho, provavelmente é induzido por *P. brasiliensis* o que permitiria ao fungo aumentar sua eficiência de parasitismo às células hospedeiras. Esses dados reforçam os resultados de indução de genes relacionados à adesão e fagocitose, descrito anteriormente, já que *P. brasiliensis* é um parasita facultativo que pode sobreviver e se replicar em macrófagos não ativados previamente (Brummer *et al*. 1989). Dessa forma, tanto o aumento de genes relacionados à fagocitose quanto aqueles envolvidos no remodelamento da matriz possivelmente contribuirão para uma invasão mais efetiva de células e tecidos pelo fungo **(Figura 10).**
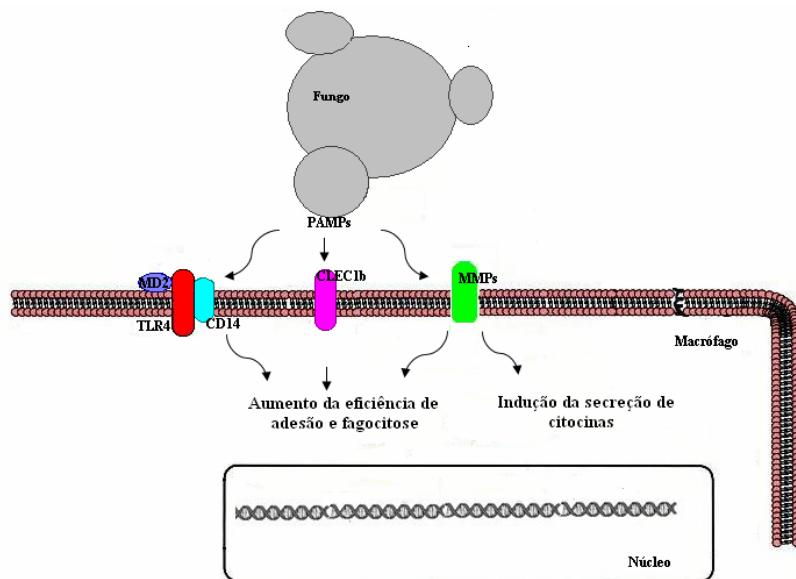
Figura 10. Proposta de modelo da modulação de genes relacionados à adesão e fagocitose de *P. brasiliensis* pelos macrófagos. Estão representadas as moléculas CD14, CLEC1b e MMPs. **CD14** se liga a moléculas na superfície do patógeno e interage com TLR4 possivelmente aumentando a eficiência de fagocitose pelos macrófagos; **CLEC1b** também atua no reconhecimento de moléculas de superfície da parede celular do patógeno aumentando a eficiência de adesão e fagocitose das leveduras;

MMPs são metaloproteinase de matriz que estão relacionadas ao remodelamento da matriz extracelular sendo possivelmene relacionadas ao aumento da eficiência de invasão das células hospedeiras pelo *P. brasiliensis,* além disso essas metaloproteinases também podem induzir a secreção de citocinas pelas células fagocíticas.

A apoptose possui uma importante função na regulação da patogênese. Genes envolvidos na indução de morte celular por apoptose foram induzidos por *P. brasiliensis* em 24 horas de infecção, incluindo caspases 2, 8 e 3. A indução de apoptose por microrganismos pode levar a evasão da resposta do macrófago. Caspases são moléculas que participam de uma cascata de eventos de clivagem que resulta no início do processo apoptótico. As Caspases 2 e 8 são denominadas caspases iniciadoras sendo responsáveis por iniciar a cascata de eventos apoptóticos, já a Casp 3 possui a função de degradar uma variedade de componentes celulares. Corroborando com os dados encontrados neste trabalho a indução de apoptose foi observada na fase inicial da PCM experimental sendo correlacionada como um marcador fenotípico de resistência à infecção (Vericimo *et al.*, 2006).

6.2. Regulação transcricional de macrófagos infectados com *H. capsulatum*

No presente trabalho o perfil temporal de expressão gênica de macrófagos não ativados na cinética de interação com *H. capsulatum* (2, 24 e 48h) foi avaliado também por meio da tecnologia de microarranjos de cDNA. A análise da modulação gênica global e temporal que ocorre durante a interação deste fungo com fagócitos não ativados revelou a indução de genes relacionados a uma resposta pró-inflamatória bem como a uma invasão mais efetiva dos fagócitos por esse fungo. A repressão de genes relacionados principalmente com a regulação transcricional e transdução de sinal também foi observada.

A indução de moléculas relacionadas a quimiotaxia (Cxcl4, Ccl2, Ccl3 e Ccl17) de células de defesa para o local da infecção foi verificada no presente trabalho principalmente em 48 horas de interação dos macrófagos com *H. capsulatum.* A quimiocina CCL3 além de atrair neutrófilos também induzem a síntese por macrófagos de outras citocinas pró-inflamatórias tais como IL-1, IL-6 e TNF, o que sugere que a modulação deste gene possivelmente desempenha um importante papel na ativação desses fagócitos (Figura 11). Recentemente, foi observado em modelo murino que no processo de formação de granuloma induzido por leveduras de *H. capsulatum* ocorre um aumento na secreção de algumas dessas

quimiocinas além de outras que também estão envolvidas na quimiotaxia de neutrófilos (KC e CCL3) e monócitos (CCL5, CCL2, CXCL10 e CXCL9), tendo sido esta secreção correlacionada com a presença de granulomas bem formados (Heninger *et al.,* 2006).

A citocina TNF-α, assim como foi observado nos experimentos de infecção dos macrófagos com *P. brasiliensis*, também foi detectada no sobrenadante dos macrófagos durante a interação com *H. capsulatum* analisados neste trabalho. Recentemente Heninger *et al*. (2006) verificou que a principal fonte de TNF-α no granuloma de camundongos infectados com *H. capsulatum* são os macrófagos. Adicionalmente, tem sido demonstrado que tratamento terapêutico anti-TNF-α induz reativação de infecção latente por *H. capsulatum* em alguns pacientes, enfatizando a função deste mediador no controle deste patógeno e prevenção de sua disseminação (Wynn, 2005). Embora a modulação da expressão de TNF-α não tenha sido observada no presente trabalho a secreção da proteína foi detectada no sobrenadante em 48h. Dessa forma, estes dados sugerem que provavelmente a modulação do gene que codifica para esta citocina deve ocorrer em tempos diferentes do que foi considerado no presente estudo ou então que a regulação deste gene ocorre a nível pós-transcricional.

As proteínas TRAF compreendem a segunda família de transdutores de sinais que interagem com membros da família de receptores de TNF. No presente trabalho observou-se em 24 horas de interação dos macrófagos com as leveduras de *H. capsulatum* a repressão do gene Traf6 seguida de uma indução de sua expressão em 48 horas. TRAF6, assim como outros membros desta família de receptores de TNF, possuem como propriedade biológica a capacidade de ativação do gene que codifica para o fator de transcrição NFKB (Rothe *et al.,* 1994). Além da propriedade que a proteína TRAF6 tem de ativação celular via indução por TNF-α, a ligação da citocina IL-1 com seu receptor (IL-1R) induz a associação de TRAF6 com a serina/treonina quinase IRAK levando a uma cascata de sinalização que induz a expressão de Nfkb (Cao *et al*., 1996)**.** O NFKB é uma molécula chave na indução de genes que codificam para várias citocinas envolvidas na indução de migração e ativação celular tais como CCL2 e TNF-α. A indução do gene relacionado ao fator de transcirção NFKB não foi observada nos macrófagos infectados com *H. capsulatum* durante os tempos considerados no presente estudo. Entretanto, observou-se o que o gene que codifica para o repressor do fator de transcrição Nfkb foi reprimido em 48 horas, indicando que não deve ocorrer a repressão de Nfkb durante a cinética de interação dos macrófagos com *H. capsulatum*. Dessa forma, pode-

se sugerir que embora a expressão diferencial de Nf-kb não tenha sido observada no presente trabalho, sua regulação provavelmente pode ocorrer a nível pós-transcricional. Dessa forma, a indução de genes que codificam para CCL2 e TRAF6, observados neste estudo, poderia estar sendo regulada pelo fator de transcrição Nfk-b.

Os receptores toll-like presentes nas células hospedeiras de mamíferos tem sido relacionados ao processo de resistência à infecção de vários microrganismos (Takeuchi *et al.,* 1999; Akira & Ulevitch, 2003; Aderem, 2000). A repressão de TLR2 foi observada em macrófagos infectados com *H. capsulatum* em 48 horas. TLR2 tem sido associado a uma fagocitose mais eficiente de *A. fumigatus* por macrófagos murinos (Luther *et al*., 2007). Além disso, Murciano *et al.* (2007) verificaram também que a produção de TNF-α por macrófagos infectados com *C. albicans* é dependente da via de sinalização induzida por TLR2. Neste sentido, a repressão de TLR2 observada nos macrófagos infectados com *H. capsulatum* provavelmente deve fazer parte de um mecanismo utilizado pela célula hospedeira para controlar a fagocitose e a resposta inflamatória já que uma produção exacerbada de citocinas no local da infecção, embora aumente a eficiência de destruição do patógeno, pode causar sérios danos para o hospedeiro.
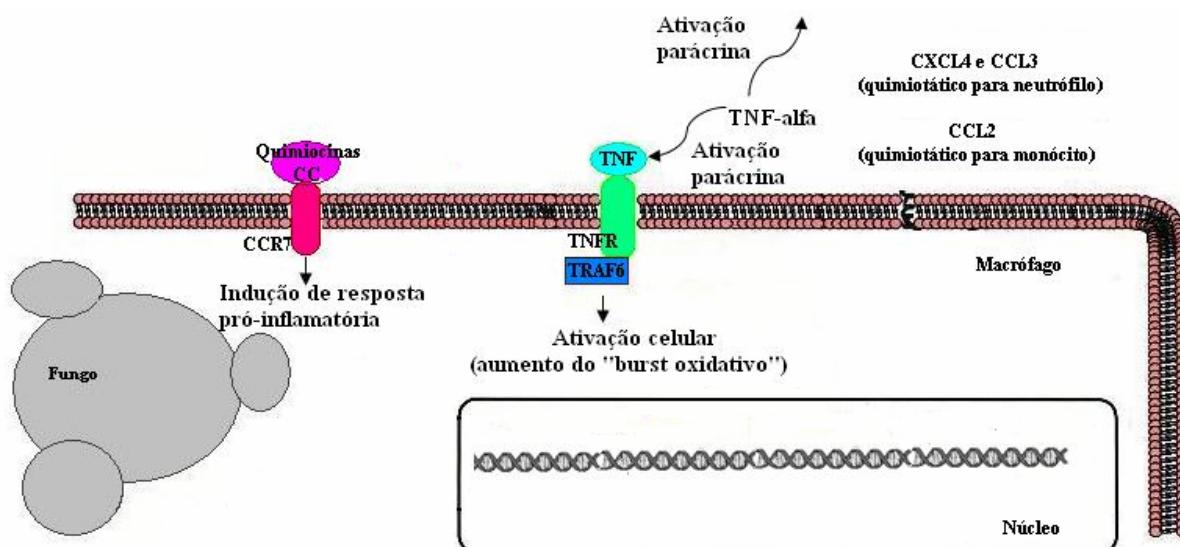

Figura 11. Proposta de modelo da modulação de genes relacionados ao processo pró-inflamatório de macrófagos murinos durante a interação com *H. capsulatum*. Está representada na figura a indução dos genes que codificam para as seguintes proteínas: **Quimiocinas:** **CXCL4** e **CCL3** (C-X-C ligante 4 e C-C ligante 3 -quimiotáticos para neutrófilos); **CCL2** (C-C ligante 2 -quimiotático para monócitos); **TNF**-alfa induz a migração de células fagocíticas para o local da infecção e a ativação desta células (ativação parácrina). Além disso, esta citocina pode ativar a própria célula responsável

por sua secreção (ativação autócrina); **Receptores de quimiocinas: TRAF6.** Interage com o receptor de TNF alfa para ativar o macrófago; **CCR-7**. Este receptor de quimiocinas CC está relacionado à indução da resposta pró-inflamatória.

A fagocitose de leveduras não opsonizadas de *H. capsulatum* ocorre principalmente via LFA-1, CR3 e 150,95 (Bullock & Wright, 1987). No presente trabalho macrófagos foram co-cultivados com células de levedura de *H. capsulatum* previamente opsonizadas com soro fresco de camundongo. A opsonização das leveduras deste patógeno, realizada neste trabalho, provavelmente deve refletir a modulação da expressão gênica dos macrófagos que mais se aproxima do que ocorre na interação *in vivo,* onde o fungo se encontra em contato com as proteínas do hospedeiro. Neste sentido, observou-se, neste trabalho, a indução do gene que codifica para outras moléculas relacionadas a adesão e fagocitose, como por exemplo, a molécula de Procolágeno, tipo XXVII, alfa 1 (Col27a1) (Figura 12). Wang e colaboradores (2003) identificaram por microarranjos de macrófagos infectados com *M. tuberculosis* a indução do gene que codifica para a molécula de colágeno tipo XIX. Em camundongos suscetíveis Keller e colaboradores (2004) verificaram também por microarranjos que durante a infecção com *M. tuberculosis* observa-se a repressão de genes que codificam para as moléculas de procolágeno tipo VI e tipo I. Dessa forma, visto que em camundongos suscetíveis a modulação deste gene é negativa, provavelmente a indução do gene Col27a1 pelos macrófagos durante a interação com *H. capsulatum,* observada no presente estudo, poderia estar correlacionada a uma resposta mais efetiva contra ao patógeno. Outro gene, também relacionado a uma proteína de membrana, e que teve sua expressão induzida em macrófagos durante a interação com *H. capsulatum* foi o Itga5 (integrina alfa 5). A proteína ITGA5 tem sido correlacionada com migração celular de macrófagos (Seftor *et al*., 1992), o que suge que a indução deste gene observada neste trabalho possa estar relacionada à indução de migração de células fagocíticas para o local da infecção. Provavelmente, este é um mecanismo induzido pelo fungo para invadir outras células ou reflete uma resposta do hospedeiro na tentativa de eliminação do patógeno do sítio inicial da infecção. A indução de outros genes também relacionados a proteínas de membrana, Tmem 115 e Tmem 48 foi verificada em 24 e 48 horas, respectivamente. Este gene codifica para proteínas transmembranas, entretanto não há relatos na literatura sobre sua função na fagocitose de microrganismos. A indução dos genes que codificam para TMEM sugere que provavelmente

essas moléculas possam desempenhar um papel importante na fagocitose e/ou ativação dessas células, sendo, entretanto, necessários estudos posteriores para analisar e compreender a função destas proteínas na interação com o patógeno.

Allen & Deep (2005) demonstraram que apoptose de célula T induzida por *H. capsulatum* é um requisito para o desenvolvimento eficiente de uma resposta antifúngica. Adicionalmente, Heninger *et al.* (2006) caracterizaram a presença de células apoptóticas em granulomas induzidos por *H. capsulatum* e verificaram que tanto células T quanto macrófagos contribuem para a ocorrência relativamente alta de apoptose no infiltrado de células do granuloma. Entretanto, Medeiros *et al.* (2002) verificaram aumento da expressão de Mac-1 (envolvido na fagocitose do patógeno) e diminuição de apoptose em monócitos de camundongos infectados com *H. capsulatum*, sugerindo que este é um mecanismo utilizado pelo fungo na tentativa de escapar do sistema imune e sobreviver no interior do fagócito. Nos experimentos de infecção com *H. capsulatum* observou-se a repressão dos genes pró-apoptóticos Casp2 e Casp8. Dessa forma, pode-se sugerir que provavelmente, no estágio inicial de internalização das leveduras, onde o macrófago não está ativado, o fungo pode induzir uma repressão inicial de apoptose para facilitar sua invasão e manutenção no microambiente das células hospedeiras.
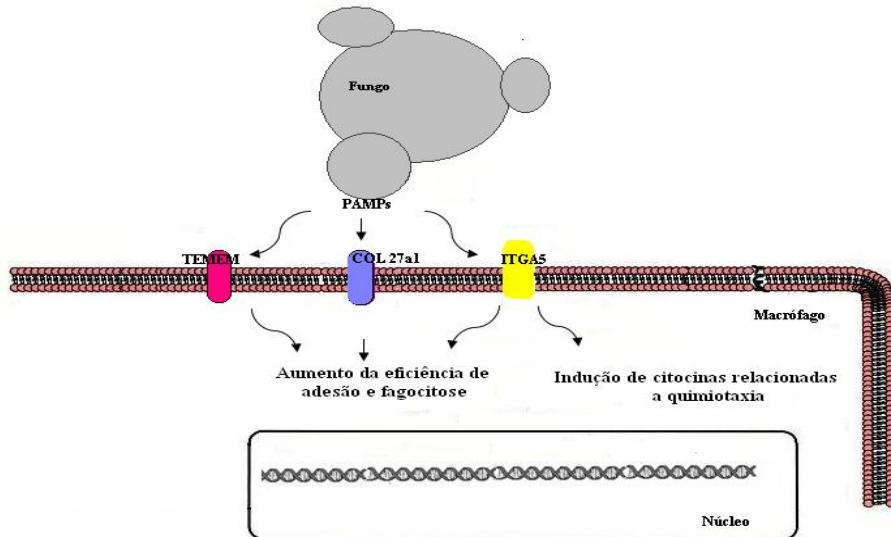
Figura 12. Proposta de modelo da modulação de genes relacionados à adesão e fagocitose de *H.capsulatum* pelos macrófagos. Estão representadas as moléculas TEMEM, COL27a1 e ITGA5. TEMEM é uma proteína de membrana de função ainda não conhecida; COL27a1 atua no reconhecimento de moléculas de superfície da parede celular do patógeno; ITGA5 é correlacionada com a indução de migração celular de macrófagos.

6.3. Análise comparativa da resposta do hospedeiro frente à infecção causada por *P. brasiliensis* ou *H. capsulatum*

A análise do perfil transcricional dos macrófagos murinos durante a interação tanto com *P. brasiliensis* quanto com *H. capsulatum* revelou que os dois patógenos interagem com os macrófagos modulando genes envolvidos no processo pró-inflamatório, proteínas relacionadas à membrana, regulação da transcrição, transdução de sinal e apoptose (Figura 13). Na infecção dos macrófagos com levedura de *P. brasiliensis* o maior número de genes induzidos foi verificado em 24 horas enquanto que nos experimentos de interação com *H. capsulatum* essa modulação foi mais expressiva em 48 horas. Estes resultados indicam que os mecanismos sensoriais e de resposta do macrófago a esses fungos é temporalmente diferente, embora as categorias funcionais e alguns dos genes induzidos tenham sido similares.

Genes relacionados à transdução de sinal tais como proteínas quinases e de transporte de vesículas foram induzidos de forma bem semelhante em todos os tempos de interação dos macrófagos com esses dois fungos. Essas proteínas são elementos essenciais que controlam processos celulares fundamentais, incluindo crescimento, diferenciação e função do citoesqueleto (Cobb and Goldsmith, 1995). Os genes de macrófagos relacionados a transdução de sinal, como por exemplo a proteína TXK foi induzida tanto por *P. brasiliensis* quanto por *H. capsulatum.* A proteína TXK é uma tirosina quinase da família Tec que possui a capacidade de se ligar à região promotora do gene IFN-γ levando à regulação positiva de sua transcrição (Takeba *et al*., 2002). IFN-γ realiza uma função central na imunidade contra *P. brasiliensis* e *H. capsulatum.* Durante a formação do granuloma, IFN-γ modula a expressão de quimiocinas e receptores de quimiocinas que determinam as células que irão migrar para o pulmão (Souto *et al*., 2003; Mamoni *et al*. (2005); Heninger *et al*., 2006). Esta citocina é produzida em altos níveis pelas células que infiltram no pulmão de camundongos infectados com estes patógenos (Souto *et al*., 2003; Heninger *et al*., 2006). Adicionalmente, a produção local desta citocina ativa os macrófagos para produzirem radicais reativos cruciais para o controle da disseminação das leveduras para outros órgãos ou tecidos. O aumento da expressão do gene de macrófago que codifica para TXK induzido por *P. brasiliensis* e *H. capsulatum* sugere que esta molécula possui um papel importante na ativação da célula hospedeira provavelmente regulando positivamente a transcrição de IFN-γ. Simultaneamente à indução do gene Txk observou-se também neste trabalho uma intensa modulação positiva induzida por esses fungos de Map quinases e genes relacionados a transporte de vesículas dos

macrófagos. Dessa forma, esses dados obervados no presente trabalho indicam que provavelmente tanto *P. brasiliensis* quanto *H. capsulatum* induzem uma intensa reorganização celular dos macrófagos no estágio inicial da infecção para responder a invasão pelo patógeno. Por outro lado, pode-se sugerir também que os fungos induzem genes que irão favorecer sua entrada no fagócito, tais como os genes relacionados a adesão e fagocitose que poderão favorecer os patógenos invadirem novas células.

A ligação de patógenos com moléculas da matriz extracelular do hospedeiro transduz sinais que irão culminar na ativação de fatores de transcrição responsáveis por ativar genes relacionados a produção de citocinas, quimiocinas, receptores de quimiocinas e moléculas de adesão que poderão auxiliar na fagocitose do microrganismo e ativação dos fagócitos. Neste sentido, genes relacionados à ativação transcricional foram modulados tanto por macrófagos infectados com *P. brasiliensis* quanto com *H. capsulatum*. A expressão gênica diferencial de NfkB, observados neste trabalho, e de outras moléculas que regulam este fator de transcrição, sugere que um dos mecanismos de ativação de genes relacionados a citocinas pró-inflamatórias provavelmente é regulado por NfkB. De fato, a modulação de genes envolvidos no processo inflamatório e que são regulados por Nfkb durante a interação dos macrófagos tanto com *P. brasiliensis* quanto com *H. capsulatum* foi observada no presente trabalho. Adicionalmente, o gene que codifica para o repressor de Nfkb teve sua expressão modulada negativamente nos macrófagos em 48 horas de interação com *H. capsulatum*, o que sugere que este fungo regula negativamente esta molécula para manter uma resposta favorável a inflamação. Sendo assim, quando comparadas as respostas sobre a regulação do gene Nfkb nos dois patógenos conclui-se que são similares, embora provavelmente ocorram por mecanismos moleculares diferentes. Em *P.brasiliensis* ocorre a indução de Nfkb enquanto em *H.capsulatum* ocorre a repressão do gene que codifica para o repressor de Nfkb o que também irá gerar provavelmente uma regulação positiva de genes relacionados ao processo pró-inflamatório.

A indução de genes que codificam para quimiocinas e que estão relacionadas a quimiotaxia sugere que a migração de células (monócitos, neutrófilos), poderia favorecer a invasão e manutenção do fungo no interior do fagócito enquanto estes não estão ativados. Adicionalmente, a indução de genes relacionados a proteínas de membrana e que são envolvidas principalmente numa fagocitose mais efetiva dos fungos (*P. brasiliensis* e *H. capsulatum*) pelos macrófagos murinos, observada neste trabalho, poderia ser também um

mecanismo induzido por estes microrganismos para aumentar sua eficiênica de invasão à célula hospedeira. Por outro, verificou-se também a indução da secreção da citocina TNF-α que induz a ativação autócrina e parácrina de macrófagos, indicando que esta célula fagocítica também começa a responder a invasão pelos fungos, no sentido de induzir o aumeto da produção de radicais oxidativos para tentar eliminar o microrganismo invasor. Embora a maioria dos genes regulados durante a interação dos macrófagos com *P. brasiliensis* apresentaram funções semelhantes, observou-se neste trabalho que os genes que codificam para proteínas pró-apoptóticas, Casp2 e Casp8 foram diferentemente modulados por esses dois patógenos. A expressão gênica dessas moléculas foi modulada positivamente por macrófagos infectados com *P. brasiliensis* e negativamente por *H. capsulatum.* Conforme mencionado anteriormente, a regulação da expressão de genes de macrófagos induzida por esses patógenos é temporalmente diferente. Dessa forma, pode-se sugerir que a modulação positiva de genes envolvidos na indução de apoptose pode ocorrer de forma mais tardia. A indução de fagocitose por esses dois fungos tem sido correlacionada com resistência a infecção. Provavelmente *H. capsulatum* consegue inicialmente induzir a repressão deste gene na tentativa de permanecer por mais tempo no microambiente do fagócito. Outra molécula diferentemente regulada nos macrófagos durante a interação com esses fungos foi a que codifica para a proteína CD14, e que é relacionada a uma maior capacidade de invasão do fungo às células hospedeiras. Esta molécula foi induzida em macrófagos durante a interação com *P. brasiliensis* e reprimida durante a interação com *H. capsulatum*, o que sugere que os macrófagos modulam negativamente este gene possivelmente na tentativa de diminuir a invasão por estes patógenos a novas células.

A abordagem cinética usada neste trabalho para analisar o perfil transcricional de macrófagos murinos durante a interação tanto com *P. brasiliensis* quanto por *H. capsulatum* por meio de microarranjos de cDNA proporcionou um melhor entendimento dos eventos que culminam na ativação coordenada e temporal de moléculas de defesa durante esta interação. Os dados deste trabalho indicam que as leveduras de *P. brasiliensis* e *H. capsulatum* induzem nos macrófagos uma modulação gênica dinâmica de moléculas que podem favorecer inicialmente a sobrevivência deste fungo no interior dos fagócitos. Por outro lado, observa-se nesta interação que os macrófagos parecem responder induzindo genes relacionados a atividade fungicida possivelmente na tentativa de conter a disseminação do fungo.
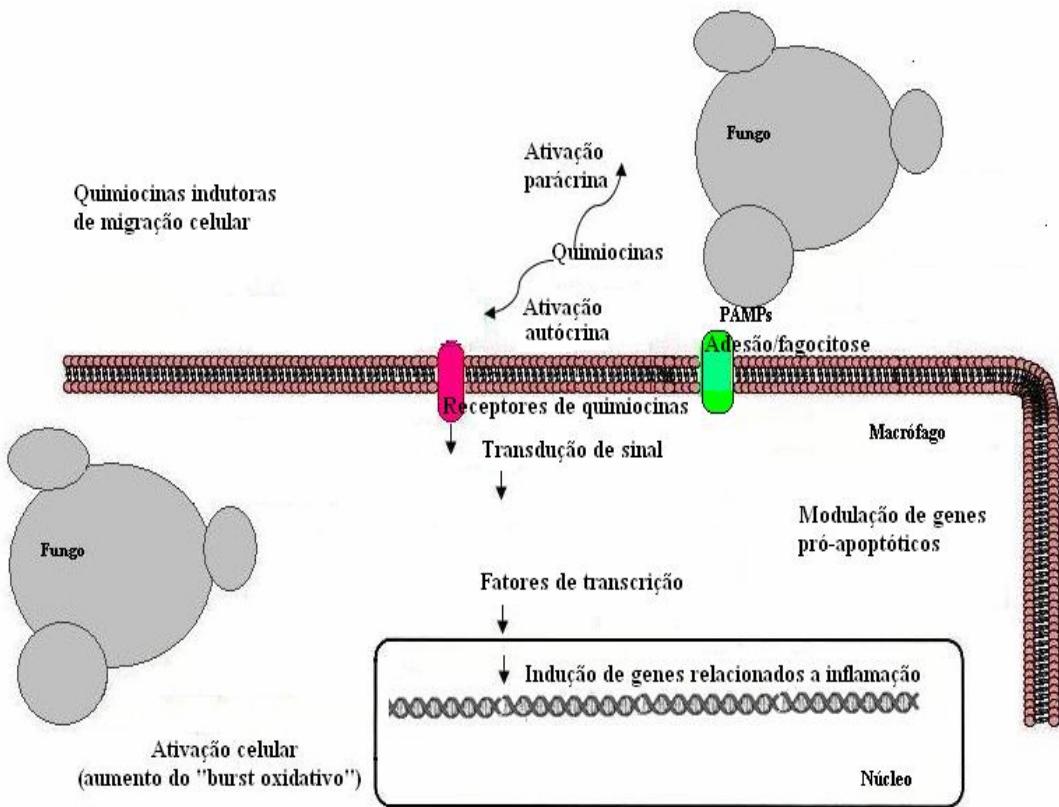
Figura 13. Proposta de modelo da modulação global de genes de macrófagos murinos durante a interação com *P. brasiliensis* e *H. capsulatum*. Os fungos dimórficos *P. brasiliensis* e *H. capsulatum* induzem nos macrófagos murinos a modulação de genes relacionados às mesmas 5 categorias funcionas: inflamação, proteínas de membrana, transdução de sinal, fatores de transcrição e apoptose. **Inflamação**: indução de genes relacionados a quimiotaxia de monócitos e macrófagos e receptores de quimiocina, além de genes envolvidos na ativação celular; **Proteína de membrana**: modulação de genes envolvidos na adesão e fagocitose que reconhecem as moléculas da superfície da parede celular do fungos (PAMPs) e aumentam a eficiência de fagocitose e/ou ativação do macrófago; **Fatores de transcrição**: envolvidos na modulação de genes relacionados ao processo inflamatório; **Apoptoses**: a apoptose está envolvida no controle a nfecção por estes patógenos. A modulação dos mesmos genes pró-apoptóticos foi diferentemente regulado pelos dois patógenos. *P. brasiliensis* induz e *H. capsulatum* reprimiu a expressão de genes pró-apoptóticos durante a cinética de interação.

6.4. Análise global da interação macrófago-*P. brasiliensis* – adaptação transcricional do patógeno e do hospedeiro

Recentemente, nosso grupo utilizou a tecnologia de microarranjos de cDNA para avaliar a resposta transcricional de *P. brasiliensis* no interior de macrófagos em 6 horas de infecção (Tavares *et al.*, 2007). No presente trabalho foi analisado o perfil de expressão gênica dos macrófagos infectados com o *P. brasiliensis* em 6 horas com o objetivo de avaliar a resposta molecular do hospedeiro. A indução de genes relacionados ao processo pro-inflamatório (Ccl21, Cxcl1 and Ccl22) e a fagocitose (Clec1b) foi observado em seis horas, consistente com um aumento na internalização de *P. brasiliensis* nesse mesmo tempo de infecção. Desta forma, considerando as análises de expressão gênica diferencial para o patógeno e o hospedeiro, em 6 horas, é possível propor um modelo para a interação *P. brasiliensis*-macrófago (figura) que pode sugerir os principais eventos moleculares ocorridos no estágio inicial da interação.

Em resposta ao microambiente inóspito do macrófago, *P. brasiliensis* induz genes tais como aqueles relacionados à detoxificação de radicais oxidativos (*sod3* and *hsp60*), biossíntese de aminoácidos (*metG*) e os relacionados a via glicolítica (*pbgapdh, pgk, gpmA* e *eno*). A proteína SOD3 (provável proteína de membrana GPI-ancorada) participa da eliminação de ânios superóxidos (Castro *et al.,* 2005). Dessa forma, pode-se sugerir que *sod3* é positivamente regulada pelo fungo para favorecer uma melhor acessibilidade aos derivados de ânion superóxido e subsequente detoxificação de intermediários reativos de oxigênio produzidos pelo hospedeiro no ambiente do fagolisossoma. Adicionalmente, o gene que codifica para a *hsp60* também foi induzido pelas leveduras de *P. brasiliensis* fagocitadas pelos macrófagos. Uma das funções protetoras descritas para a HSP60 está relacionada a sua habilidade de bloquear a liberação de ferro ferroso reduzido, provenientes da oxidação de proteínas mitocondriais (Cabiscol *et al.*, 2002). Neste sentido, provavelmente a indução de *hsp60* por *P. brasiliensis* exposto a radicais oxidativos produzido pelos macrófagos pode ter uma função protetora similar (Tavares *et al*., 2007). Além disso, genes envolvidos com a via glicolítica também foram modulados pelo fungo após sua fagocitose pelo macrófago. A fosfofrutoquinase (PFKA) é uma enzima regulatória chave da via glicolítica e sua expressão foi reprimida no fungo internalizado pelas células fagocíticas. Adicionalmente, genes codificando outras quatro enzimas desta via também foram reprimidos (*pbgapdh, pgk, gpmA* e *eno*), sugerindo que as leveduras de *P. brasiliensis* se encontram em um ambiente com

diminuída concentração de nutrientes (Tavares *et al*., 2007). No presente trabalho, observou-se que os macrófagos ao mesmo tempo induzem genes relacionados à inflamação (quimiocinas e citocinas) e a fagocitose, provavelmente na tentativa de conter a infecção pelo fungo. A indução desses genes além de levar ao aumento da fagocitose do fungo pelos macrófagos induz a migração de células para o local da infecção favorecendo a invasão de novas células hospedeiras. O fungo *P. brasiliensis* é um parasita facultativo que pode sobreviver e se replicar em macrófagos não ativados previamente (Brummer *et al*. 1989). Dessa forma, a indução da expressão de genes pelo fungo importantes para sua adaptação no microambiente do macrófago aliado a indução de moléculas do macrófago relacionados a quimiotaxia e fagocitose sugere que a invasão de novas células pelo fungo pode ser uma estratégia favorável para esse patógeno se manter viável e se multiplicar no hospedeiro.
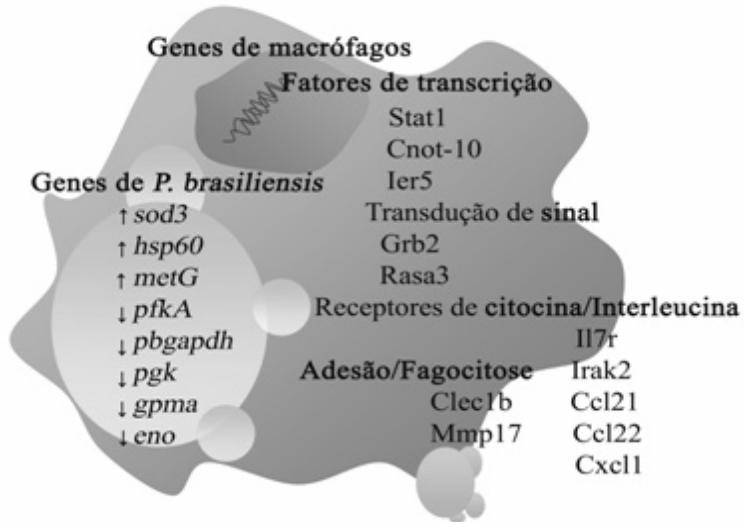
Figura 14. Modelo proposto para a expressão gênica diferencial inicial na interação macrófago-P. brasiliensis. Os resultados para os seguintes genes são mostrados: *P. brasiliensis*. As setas indicam indução (↑) ou repressão gênica (↓). Expressão diferencial de genes relacionados a estresse. *sod3* (superóxido dismutase 3)*, hsp60* (proteína heat shock 60)*; pfkA* (fosfofrutoquinase A); *metG* (cistationina β-liase); *pbgapdh* (gliceraldeíde-3-fosfate desidrogenase)*; pgk* (fosfoglicerato quinase)*; gpma* (fosfoglicerato mutase)*; eno* (enolase)*.* **Macrófago.** Indução gênica de moléculas de defesa inata do hospedeiro. **Receptores de citocinas e interleucina**: IL7r (Receptor de interleucina-7); Irak2 (Similar to interleukin-1 receptor-associated kinase 2); Ccl21 and Ccl22 (C-C ligantes de quimiocina 21 e 22); Cxcl-1 (C-X-C ligante 2); **Adesão**: Clec1b (C- receptor tipo lectina 1 b) e Mmp17 (Metaloproteinase de matriz 17); **Transdução de sinal:** Grb2 (Fator do receptor de crescimento ligado a proteína 2); Rasa3 (Ras p21 ativador de proteína 3); **Fatores de transcrição:** Stat1 (Similar ao receptor de interleucina-1 associado a quinase 2); Cnot10 (Complexo de transcrição CCR4-NOT , subunidade 10) and Ier5 (resposta inicial imediata 5).

7. SUMÁRIO DOS RESULTADOS

1- Modulação global de adaptação induzida pelos dois patógenos.

2- Modulação de genes-chaves como Nfkb que provavelmente funcionará como fator de trancrição que irá orquestrar a modulação geral de genes para induzir a resposta pró-inflamatória ao longo da cinética de interação analisada.

3- Em paralelo ocorre uma forte indução de genes que codificam para proteínas que irão participar do processo de inflamação tais como migração celular e ativação da capacidade microbicida dos macrófagos, ao longo da cinética de interação analisada.

4- Ocorre em seguida um aumento de proteínas-chave de membranas tais como Clec, metaloproteinases, integrina, que provavelmente irão aumentar a capacidade de invasão e internalização dos patógenos pelos macrófagos.

5- A maioria dos eventos mencionados acima ocorre nos dois sistemas de interação patogeno-hospedeiro, utilizados neste trabalho.

6- Na interação de *P. brasiliensis* com os macrófagos foi também verificado a indução em tempos mais tardios (24 h) de genes que induzem o processo de apoptose dos macrófagos, indicando que este pode ser o mecanismo utilizado por esses fagócitos na tentativa de conter a disseminação do fungo.

7- Para o caso da interação *P. brasiliensis* - macrófagos, o modelo de interação proposto sugere que o fungo se adaptou para sobreviver no microambiente do macrófago, induzindo genes relacionados à detoxificação, metabolismo de aminóacidos e de carboidratos.

8. CONCLUSÃO

Este trabalho permitiu acessar a modulação geral de genes de macrófagos envolvidos principalmente com o processo pró-inflamatório durante a interação com leveduras de *P. brasiliensis* e *H. capsulatum*.

9. PERSPECTIVAS

Considerando os resultados alcançados neste trabalho tem-se como perspectivas os seguintes pontos:

1- Analisar o padrão de adaptação de expressão gênica dos macrófagos em condições de ativação com IFN-γ para verficar se o perfil de resposta é similar ao observado na condição de macrófagos não-ativados. Provavelmente deverá ser obtido um padrão diferente, por exemplo, na expressão de genes envolvidos na produção de radicais de espécies reativas (ROS e RNS), na tentativa de conter a disseminação do patógeno. Isto não foi observado nos resultados apresentados neste trabalho muito provavelmente devido a não-ativação prévia dos macrófagos;

2- Analisar o padrão de expressão gênica de *P. brasiliensis* e de *H. capasulatum* durante a cinética de infecção, visando entender temporalmente a modulação e adaptação destes dois patógenos tanto com macrófagos não-ativados quanto com ativados;

3- Para tornar esta análise mais próxima da realidade da interação patógeno-hospedeiro, o nosso grupo está padronizando e realizando estes experimentos utilizando células de pulmão humano em cultura (A549) visando avaliar os resultados de modulação de expressão gênica tanto do patógeno como do hospedeiro, durante a cinética de infecção.

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INSTITUT PASTEUR

Original article

Early transcriptional response of *Paracoccidioides brasiliensis* upon internalization by murine macrophages

Aldo Henrique F.P. Tavares [a,1], Simoneide S. Silva [a,1], Alessandra Dantas [a],
Élida G. Campos [a], Rosângela V. Andrade [a], Andréa Q. Maranhão [a], Marcelo M. Brígido [a],
Danielle G. Passos-Silva [b], Ana L. Fachin [c], Santuza M.R. Teixeira [b],
Geraldo A.S. Passos [c,d], Célia M.A. Soares [e], Anamélia L. Bocca [a],
Maria José A. Carvalho [a], Ildinete Silva-Pereira [a], Maria Sueli S. Felipe [a,*]

[a] *Departamento de Biologia Celular, Laboratório de Biologia Molecular, Universidade de Brasília-Unb Brasília,
Campus Darcy Ribeiro-Asa Norte 70910-900, Brasília, DF, Brazil*
[b] *Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, 31270-901, Belo Horizonte, MG, Brazil*
[c] *Departamento de Genética, Universidade de São Paulo, 14040-900, Ribeirão Preto, SP, Brazil*
[d] *Faculdade de Odontologia, Universidade de São Paulo, 14040-900, Ribeirão Preto, SP, Brazil*
[e] *Departamento de Bioquímica, Universidade Federal de Goiás, 74001-970, Goiânia, GO, Brazil*

Abstract

Paracoccidioides brasiliensis, a thermal dimorphic fungus, is the etiologic agent of the most common systemic mycosis in Latin America, paracoccidioidomycosis. The yeast form of *P. brasiliensis* acts as a facultative intracellular pathogen being able to survive and replicate within the phagosome of nonactivated murine and human macrophages. This ability has been proposed to be crucial to the development of disease. Thus, *P. brasiliensis* may have evolved mechanisms that counteract the constraints imposed by phagocytic cells. By using cDNA microarray technology we evaluated the early transcriptional response of this fungus to the environment of peritoneal murine macrophages in order to shed light on the mechanisms used by *P. brasiliensis* to survive within phagocytic cells. Of the 1152 genes analyzed, we identified 152 genes that were differentially transcribed. Intracellularly expressed genes were primarily associated with glucose and amino acid limitation, cell wall construction, and oxidative stress. For the first time, a comprehensive gene expression tool is used for the expression analysis of *P. brasiliensis* genes when interacting with macrophages. Overall, our data show a transcriptional plasticity of *P. brasiliensis* in response to the harsh environment of macrophages which may lead to adaptation and consequent survival of this pathogen.
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Keywords: Paracoccidioides brasiliensis; Intracellular; Macrophage; Microarray

1. Introduction

Paracoccidioides brasiliensis, a thermal dimorphic fungus, is the etiologic agent of the most common systemic mycosis in Latin America, paracoccidioidomycosis (PCM). Epidemiological and experimental data suggest that humans probably become infected via inhalation of airborne conidia derived from the saprophytic phase [1]. The first defense line faced by *P. brasiliensis* during host invasion is the lung resident macrophages. Despite being phagocytized, *P. brasiliensis* conidia transform into the parasitic yeast form that subvert the normally harsh intraphagosomal environment, surviving and replicating in nonactivated murine and human macrophages. Only upon induction of cell-mediated immunity, mainly via gamma interferon production, are macrophages activated and exhibit full fungicidal capacity [2,3]. It

has been proposed for PCM and other systemic mycoses, such as histoplasmosis and coccidioidomycosis, that fungal intracellular parasitism is a major event for disease establishment and progression in susceptible hosts, by enabling fungal latency and/or dissemination from the lungs to several organs. In addition, intracellular shelter may also confer protection from complement, antibodies and other innate defense mechanisms [4].

Several studies reveal that facultative intracellular bacteria and fungi alter their gene expression programs in response to the macrophage environment [5−7]. The understanding of this process should present key elements on how these pathogens manage to survive and replicate intracellularly and eventually lead to the development of new antimicrobial drugs. Although some efforts have recently been done to study the complex *P. brasiliensis*−host interaction [8], no report has focused on the fungus' differently expressed genes inside macrophages. In the present study, we used a DNA microarray consisting of 1152 cDNA clones derived from *P. brasiliensis* expressed sequence tag (EST) data bank [9] in order to define the transcript profile of this fungus co-cultured with murine macrophages for 6 h, an early time point of *P. brasiliensis* yeast cell internalization [2]. The data presented here may provide a foundation for a better understanding of the *P. brasiliensis*−host cell interaction.

2. Materials and methods

2.1. Isolation and cultivation of murine peritoneal macrophages

Balb/c mice [2] were intraperitoneally injected with 2 ml of sterile 3% sodium thioglycolate. After 72 h, the animals were killed and their cells recovered by peritoneal lavage with 10 ml of ice-cold RPMI-1640 tissue culture medium (Sigma), supplemented with 20 mM HEPES, 1.5 g/l sodium bicarbonate, 25 μg/ml gentamicin and 10 U/ml heparin. Peritoneal cells were dispensed into 150 cm² cell culture flasks (2×10^7 cells/flask) and incubated at 37 °C for 1 h. After non-adherent cells were removed by washing, the adherent cells were incubated in RPMI supplemented as above, plus 10% heat-inactivated fetal calf serum at 37 °C for 16 h.

2.2. Infection of murine macrophages cultures with P. brasiliensis

P. brasiliensis yeast cells were suspended in RPMI medium containing 20% fresh mouse serum from the same normal BALB/c mice used to obtain peritoneal macrophages. The opsonization protocol was carried out by incubation of yeast cell suspensions at 37 °C for 30 min. Macrophage monolayers were infected, as previously described [2], with 4×10^6 yeast cells, representing a yeast-to-macrophages ratio of 1:5. Incubation was done at 37 °C in a humidified 5% CO_2 atmosphere. The percentage of internalized *P. brasiliensis* yeast cells over 12 h of *ex vivo* infection was determined and a 6-h period of infection was selected because it represents an early time point

of fungal cell internalization by macrophages (data not shown).

2.3. Total RNA extraction of P. brasiliensis yeast cells internalized by murine macrophages

At 6 h after infection, extracellular and weakly adherent fungi were removed by washing with pre-warmed RPMI. Macrophages were then lysed with a guanidine thiocyanate based solution [10] and intact fungi were harvested by centrifugation and immediately followed by Trizol total RNA extraction (Invitrogen) according to the manufacturer's instruction. Total RNA from *in vitro* grown control yeast cells was also extracted with Trizol.

2.4. cDNA microarray construction, hybridization and data analysis

Two sets of microarrays containing a total of 1152 PCR products corresponding to *P. brasiliensis* cDNAs were spotted in duplicate onto Hybond N⁺ nylon membranes (GE Health Care, UK) as previously described [9]. Membranes were first hybridized against the T3 [γ-³³P] ATP-labeled oligonucleotide. The amount of DNA deposited in each spot was estimated by the quantification of vector hybridization signals. After vector probe stripping, membranes were used for hybridization against [α-³³P]dCTP labeled cDNA complex probes. The latter were prepared by reverse transcription, using oligo(dT)$_{12−18}$ primer, 10 μCi/μl (≥3000 Ci/mM) of [α-³³P]dCTP and 10 μg of total RNA isolated from *P. brasiliensis* yeast cells internalized by murine macrophages or *in vitro* grown yeast cells. Due to the limited quantity of total RNA extracted in a single macrophage infection experiment (∼3 μg), gene expression data analyzed here resulted from three independent sets of pooled RNA. Each pool consisted of 3−4 macrophage infection experiments. Thirty to fifty million cpm of [α-³³P]cDNA complex probe were hybridized against microarrays at 65 °C for 48 h. Membranes were washed and exposed to radiosensitive imaging plates for 48 h, which were scanned in a PhosphorImager (Cyclone, Packard Instruments) to capture the hybridization signals. After normalization, SAM (Significance Analysis of Microarrays) software was used to assess the significant variations in gene expression between experimental and control conditions. Briefly, this method is based on *t* test statistics, specially modified to high throughput analysis. Significantly modulated genes were identified by passing both a statistical (*q*-value < 5%; FDR < 5%) and a fold-variation (≥2-fold up or down) cutoff. Microarray data have been deposited in ArrayExpress under accession numbers A-MEXP-558 and E-MEXP-823.

2.5. Real time RT-PCR validation of differentially modulated genes

An independent and the same RNA samples used for microarray experiments were used for real time RT-PCR. Equal amounts of RNA (0.5 μg) were reverse transcribed using oligo(dT)$_{12−18}$ primer and submitted to real time PCR.

Amplification assays were carried out with a 7900HT Sequence Detection System ABI PRISM instrument (Applied Biosystems, USA) in 12 μl reactions containing 0.4 μM of each primer (listed in Table 1), 6 μl of SYBR Green PCR Master mix (2×) and 0.2 μl of template cDNA. After initial denaturation at 95 °C for 10 min, amplifications were done for 40 cycles at 95 °C/15 s and 60 °C/1 min. The comparative CT (crossing threshold) method, employing the constitutive *P. brasiliensis* α-tubulin gene expression data for normalization, was used to evaluate the fold change of each interest gene in macrophage-internalized sample when compared to control. Two to three replicates of RT-PCR experiments were done for all analyzed genes.

2.6. Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) produced by P. brasiliensis infected macrophage

After 6 h of incubation, supernatants from *P. brasiliensis* infected macrophage cultures were removed and the cells were washed with Krebs−Ringer buffer. O_2^- release was determined by superoxide dismutase (SOD) inhibitable reduction of ferricytochrome *c* [11]. *P. brasiliensis* infected macrophage monolayers were incubated at 37 °C for 1.5 h in Krebs−Ringer buffer containing 80 μM ferricytochrome *c* (Type III; Sigma) with or without phorbol 12-myristate 13-acetate (PMA, 5 μg/ml; Sigma) in order to stimulate O_2^- release. Control wells contained cytochrome *c* without *P. brasiliensis* (uninfected cultures) or *P. brasiliensis* infected macrophage plus 40 μg/ml of SOD (Sigma). After incubation, supernatants were collected and the absorbance measured at 550 nm. The background absorbance of cell-free wells containing only cytochrome *c* was subtracted. O_2^- produced was calculated and normalized to nanomoles per 5×10^5 macrophage cells.

H_2O_2 was measured by the horseradish peroxidase-dependent oxidation of phenol red method [11]. Infected and control macrophage monolayers were incubated at 37 °C for 1.5 h in phosphate buffer containing 0.56 mM phenol red and 20 U/ml peroxidase (Type II, Sigma), with or without PMA 5 μg/ml. H_2O_2 produced was measured at 620 nm after mixing with 10 μl of 1 M NaOH per well. H_2O_2 was quantified by comparison with a standard curve prepared with known concentrations of H_2O_2. The amount of H_2O_2 produced was expressed as nanomoles per 5×10^5 macrophage cells. Results were expressed as the mean ± SEM for two triplicate

experiments. Differences between the control and experimental groups were evaluated by paired two-tailed Student's *t* test. A probability of $p < 0.05$ was considered significant.

2.7. P. brasiliensis sod3 expression following in vitro treatment with H_2O_2 and menadione

After 7 days of growth in Fava-Netto medium, yeast cells were harvested and cultivated into YPD medium with or without 50 mM menadione (Sigma) or 50 mM H_2O_2, at 36 °C, 140 rpm, for 0, 30, 50 and 60 min.

Approximately 13 μg of denatured RNA was separated by electrophoresis and transferred to a nylon membrane, as previously described [9]. PCR-product corresponding to *P. brasiliensis sod3* cDNA was radiolabeled with [α-^{32}P]dATP employing the random primer kit (Invitrogen), purified and used in the hybridization experiments. Membranes were washed to a final stringency of $2 \times SSPE−0.1\%$ SDS at 65 °C for 25 min, exposed to a storage phosphor screen and visualized using Typhoon 9210 PhosphorImager (Amersham Pharmacia). Hybridization signal intensities were quantified (ImageQuant, Molecular Dynamics) using the ethidium bromide stained 18S ribosomal RNA as a control of sample load.

3. Results

3.1. Transcriptional response of P. brasiliensis yeast cells upon internalization by murine macrophages

Gene expression analysis of internalized *P. brasiliensis* was performed using a DNA microarray consisting of 1152 cDNA clones related to putative virulence factors, general metabolism enzymes, heat shock proteins, cell wall construction enzymes and others [9]. Microarray analysis showed the differential expression of 152 *P. brasiliensis* genes in response to the macrophage microenvironment (for complete list see Supplemental Material). Based on previous findings of bacterial and fungal−macrophage interaction microarray studies [5−7], we selected up- and down-regulated genes for discussion (Table 2). Among those up-regulated genes, we found genes encoding proteins related to oxidative (Cu,Zn superoxide dismutase 13-fold) and nutritional (cystathionine beta-lyase 3.2-fold) stressors. *In silico* analysis previously performed by our group [12] showed that the Cu,Zn superoxide dismutase (SOD) gene

Table 1
Primers used for real time RT-PCR validation of microarray data[a]

PbAEST[b]	Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
50	*sod3*	CTGTTCGCTGGGCTTTGC	TCAGTAGTGACGGCTTCCATCAT
269	*hsp60*	TATCACCACCACTGAAGAGATTGC	GCTTGCCAACATGGGTATCA
281	*metG*	GTTTCACGTCATTTCCCCTTATCT	CAGCCCCAGCCATTGTTG
3775	*fks*	AATTATTGCCTTCGCTCCCATA	GCCATCGCGAAGAGAACTG
2976	*fmdS*	CCGTCAGCCGAGGTCCTA	GCATTGGCCGAGAGGAGAT
5491	*eno*	GTAACCGAGACTGGGCTTCATC	GCCTCATGCTGACCGGTAGA

[a] All primer pairs were designed using the Primer Express software (Applied Biosystems) based on the sequences obtained from the *P. brasiliensis* transcriptome database [9].
[b] *P. brasiliensis* assembled EST sequence [9].

Table 2
Selected up- and down-regulated genes of *P. brasiliensis* yeast cells phagocytized by murine macrophage

	PbAEST[a]	Annotated function	EC number	Fold change[b]	Ortholog gene	Accession number/best hit organism/E-value[c]
Oxidative stress	50	Cu,Zn superoxide dismutase	1.15.1.1	13.8	*sod1*	ABB36775/*Coccidioides posadasii*/1e-44
Amino acid biosynthesis	281	Cystathionine beta-lyase	4.4.1.8	3.2	*metG*	AAB03241/*Emericela nidulans*/e-104
Heat shock protein	269	60 kDa heat shock protein	–	2.0	*hsp60*	O60008/*P. brasiliensis*/e-140
Cytochrome activity	284	Cytochrome oxidase *c*, subunit VIII	–	3.2	*qcr8*	EAS30263/*Coccidioides immitis*/9e-32
	500	Mitochondrial copper transporter	–	2.0	*cox17p*	AAY64186/*P. brasiliensis*/3e-16
Histones	1230	Histone H3	–	4.2	*hht2*	CAA39154.1/*E. nidulans*/2e-68
	1873	Histone H4	–	8.1	*hhf2*	AAL38972.1/*Neurospora crassa*/8e-39
	2069	Histone H2A	–	3.4	*htz1*	CAA99011.1/*Saccharomyces cerevisiae*/1e-20
Translation machinery	1006	40S ribosomal protein S15	–	2.3	*rps15*	NP_014602/*S. cerevisiae*/2e-46
	654	40S ribosomal protein S24	–	2.3	*rps24*	NP_010997/*S. cerevisiae*/3e-38
	857	40S ribosomal protein S27	–	2.7	*rps27*	NP_012766/*S. cerevisiae*/2e-32
	1484	40S ribosomal protein S31	–	4.8	*rps31*	NP_013268/*S. cerevisiae*/2e-55
	195	60s ribosomal protein L12	–	2.0	*rpl12*	NP_010860/*S. cerevisiae*/9e-53
	477	60S ribosomal protein L21A	–	2.4	*rpl21*	NP_009750/*S. cerevisiae*/4e-56
	3213	60S ribosomal protein L24	–	2.7	*rpl24*	NP_011484/*S. cerevisiae*/1e-32
	3528	60S ribosomal protein L27	–	2.7	*rpl27*	NP_010759/*S. cerevisiae*/2e-33
	1317	60S ribosomal protein L28	–	3.7	*Rpl28*	NP_013176/*S. cerevisiae*/1e-83
	5077	60S ribosomal protein L41	–	2.1	*rpl43*	NP_015368/*S. cerevisiae*/9e-28
	2599	60S ribosomal protein L8	–	2.3	*rpl8*	NP_013055/*S. cerevisiae*/1e-103
	551	60S ribosomal protein L35	–	2.8	*rpl35*	NP_010090/*S. cerevisiae*/2e-22
	81	60S ribosomal protein L42	–	2.1	*rpl42*	NP_014237/*S. cerevisiae*/4e-52
Glycolysis	3624	phosphofructokinase	2.7.1.11	−3.6	*PfkA*	Q4W9B8/*Aspergillus fumigatus*/1e-40
	3698	Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	−2.4	*Pbgadph*	AAP42760/*P. brasiliensis*/1e-34
	42	Phosphoglycerate kinase	2.7.2.3	−2.2	*pgk*	XP_658850/*Aspergillus nidulans*/2e-174
	4820	Phosphoglycerate mutase	5.4.2.1	−5.3	*gpmA*	XP_754690/*A. fumigatus*/5e-121
	2976	Enolase	4.2.1.11	−5.2	*eno*	AAK49451.1/*A. fumigatus*/7e-69
Cell wall	3775	1,3 β-glucan synthase	2.4.1.34	−3.5	*fks*	AAD37783.1/*P.brasiliensis*/1e-60
Nitrogen metabolism	5491	Formamidase	3.5.1.49	−22	*pbfmd*	AAN87355/*P. brasiliensis*/1e-111

[a] [9] and http://www.biomol.unb.br/Pb-eng.
[b] Positive and negative numbers represent up- and down-regulated genes, respectively.
[c] BLASTx searches were performed at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/).

(PbAEST 50) deduced amino acid sequence corresponds to a putative glycosylphosphatidylinositol (GPI)-anchored SOD, which shows high sequence similarity to GPI-SOD5 of *C. albicans* [13,14]. In view of two other SOD-related PbAESTs already described for *P. brasiliensis* [9], the PbAEST 50 was designated *sod3* (GenBank accession number DQ832188). The heat shock protein 60 (*hsp60*) encoding gene was also up-regulated. It has been shown that besides its chaperone activity, HSP60 protein has an important role in protection against oxidative stress [15]. Other genes that presented an increased expression were related to the mitochondrial electron transport chain (*qrc8* and *cox17p*; 3.2- and 2-fold, respectively). Interestingly, the up-regulation of three histone-related genes suggests that *P. brasiliensis* may respond to the macrophages' intracellular environment by remodeling the chromatin. Genes encoding ribosomal subunits were the majority of up-regulated genes with fold changes ranging from 2 to 4.8.

Among the repressed genes, five encode proteins associated with glycolysis/glyconeogenesis, suggesting a complex carbon depleted environment within macrophages. The genes encoding 1,3-β-D-glucan synthase (*fks*) and formamidase (*pbfmd*)

were also repressed (−3.5 and −22-fold, respectively). Noteworthy is the fact that 1,3-β-D-glucan synthase synthesizes β-glucan, a central polysaccharide component of fungal cells that has immunostimulatory activity [16,17]. In addition, 23 genes of unpredicted function (15% of the differential expressed genes) were also down-regulated.

3.2. Validation of the microarray data

An independent and the same RNA samples employed in the microarray experiments were used to validate our microarray data by real time RT-PCR. We selected three *P. brasiliensis* genes that were up-regulated (*sod3*, *hsp60* and *metG*) and three that were down-regulated (*fks*, *fmdS* and *eno*) following internalization by murine peritoneal macrophages. Table 3 shows that the expression profile of all these genes, when analyzed by real time RT-PCR, correlated with the profile observed in the microarray experiments. The magnitude of fold changes differed between the methodologies, a result expected due to their technical differences regarding normalization protocol, kinetics and sensitivity.

Table 3
Real time RT-PCR validation of microarray data

PbAEST	Gene	Fold change[a,b]	
		Microarray	RT-PCR[c]
50	sod3	13.0	3.9
269	hsp60	2.0	6.0
281	metG	3.2	1.4
3775	fks	−3.5	−3.4
2976	eno	−5.2	−1.2
5491	fmdS	−22.0	−5.2

[a] Fold change values were determined after normalization of each gene to the α-tubulin gene by using the comparative threshold method.
[b] Positive and negative numbers represent up- and down-regulated genes, respectively.
[c] Mean fold change of 2−3 experiments done in duplicate.

3.3. Superoxide anion and hydrogen peroxide release by P. brasiliensis-infected macrophage cells

Since our microarray expression data suggested that oxidative stress seemed to be an important determinant on the regulation of gene expression of intracellular *P. brasiliensis* (*sod3* and *hsp60* up-regulation), we sought to investigate the release of O_2^- and H_2O_2 by *P. brasiliensis* infected macrophages.

Table 4 shows that infection of Balb/c peritoneal macrophages with *P. brasiliensis* caused a significant increase of more than 4 and 5 times in O_2^- and H_2O_2 production, respectively, when compared with uninfected macrophages. Thus, our results suggest that *P. brasiliensis* cells were under oxidative attack, which may have triggered the induction of important genes for cellular protection. In addition, upon stimulation by PMA, macrophage cultures increased O_2^- and H_2O_2 production with no significant differences between infected and uninfected macrophages, suggesting a similar response capacity for the production of these reactive oxidative intermediates.

3.4. Northern-blot analysis of sod3 gene upon in vitro P. brasiliensis exposure to ROS-producing agents

Considering the up-regulation of *C. albicans* *sod5* under *in vitro* treatment with O_2^- generating agents as well as H_2O_2

Table 4
Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) released by peritoneal murine macrophage infected or not with *P. brasiliensis*, in the presence or absence of PMA[a]

Condition	O_2^- (nmoles/5×10^5 cells)	H_2O_2 (nmoles/5×10^5 cells)
Pb[b]-infected macrophages	5.5 ± 0.5[c]	4.2 ± 0.4[c]
Uninfected macrophages	1.3 ± 0.6	0.8 ± 0.3
Pb-infected macrophages + PMA	49.3 ± 3.7	43.1 ± 2.7
Uninfected macrophages + PMA	52.8 ± 2.6	40.1 ± 1.7

[a] Mean ± standard error of the mean of averages of triplicates of two experiments. O_2^- release was determined by SOD inhibitable reduction of ferricytochrome *c* and H_2O_2 by the horseradish peroxidase-dependent oxidation of phenol red.
[b] *P. brasiliensis*.
[c] Statistically different ($p < 0.01$) when compared with uninfected macrophages as calculated by Student's *t* test.

[14], we investigate the *sod3* transcript levels in response to *P. brasiliensis in vitro* exposure to menadione (intracellular O_2^- generator) and H_2O_2. Fig. 1. shows that under the influence of high concentrations of menadione (Fig. 1A; 50 mM for 60 min) or H_2O_2 (Fig. 1B; 50 mM for 50 min), *sod3* gene expression was induced about three and four times higher than control, respectively. Our northern blot results are consistent with up-regulation of *sod3* gene in internalized *P. brasiliensis* in response to the significant O_2^- and H_2O_2 production by macrophages, strongly suggesting that *P. brasiliensis* may be able to respond to the oxidative burst *in vivo*.

4. Discussion

The macrophage phagosome is believed to be a poor source of glucose and amino acids [6,7]. Such nutrient deprivation inside the macrophage induces a similar adaptive response of intracellular bacterial and fungal pathogens. Shortly following phagocytosis, *Listeria monocytogenes* and *C. albicans* have a strong reduction in the expression of genes involved in glycolysis [5,7]. We show here that early phagocytized *P. brasiliensis* also sense and respond to the glucose depleted environment of the macrophage phagosome. The gene encoding the key regulatory enzyme phosphofructokinase (*pfkA*) was repressed threefold. Furthermore, four downstream enzymes in the glycolysis/glyconeogenesis pathway were also down-regulated in internalized *P. brasiliensis*. Surprisingly, in our microarray data, we did not observe an up-regulation of isocitrate lyase gene (*icl*), which encodes one of the glyoxylate cycle key enzymes. Induction of the glyoxylate cycle upon phagocytosis has been described as an important pathogen adaptation to the glucose-poor environment within macrophages since it facilitates the assimilation of two-carbon compounds [6,7]. A possible explanation to our microarray results concerning *icl* expression may be that *in vitro* grown control *P. brasiliensis* yeast cells already present a relatively high *icl* expression level, as previously described by our group [9].

Another important pathogen metabolic adaptation in response to phagocytosis is the induction of amino acid biosynthetic genes [6,7]. Specifically, genes involved in methionine biosynthesis are induced in *C. albicans* in response to neutrophil contact [13]. In agreement with these studies, the *P. brasiliensis* cystathionine β-lyase encoding gene (*metG*), whose product is engaged in methionine production, was induced upon phagocytosis by murine macrophage. Besides being critical for protein biosynthesis, methionine in the form of *S*-adenosylmethionine is the main methyl donor of numerous vital methylation reactions in all organisms [18]. The importance of cystathionine β-lyase activity was reported in a mouse model of systemic infection with *Salmonella enterica* serovar *Typhimurium*. This bacterium lacking the *metG* gene was less virulent than the wild-type [19]. In this sense, up-regulation of *metG* gene would confer an important adaptative response of *P. brasiliensis* to survive inside macrophages, where concentrations of amino acids are relatively low.

Of particular interest for PCM pathogenesis are the genes involved in the synthesis of the cell wall polysaccharides. In
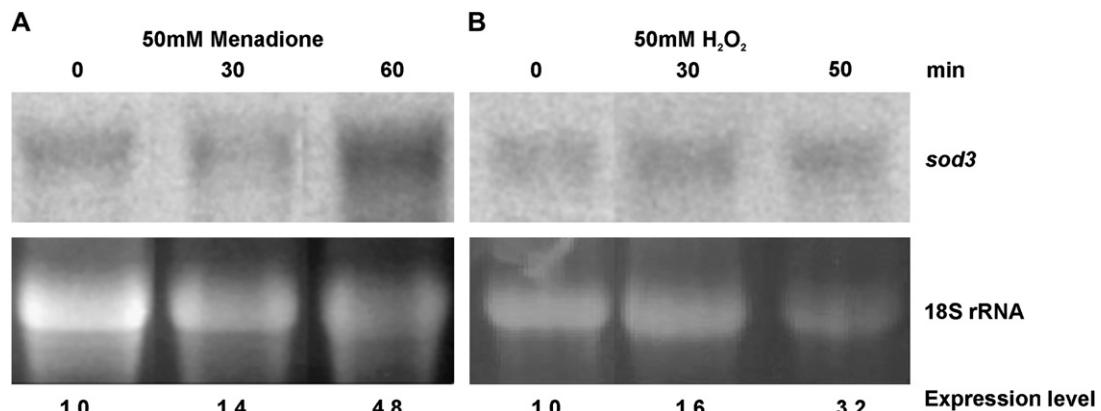
Fig. 1. Northern blot analysis of *P. brasiliensis sod3* transcription following *in vitro* treatment with menadione and H$_2$O$_2$. Equal amounts of total RNA from *P. brasiliensis* yeast cells treated with 50 mM menadione (A) or 50 Mm H$_2$O$_2$ (B) for different periods of time were electrophoresed, blotted and probed with PCR-amplified gene fragment for *sod3*. Hybridization signal intensities were quantified using the ethidium bromide stained 18S ribosomal RNA as a control of sample load.

the present study, the gene encoding β-glucan synthase (*fks*) was among those *P. brasiliensis* genes that were down-regulated in response to the macrophage environment. It has been shown for *P. brasilieneis* [20] and other fungi [21], that virulence of different isolates in experimental mouse infection is related to the relative amounts of α-glucan to β-glucan present in the cell wall of these fungi (i.e., the cell wall of avirulent yeast isolates contains less α-glucan and more β-glucan). The apparent mechanism of β-glucan host protection may be related to its capacity to stimulate the production of several inflammatory mediators, including tumor necrosis factor alpha (TNF-α). Specifically, purified β-glucan from the *P. brasiliensis* cell wall elicits the production of TNF-α in murine and human macrophages and has been employed as an immunostimulant in the treatment of human PCM [16,17]. Since TNF-α is a pivotal cytokine involved in leukocyte migration, macrophage activation and has been associated with resistance to *P. brasiliensis* [3,22], our results suggest that *fks* gene down-regulation may be an important adaptative mechanism of *P. brasiliensis* to reduce the inflammatory response elicited by its β-glucan. Such a mechanism may be involved in the well characterized ability of this fungus to remain latent for long periods of time [1].

After internalization, macrophages expose pathogens to a group of toxic antimicrobial molecules, including reactive oxygen intermediates (ROI) generated by the phagocyte NADPH oxidase system, and reactive nitrogen intermediates (RNI) generated by the inducible nitric oxide synthase (iNOS). It has been shown that fungicidal mechanism against *P. brasiliensis* is dependent on iNOS-generated RNI, since inhibitors of iNOS activity precluded this process [23]. In addition, iNOS2 gene-disrupted mice are highly susceptible to *P. brasiliensis* infection [24]. On the other hand, the role of ROI in host protection in PCM seems to be less important. *P. brasiliensis* is relatively resistant to killing by ROI *in vitro* [25] and survive within nonactivated macrophages and neutrophils, where the oxygen radicals could be expected to have a more prominent role [2,3,25]. In this sense, the significant

production of O$_2^-$ and H$_2$O$_2$ by *P. brasiliensis* infected macrophages corroborates the idea that an efficient adaptive system for detoxification of NADPH oxidase-dependent oxidative burst products may be operating in this fungi in order to promote its survival within phagosomes. In fact, our microarray data revealed two oxidative stress related genes (*sod3* and *hsp60*) that were up-regulated in phagocytized *P. brasiliensis*. Further, genes related to the cytochrome electron transport system, which is important for ROI detoxification, were also induced.

The most significantly induced gene in our microarray analysis, *sod3*, encodes for a putative Cu,Zn SOD, which is an enzyme involved in the elimination of superoxide anions. The importance of Cu,Zn SOD in fungal virulence and viability has been recently addressed. *C. albicans* cells lacking *sod1*, which encodes the cytoplasmatic localized SOD, are unable to survive the fungicidal attack of a macrophage cell line and have attenuated virulence in a mouse model of infection [26]. Similar results were obtained with *C. neoformans* [27], implicating SOD in protection against host-derived oxygen radicals. *In silico* analysis showed that the *P. brasiliensis sod3*-deduced amino acid sequence corresponds to a putative membrane GPI-anchored Cu,Zn SOD [12], which would provide a better accessibility to host-derived superoxide anions and subsequently rapid detoxification of the ROI. This idea is reinforced by *C. albicans* GPI-anchored Cu,Zn SOD gene, which is induced *in vitro* by oxidative species generators and upon internalization by neutrophils [13,14]. Furthermore, null mutants of *C. albicans sod5* are more sensitive to neutrophils and mouse killing [14]. In this context, the up-regulation of *sod3* expression in internalized *P. brasiliensis* and *in vitro* menadione treated cells provide evidences that *sod3* may be needed for the elimination of exogenously and endogenously generated superoxides. Additionally, as described to other fungi [28,14], we also demonstrated that *in vitro* H$_2$O$_2$ treated *P. brasiliensis* yeast cells showed *sod3* gene induction. The major role of SOD activity, which converts O$_2^-$ into H$_2$O$_2$ and molecular oxygen, is the avoidance of O$_2^-$ dependent

Fe^{+3} to Fe^{+2} reduction. The ferrous iron reacts with H$_2$O$_2$ generating the highly toxic hydroxyl radical via Fenton reaction. In this sense, we believe that *sod3* gene induction by H$_2$O$_2$ should protect *P. brasiliensis* from hydroxyl radical production, limiting further oxidative damage.

HSP60 is a mitochondrial chaperone, homologous to *Escherichia coli* GroEL, that promotes the correct folding of several proteins imported into the mitochondrial matrix [29]. In addition to its function as a chaperone, HSP60 is involved in cellular responses to oxidative stress [15]. Mutant strains of *S. cerevisiae* producing various amounts of this protein were exposed to menadione and H$_2$O$_2$. It was observed that cells presenting higher levels of HSP60 were more resistant to these oxidative agents. The HSP60 protective role was related to its ability to block the release of reduced ferrous iron from the oxidation of mitochondrial proteins, thus limiting the Fenton reaction [15]. The induction of *hsp60* gene in *P. brasiliensis* exposed to the oxidative radicals produced by macrophages may have a similar protective role. In addition, GroEL was among the most highly up-regulated genes during *L. monocytogenes* infection of murine macrophages [5], suggesting a conserved strategy in fungi and bacteria for intracellular host cell survival.

Our study provides the first gene expression analysis of *P. brasiliensis* response to the intracellular environment of murine macrophages. The differentially expressed genes identified in this report are potential targets for molecular studies of virulence in an experimental infection model. Only recently have molecular tools necessary for such studies become available [30], which may help unravel in the near future some secrets of this important fungal pathogen.

Acknowledgments

Appendix A. Supplementary Material

Supplementary material can be found, in the online version, at doi:10.1016/j.micinf.2007.01.024.

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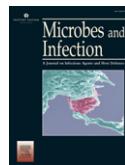
Original article

Transcriptional response of murine macrophages upon infection with opsonized *Paracoccidioides brasiliensis* yeast cells

Simoneide S. Silva [a,f,1], Aldo Henrique F.P. Tavares [a,f,1], Danielle G. Passos-Silva [b,f], Ana L. Fachin [e,f], Santuza M.R. Teixeira [b,f], Célia M.A. Soares [e,f], Maria José A. Carvalho [a,f], Anamélia L. Bocca [a,f], Ildinete Silva-Pereira [a,f], Geraldo A.S. Passos [c,d,f], S. Felipe Maria Sueli [a,f,*]

[a] *Departamento de Biologia Celular, Universidade de Brasília, Brasília, DF 70910-900, Brazil*
[b] *Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil*
[c] *Departamento de Genética, Universidade de São Paulo, Ribeirão Preto, SP 14040-900, Brazil*
[d] *Faculdade de Odontologia, Universidade de São Paulo, Ribeirão Preto 14040-900, SP, Brazil*
[e] *Departamento de Bioquímica, Universidade Federal de Goiás, Goiânia, GO 74001-970, Brazil*
[f] *Unidade de Biotecnologia, Universidade de Ribeirão Preto, Ribeirão Preto, SP 14096-380, Brazil*

Abstract

Paracoccidioides brasiliensis is the etiologic agent of the Paracoccidioidomycosis the most common systemic mycosis in Latin America. Little is known about the regulation of genes involved in the innate immune host response to *P. brasiliensis*. We therefore examined the kinetic profile of gene expression of peritoneal macrophage infected with *P. brasiliensis*. Total RNA from macrophages at 6, 24 and 48 h was extracted, hybridized onto nylon membranes and analyzed. An increase in the transcription of a number of pro-inflammatory molecules encoding membrane proteins, metalloproteases, involved in adhesion and phagocytosis, are described. We observed also the differential expression of genes whose products may cause apoptotic events induced at 24 h. In addition, considering the simultaneous analyses of differential gene expression for the pathogen reported before by our group, at six hours post infection, we propose a model at molecular level for the *P. brasiliensis*-macrophage early interaction. In this regard, *P. brasiliensis* regulates genes specially related to stress and macrophages, at the same time point, up-regulate genes related to inflammation and phagocytosis, probably as an effort to counteract infection by the fungus.
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Keywords: Paracoccidioides brasiliensis; Macrophage; cDNA microarray; Host-pathogen interaction

1. Introduction

Paracoccidioides brasiliensis is a facultative intracellular fungus that causes Paracoccidioidomycosis (PCM), an important endemic disease in Latin America, especially in Brazil

* Corresponding author : Laboratório de Biologia Molecular, Departamento de Biologia Celular, Universidade de Brasília-Unb Brasília, Campus Darcy Ribeiro-Asa Norte, Brasília, DF 70910-900, Brazil. Tel.: +55 61 307 2423; fax: +55 61 349 8411.

E-mail address: msueli@unb.br (S.F. Maria Sueli).

[1] S.S.S and A.H.F.P.T contributed equally to this work.

[1]. Animal models suggest that the natural infection route is by inhalation of airborne conidia derived from the mycelial saprophytic form of the fungus [2]. Once in the lungs it undergoes a thermally controlled dimorphic transition to the yeast form, which is an essential step for the establishment of the infection [3].

As observed in other systemic mycoses, the most relevant defence of the host against PCM is the cellular immunity mediated mainly by γ-interferon activation of macrophages [4]. Conversely, the observation of susceptible hosts has revealed that non-activated macrophages serve as a protected environment

wherein the fungus can survive and replicate, thus leading to fungal dissemination from the lungs to other organs [5].

Despite their important role in PCM, no high-throughput analysis of host cell genes that be modulated upon infection has been reported for *P. brasiliensis* pathogen. Recently, our group analysed by cDNA microarray technology the differential gene expression of *P. brasiliensis* recovered from macrophages upon six hours of infection [6]. However, whole lung or macrophage gene expression profiles in response to infection by *P. brasiliensis* were limited to some genes coding for cytokines and host adhesion molecules [7,8]. This is in contrast to other models of microbial infection, in which numerous studies employing high-throughput genomics have been carried out to assess large-scale changes in the transcriptional profiles of both host and pathogen in their complex interaction. As shown for bacteria and in fungus-phagocyte interactions, microarray technology enables the measurement of expression levels for hundreds to thousands of genes, providing a global understanding of key effectors related to pathogen virulence and host defence can emerge [9–13]. In the present study, we have evaluated the kinetic profile of macrophage gene expression after exposure to *P. brasiliensis* yeast cells for 6, 24 and 48 h. We have been able to identify a total of 118 genes that are differentially expressed at least one time point. Those genes are related to several immune processes such as inflammation, cell membrane, transcriptional regulation, signal transduction and apoptosis. In addition we discuss the host-pathogen interaction in the light of recent results on *P. brasiliensis* gene expression patterns upon phagocytosis by peritoneal macrophages [6]. Thus our data add to the current understanding of early host gene expression upon infection by *P. brasiliensis* and suggest an interaction model for *P. brasiliensis*-infected macrophages.

2. Materials and methods

2.1. Isolation, cultivation and infection of murine peritoneal macrophages with P. brasiliensis yeast cells and total RNA extraction of host cells

The clinical isolate of *P. brasiliensis*-Pb01 (ATCC-MYA-826) was used for macrophages infection. Isolation, cultivation and infection of murine peritoneal macrophages protocol were carried out as previously described [6]. Cultures were incubated at 37 °C under a humidified, 5% CO_2 atmosphere. At 6, 24 and 48 h upon infection, extracellular fungi were removed by exhaustive washing with RPMI pre-warmed to 37 °C. Macrophages were then lysed and total RNA was extracted with the Trizol® reagent (Invitrogen, USA). Total RNA from control peritoneal macrophages was also extracted using the same protocol. The infection of macrophages with yeast *P. brasiliensis* was repeated three times for each point of co-cultivation, the macrophage RNA was extracted individually and pooled in order to use it in the cDNA microarrays and Real Time-PCR analysis. For cytokine dosage, peritoneal macrophage cells (5×10^5) and serum-opsonized fungal cells (1×10^5) were prepared and incubated as above. At 6, 24 or

48 h after infection, supernatants were collected and stored at −20 °C.

2.2. Construction and hybridization of cDNA microarrays and data analysis

Mouse expressed sequence tags (ESTs) cDNA clones were obtained from thymus 2NbMT normalized library and available in the I.M.A.G.E. Consortium (http://image.llnl.gov/image/html/iresources.shtml). cDNA inserts were cloned in three vectors (pT7T3D, pBluescript and Lafmid) and were amplified in 384- or 96-well plates using vector-PCR amplification with the following primers, which recognize the three vectors, LBP 1S 5′-GTGGAATTGTGAGCGGATACC-3′ forward and LBP 1AS 5′-GCAAGGCGATTAAGTTTGG-3′ reverse. Distilled water and a poly $(A)_{80}$ DNA segment were used as negative controls. The products were then spotted in duplicate onto 2.5×7.5-cm Hybond® N^+ nylon membranes (GE Health Care, USA) with a Generation III Array Spotter (GE Healthcare, USA). A complete file providing all genes present in the microarrays used in this study is available online at (http://rge.fmrp.usp.br/passos/nylon_array/mtb1). Each microarray contained 624 murine genes known to be implicated in immune processes, with a predominance of those involved in the innate response. The membranes were first hybridized with the LBP 1AS [γ-^{33}P]dCTP labeled oligonucleotide. Upon vector probe stripping membranes were used for hybridization against [α-^{33}P]dCTP-labelled (GE Health Care, USA) cDNA complex probes. Microarray experiments were carried out in triplicate for each time and for infected and non-infected conditions. After normalization, the Significance Analysis of Microarrays software (SAM- http://www-stat.standord.edu/~tibs/SAM) were used to assess the significant variations in gene expression between experimental and control conditions. Significantly modulated genes were identified by passing both a statistical (*q* value <5%; FDR <5%) and a fold-variation (≥1.5-fold up or down) cutoff. Microarray data have been deposited in ArrayExpress under accession numbers A-MEXP-744 and E-MEXP-1093.

2.3. Real-time RT-PCR validation of differentially modulated genes

The same RNA samples used for microarray experiments were used for Real-time RT-PCR. The comparative CT (crossing threshold) method, employing the constitutive ribosomal Rps9 macrophage gene was used in order to normalization (amplified by the primer pair forward 5′-CGCCAGAAGCTG GGTTTGT-3′ and reverse 3′-CGAGACGCGACTTCTCGA A-5′) of the expression value (fold-change) of each gene of interest in the macrophage infected sample in comparison to the non-infected control. Other primers used included those for *Clec1b* (forward 5′- CTCTTCTTGGTGGCGTGTGA-3′, reverse 3′- AACAACCAGCCCCATGGA-5′), *Nfkb* (forward 5′ AGCCAGCTTCCGTGTTTGTT-3′, reverse 3′-AGGGTTTCG GTTCACTAGTTTCC-5′), *Nkrf* (forward 5′ ACCTTTCAAC CTACGATGGTCAGA-3′, reverse 3′- GAGCTCTCACATGG

3

AATTTGGAA-5′), and *Tnf-α* (5′-GTACCTTGTCTACTCC CAGGTTCTCT-3′, reverse 3′-GTGGGTGAGGAGCACGTA GTC-5′). Real time RT-PCR experiments were done in duplicate for two times for all analyzed genes. All primer pairs were based on the sequences obtained from the mouse transcriptome database (http://www.informatics.jax.org) and designed with the Primer Express software (Applied Biosystems).

2.4. Cytokine quantification

Supernatants from 6, 24 and 48 h of incubation were frozen and stored at −20 °C and thawed immediately prior to each assay. Secreted TNF-α and IL-12 was assayed by a commercial ELISA kit (Pharmigen). Statistical analysis were done using one-way ANOVA with Dunnett's post test and performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA.

3. Results

3.1. Expression modulation of macrophages genes upon infection with P. brasiliensis

Differential gene expression was assessed on peritoneal macrophages after 6, 24 and 48 h of infection with *P. brasiliensis*.

Three independent cDNA microarrays experiments for each time were performed, and the transcript levels of 624 genes involved in the immune response were investigated. Analysis of macrophage gene expression data showed significant changes on expression of 273 genes upon infection with *P. brasiliensis*, as evaluated by the SAM program, but only those genes passing a fold-variation cut-off (≥1.5-fold up or down) were considered for further analysis. Such criteria reduced the number of differentially expressed genes to 118 (105 induced and 13 repressed), when comparing infected to non-infected macrophage at the aforementioned time points. The majority of differentially expressed genes were found at 24 h of infection with *P. brasiliensis*.

Table 1 lists the total of 28 up-regulated genes for the six-hour incubation time, which consists in the early macrophage infection events. Among them, the pro-inflammatory genes include Irak2, Il7r, Ccl21, Ccl22 and Cxcl1 cytokine-related proteins. Genes encoding membrane-associated proteins were Clec1b, Cd8 and Mmp17. Matrix metalloproteinases (MMPs) are a class of related, yet structurally distinct, zinc-dependent enzymes. They are known to be involved in the cleavage of cell-surface receptors and chemokine in/activation [14]. Up-regulated genes related to transcriptional control were Ier5, Ccr4-not subunit 10 and Stat1. Activation of signal transduction genes was represented by Grb2, Cd37 antigen

Table 1
Up-regulated macrophage genes after 6 h of infection with *P. brasiliensis* fungus

Category	Description	Gene	Clone ID[a]	Fold change
Inflammation	Similar to interleukin-1 receptor-associated kinase 2	Irak2	6474954	1.74
	Interleukin 7 receptor	Il7r	578171	2.19
	Chemokine (GC motif ligand 21a (leucine))	Ccl21	576394	1.94
	Chemokine (C-C motif ligand22)	Ccl22	577486	2.20
	Chemokine (C-X-C motif ligand 1)	Cxcl1	5321155	1.85
	Histocompatibility 2, class II antigen E beta	H2-Eb1	574155	2.51
	CD28 antigen	Cd28	576501	2.20
Membrane proteins	C-type lectin-like receptor 1, member b	Clec1b	574821	4.63
	CD8 antigen, alpha chain	Cd8	1247019	1.61
	Matrix metalloproteinase 17	Mmp17	30850	1.84
	Lymphocyte antigen 6 complex, locus A	Ly6a	581749	2.39
Transcriptional regulation	Immediate early response 5	Ier5	574793	1.83
	CCR4-NOT transcription complex, subunit 10	Cnot10	576406	2.33
	Signal transducer and activator of transcription 1	Stat1	583302	1.70
Signal transduction	Growth factor receptor bound protein 2	Grb2	575387	2.64
	CD37 antigen	Cd37	640080	1.56
	RAS p21 protein activator 3	Rasa3	582174	1.56
	T-complex protein 1	Tcp1	640974	2.39
Apoptose	Granzyme A	Gzma	572830	1.56
Transferase	Serine hydroxymethyl transferase 1 (soluble)	Shmt1	573743	1.82
	Protein O-fucosyltransferase 2	Pofut2	575950	1.59
Other functions	Gamma-glutamyl carboxylase	Ggcx	582706	1.55
	X-ray repair	Xrcc4	574242	1.59
	RNA binding motif protein 4B	Rbm4B	575510	1.80
	Arsenate reductase activity thioredoxin	—	582490	1.82
	DNA segment, Chr2	D2Ertd	575429	2.37
	Transcribed sequences	—	582106	1.91
	Transcribed sequences	—	640795	1.99

[a] Gene identification in SOURCE genome database (http://source.stanford.edu/).

and Rasa3. An induced expression has been observed for the apoptosis-related Granzyme A and the redox activation-related arsenate reductase thioredoxin. Three induced genes had no known functions. No down-regulation of genes was observed at this time point by our microarray analysis under the established statistical significance criteria.

At 24 h of macrophage infection, 59 genes were found to be differentially expressed being 54 induced and 5 repressed (Table 2). The gene encoding the cytokine Ccl21 and other inflammation-related genes such as Irak2, Pparbp and Ly86 were induced. Up-regulated genes involved in transcriptional regulation were the Stat1 inhibitor, Ccr4-not, Tcf12, Foxo1, and Nfkb. Some of the pro-inflammatory genes regulated by NFk-B include several chemokines, all of which can enhance inflammation by attracting additional inflammatory cells to the site of injury [15]. Nfk-B protein also regulates the transcription of its inhibitor Nkrf, which is also induced at 24 h according to our experiment. Genes encoding membrane-related proteins Clec1b and Ddr1, which are related to the effectiveness of phagocytosis, were induced as well as the complement components C2 and C3. The most important events in macrophage activation are mediated by signal transduction pathways and several genes implicated in this process were induced, including Mapk1, Mapk8ip3, Mapk8, Txk and Rab3d. Apoptosis is an innate host defence mechanism used to prevent proliferation of internalized microorganisms. At this time point five apoptosis-related genes were up-regulated, including those that encode pro-apoptotic caspases 2, 3 and 8 and the apoptotic inhibitors Faim2 and Casp8r.

At 48 h of infection, 31 genes were found to be differentially expressed, of whom 23 induced, 8 repressed (Table 3). The pro-inflammatory up-regulated genes included Irak2, Cxcl14 and Cxcl4. Among the induced genes related to membrane were Clec1b, Cd14, H2eb, Ly6a and Adam8. Signal transduction-related genes induced were Mapkk11, Txk and SH3-domain GRB2-like1 (Sh3gl1). At this time point we observed a higher number of down regulated genes from the following functional categories: inflammation (Ccl2), transcription regulation (Pias1i, solute carrier family 7, member 11 and RlKEN full-length enriched library) and signal transduction (Rab2 and microtubule affinity-regulatin kinase 2).

3.2. Validation of genes involved in immune response

In order to validate our microarray data, we performed Real time RT-PCR with the same pooled RNA samples from three independent experiments of macrophage infection that were used for microarray experiments. We chose to focus on the validation of three key genes that are involved in the immune response (Nfkb, Nkrf, and Clec1b) that were differentially expressed following infection with *P. brasiliensis*. Nfkb and its repressor Nkrf are regulators of inflammatory process. Clec1b is important for a more effectiveness phagocytosis by macrophages. Table 4 shows that expression profile of all these genes when analysed by Real-time RT-PCR, correlated with the profile observed in the microarray experiments. The magnitude of fold-changes differed between the methodologies, a result expected due to their technical differences regarding normalization protocol, kinetics and sensitivity.

3.3. TNF-α quantification during macrophage infection by P. brasiliensis

TNF-α itself is associated with the antimicrobial activity of macrophages and it is important cytokine in mycosis controlling [16−18]. TNF-α expression profile was analysed by Real time RT-PCR associated to ELISA-dosing, since it was not included in the cDNA microarrays membranes. Nevertheless, this did not eliminate the possibility of assessing the expression profile of this relevant cytokine. In this regard, it has been found that TNF-α has a fold up-regulation of 3 times at 24 h of infection. In addition we have confirmed by ELISA that mRNA induction resulted also in an increase of protein secretion. We observed at 6 h discrete increase of TNF-α while at 24 and 48 h of infection this cytokine was strongly secreted by infected macrophages when compared to non-infected ones (Fig. 1).

4. Discussion

In order to better understand the complex interaction between host cells and *P. brasiliensis* we have analysed the transcriptional profile of 624 previously selected murine immuno-regulatory genes following fungal infection of peritoneal macrophages for 6, 24 and 48 h. To our knowledge, this study is the first to assess the coordinated gene expression changes that occur in this innate host defence cell in response to *P. brasiliensis*. This time-lapse characterization provides a more encompassing view of events than would an analysis from a sole time point. A major concern in setting the experimental framework was the choice between opsonised and non-opsonised yeast cells. A major concern in setting the experimental framework was the choice between opsonised and non-opsonised yeast cells. When we set out to establish the co-cultivation protocol of *ex vivo* infection of macrophages with non-opsonised cells, we observed that both adhesion and internalization of *P. brasiliensis* occurred at low frequency and few numbers of macrophages containing adhered and/or internalized yeast cells. Then we decided to use opsonised cells that yielded rates of phagocytosis that allowed performing the microarrays experiments.

Our microarray data have identified many genes that are modulated in particular those related to inflammatory mechanisms, membrane proteins, transcription regulation and apoptosis. Inflammation is a powerful protective mechanism coordinated and controlled by cytokines and chemokines. Increased chemokine gene expression has been detected by microarray analysis in macrophages infected with, *Candida albicans*, *Aspergillus fumigatus* and *Mycobacterium tuberculosis* [12,13,19]. Accordingly, our results show that genes encoding the chemokines Ccl21, Ccl22 and Cxcl1, all of which are associated with pro-inflammatory response, were significantly increased in peritoneal macrophages in response to *P. brasiliensis*-Pb01, at the early stage of infection. It is relevant to

Table 2
Modulated macrophage genes after 24 h of infection with *P. brasiliensis* fungus

Category	Description	Gene	Clone ID[a]	Fold change
Up-regulated genes				
Inflammation	Chemokine (GC motif ligand 21a (leucine))	Ccl21a	576394	10.87
	Similar to interleukin-1 receptor-associated kinase 2	Irak2	6474954	1.71
	Peroxisome proliferator activated receptor binding protein	Pparbp	582634	24.58
	Lymphocyte antigen 86	Ly86	583305	5.88
	CD37 antigen	Cd37	640080	2.00
	Linker for activation of T cells	Lat	582840	2.05
	Complement component 2 (within H-2S)	C2	583642	3.47
	Complement component 3	C3	582886	1.80
Membrane proteins	C-type lectin-like receptor 1, member b	Clec1b	574821	1.80
	Discoidin domain receptor family, member 1	Ddr1	575615	3.11
	Matrix metalloproteinase 17	Mmp17	30850	1.55
	Selectin, lymphocyte	Sell	621878	4.76
	SHP2 interacting transmembrane adaptor	Sit1	576098	1.65
	CD8 antigen, alpha chain	Cd8	1247019	2.06
	T-cell receptor gamma, constant region	TcrgC	640129	1.58
Transcriptional regulation	Protein inhibitor of activated STAT 1	Pias1	577047	1.89
	CCR4-NOT transcription complex, subunit 2	Cnot2	572999	3.46
	Transcription factor 12	Tcf12	575368	2.62
	Forkhead box O1	Foxo1	574862	19.41
	P50 subunit of NFKappaB	Nfkb	575033	23.47
	NFKappaB repressing factor	Nkrf	21961	6.02
	Lymphoid enhancer binding factor 1	Lef1	575374	1.85
Signal transduction	Mitogen activated protein kinase 1	Mapk1	574250	1.73
	Mitogen-activated protein kinase 8 interacting protein 3	Mapk8ip3	576539	1.79
	Mitogen activated protein kinase 8	Mapk8	576631	3.06
	TXK tyrosine kinase	Txk	583533	1.67
	RAB3D, member RAS oncogene family	Rab3d	575981	1.52
Apoptose	Caspase 2	Casp2	573760	1.62
	Caspase 3, apoptosis related cysteine protease	Casp3	581767	1.68
	Caspase 8	Casp8	5099113	1.54
	Fas apoptotic inhibitory molecule 2	Faim2	37367	1.52
	CASP8 and FADD-like apoptosis regulator	Cflar	640265	1.54
Transferase	Methyltransferase 11 domain containing 1	Mett11d1	576502	13.57
	Methyltransferase-like 3	Mettl3	575022	3.85
	Protein O-fucosyltransferase 2	Pofut2	575950	1.64
Other functions	Splicing factor, arginine/serine-rich 1	Sfrs1	582744	1.54
	Arsenato reductase activity	—	582490	1.50
	Cyclin D3	Ccnd3	575230	1.66
	Gamma-glutamyl carboxylase	Ggcx	582706	1.66
	Kelch repeat protein	Kbtbd8	582871	1.73
	SUMO 1 activating enzyme subunit 2	Sae2	576467	2.20
	Damage-specific DNA binding protein I, 127 kDa	Ddb1	4823153	1.65
	Differentially expressed in FDCP 6	Def6	573651	2.1
	Ankyrin repeat domain 27	Ankrd27	575492	1.59
	Transcribed locus	—	583763	7.30
	Transcribed locus	—	641074	2.22
	Transcribed sequences	—	581675	2.34
	Transcribed sequences	—	575134	1.80
	Transcribed sequences	—	576644	1.53
	Transcribed sequences	—	582394	2.04
	Transcribed sequences	—	576140	1.63
	Transcribed sequences	—	576430	4.47
	Transcribed sequences	—	640779	1.98
	In multiple clusters	—	574242	1.71
Down-regulated genes				
Inflammation	Chemokine (C-X-C motif ligand 4)	Cxcl4	573339	0.26
	Chemokine (C-C motif ligand 6)	Ccl6	576828	0.52

(*continued on next page*)

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Table 2 (*continued*)

Category	Description	Gene	Clone ID[a]	Fold change
Other functions	Nuclear casein kinase and cyclin-dependent kinase substrate 1	Nucks1	573338	0.19
	F.box and leucine-rich repeat protein 5	Fbox5	575998	0.46
	Lymphocyte antigen 6 complex, locus D	Ly6d	581909	0.46

[a] Gene identification in SOURCE genome database (http://source.stanford.edu/).

mention that different gene expression patterns of cytokines IL-6, IL-10, IL-8 and TNF-α have also been reported by different strains of *P. brasiliensis* such as Pb18 and Pb265 [20,21]. Cxcl1 and Ccl22 are neutrophil and monocyte chemoattractants, respectively. Induction of genes that code for other neutrophil (KC and CCL3) and monocyte (CCL5, CCL2, CXCL10 and CXCL9) attractants were reported in the lung of mice infected with *P. brasiliensis*; this observation was correlated with an intense recruitment of phagocytes to the site of infection [22]. Despite being included in the microarray membrane, IL-12 was found not to be differentially expressed at any of the time points, which led us to confirm this finding by ELISA. Corroborating this, it was also not detected in the supernatant of infected macrophages. *P. brasiliensis* is likely to inhibit IL-12 production as a means to evade the innate and adaptive responses set up by the host. These data are in good agreement with similar analyses on other pathogens such as *Mycobacterium tuberculosis* [8] and *Histoplasma capsulatum* [23], which also suppress IL-12. This is presumably an instance of convergence of pathogen survival strategy inside macrophages. The expression of genes encoding cytokine receptor-associated proteins has also been evaluated at the present work. In this regard, we have detected an up-regulation of IL-7 receptor in early infection (6 h), which participates in the pro-inflammatory cytokines in monocytes and macrophages [24]. It follows that the induction of IL-7r is probably

Table 3

Modulated macrophage genes after 48 h of infection with *P. brasiliensis* fungus

Category	Description	Gene	Clone ID[a]	Fold change
Up-regulated genes				
Inflammation	Similar to interleukin-1 receptor-associated kinase 2	Irak2	6474954	1.64
	Chemokine (C-X-C motif ligand 14)	Cxcl14	583442	1.58
	Chemokine (C-X-C motif ligand 4)	Cxcl4	573339	1.52
Membrane proteins	C-type lectin-like receptor 1, member b	Clec1b	574821	2.66
	CD14 antigen	Cd14	5120996	1.91
	Histocompatibility 2, class II antigen E beta	H2-Eb	574155	1.63
	Lymphocyte antigen 6 complex, locus A	Ly6a	581749	1.67
	A disintegrin and metalloprotease domain 8	Adam8	582054	1.51
	Beta-2 microglobulin	B2m	576472	2.73
	Beta-2 microglobulin	B2m	576493	3.11
Signal transduction	Mitogen activated protein kinase kinase kinase 11	Mapkkk11	575211	3.00
	TXK tyrosine kinase	Txk	583533	1.61
	SH3- domain GRB2-like1	Sh3gl1	582991	1.69
	Twin filin, actin-binding protein, homolog 2	Twf2	640339	1.80
Other functions	Serine (or cysteine) proteinase inhibitor, clade H, member 1	Serpinh1	582567	1.98
	13 days embryo head cDNA	−	575324	1.73
	Aryl hydrocarbon receptor nuclear translocator-like	Arntl	582931	1.93
	B-cell translocation gene I, anti-proliferative	Btg1	574654	1.95
	Arsenate reductase (thioredoxin) activity	−	582490	2.75
	Cytochrome b-245, beta polypeptide	Cybb	583187	2.05
	Transcribed locus similar heat shock 27 kDa protein 1	−	582517	2.45
	Transcribed sequences	−	582338	2.12
	Transcribed sequences	−	575870	1.69
Down-regulated genes				
Inflammation	Chemokine (GC motif ligand 2)	Ccl2	573898	0.33
Transcriptional regulation	Protein inhibitor of activated STAT 1	Pias1	577047	0.47
	Solute carrier family, member 11	S7a11lc	575418	0.50
	RIKEN full-length enriched library	−	576643	0.29
Signal transduction	RAB2, member RAS oncogene family	Rab2	573835	0.50
	MAP/microtubule affinity-regulating kinase 2	Mark2	574316	0.36
Other functions	STT3, subunit of oligosaccharyltransferase complex	Stt3b	573897	0.36
	Insulin degrading enzyme	Ide	577628	0.37

[a] Gene identification in SOURCE genome database (http://source.stanford.edu/).

Table 4
Real-time RT-PCR validation of microarray data

Clone ID	Gene	Fold change[a]	
		Microarray	qRT-PCR[b]
574821	Clec2*	4.63	4.5
574821	Clec2***	2.65	1.7
575033	Nfk-b**	23.47	1.4
21961	Nkrf**	6.02	3.1

[a] Fold change values were determined after normalization of each gene to the Rps9 gene by using the comparative threshold method.
[b] Mean fold change of two qRT-PCR independent experiments done in duplicate, at *6 h, **24 h and ***48 h.

related to macrophage activation in response to early infection with *P. brasiliensis*, presumably in order to enhance the phagocytic and fungicidal properties of that cell. Also, we have observed an up-regulation of Irak2 in macrophages infected with *P. brasiliensis* at all time points analysed. One recently published microarray study has also shown an up-regulation of this gene in human monocytes at 4 h of infection with *A. fumigatus* [13]. The IRAK2 protein binds to the IL-1 type I receptor upon IL-1 attachment; this triggers intracellular signalling cascades leading to transcriptional up-regulation, with the Nf-κb gene as one of its targets [25]. Nf-κb gene expression leads to the transcription of a wide variety of immune response genes, including pro-inflammatory cytokines and chemokines such as TNF-α, which in turn activate components of the innate immune response by recruiting inflammatory cells to the initial sites of infection [15]. Consistently with this, we have observed in our study model an induction of the Nf-kb gene and similar observations have been made by microarray analyses of macrophages infected with *Brucella abortus*, *M. tuberculosis*, *Mycobacterium bovis* and *Salmonella typhi* [9,10,19]. TNF-α acts as an autocrine inductor and provides a second signal that induces cytotoxic activity in IFN-γ activated-macrophages by stimulating NO production [26].
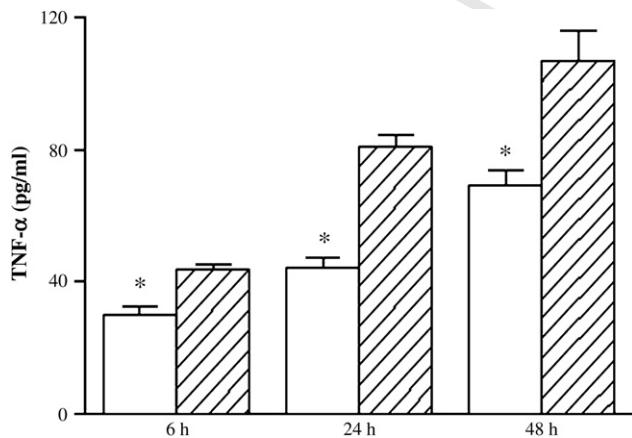
Fig. 1. TNF-α release by peritoneal macrophage in culture. Macrophages were infected with *P. brasiliensis* − Pb01. Culture supernatants were harvested after 6, 24 and 48 h. Cytokine concentrations were measured by ELISA. One-way ANOVA with Dunnett's post test was performed for three independent experiments. The open bars represents the non-infeted cells (□) and hachured bars infected cells (▨). *$p < 0.01$ when compared to not infected macrophages.

TNF-α protein was increased at all times of macrophage infection with *P. brasiliensis* as shown in this work. Nevertheless, induction of the gene was observed just at the 24-htime point. It follows that the protein measurement at six hours is probably a result of post-transcriptional regulation. This cytokine has been reported to play an important role in controlling fungal infections [16,17]. A co-culture of hamster peritoneal macrophages and *P. brasiliensis* [27] presented high levels of TNF-α, which were correlated with an increase in macrophage activation and in fungicidal activity and a check on the dissemination of the fungus. In good keeping with these findings, TNF-α deficient mice were incapable of setting a granulomatous response to *P. brasiliensis* and of controlling infection by this fungus [17].

Macrophage genes encoding membrane proteins involved in adhesion and phagocytosis were also induced by *P. brasiliensis*. In this context, we have observed significant induction of Cd14 and Clec1b. CD14 is a glycosyl-phosphatidylinositol-anchored glycoprotein expressed on leukocytes and is the major receptor responsible for the effects of LPS and various bacterial products such as peptidoglycan and lipoarabinomannan on macrophages, monocytes, and neutrophils. Previous studies have shown the involvement of CD14 in the recognition of fungi by macrophages. Induction of the Cd14 gene in macrophages engaged at phagocytising different strains of *M. avium* at 6 and 24 h of infection have been also observed [11]. This differential expression pattern is probably modulated by the pathogen and its ultimate result is an improved effectiveness of invasion and/or macrophage activation. In addition, the gene for the C-type lectin-like receptor 1b (Clec1b), which is also membrane-related, was induced at all times of macrophage infection with *P. brasiliensis*, which has been confirmed by real-time RT-PCR for the six- and 48-h time points. Clec1b is able to recognise mannose residues present on the surface of a wide spectrum of microorganisms, including *P. brasiliensis* [28]. Recently, additional evidence of its importance for the phagocytosis of *P. brasiliensis* has been obtained by the treatment of macrophages with an analogous methyl-mannoside that reduced the frequency of internalized fungus [29]; in this context, our results are in accordance with this report showing that the up-regulation of Clec1b is probably important for a more effective fungal internalization. Additionally, an induction of other cellular adhesion molecules (ICAM-1, VCAM-1, CD18 and Mac-1) in lungs of mice infected with *P. brasiliensis* have been observed participating in the inflammatory process and therefore in the pathogenesis of paracoccidiodomycosis [7,8]. Genes encoding for the related metalloproteases Mmp17 and Adam8 were significantly induced in peritoneal macrophages in response to *P. brasiliensis*. Matrix metalloproteases are known to be involved in tissue remodelling, cleavage of cell-surface receptors and chemokine activation [14]. Microarray analyses of macrophage infected by different pathogens such as *M. tuberculosis*, *M. bovis*, *S. typhi* and *A. fumigatus* have revealed an up-regulation of the genes encoding the metalloproteases MMP1, MMP9, MMP10 and MMP14 [9,10,13]. Considering their important role in collagen and cellular matrix remodelling, it

has been suggested that induction of these metalloproteases might be an important mechanism to facilitate subsequent fungal invasion. Thus, the up-regulation of Mmp17 and Adam8 observed probably increase the effectiveness of host cell parasitism. These data fit with the above described induction of adhesion and phagocytosis-related genes, since *P. brasiliensis* is a facultative parasite that can survive and replicate in non-activated macrophages [5]. Both an increased phagocytosis (Cd14 and Clec1b) and the stimulation of matrix remodelling (Mmp17 and Adam8) are expected to contribute to a more efficient invasion by the fungus of cell and tissue alike.

Apoptosis plays a significant role in the regulation of pathogenesis. Several genes involved in the induction of cell death apoptosis have been found to be responsive and up-regulated in *P. brasiliensis* at 24 h of infection, including caspases 2, 8 and 3. However, the genes coding for Caspase inhibitor 8 and Fas inhibitor were also induced in our model. Induction of apoptosis by microorganisms may allow evasion of the macrophage response. Conversely, resistance by macrophage to organism-induced apoptosis may confer an enhanced host response [10]. Caspases are involved in a cascade of cleavage events that result in the initiation of apoptosis. Caspases 2 and 8 act as upstream transducers that cleave downstream caspases in response to apoptotic stimuli, such as Casp3 which leads to cellular events that produce apoptosis. Accordingly, induction of apoptosis has been reported during the early phase of experimental Paracoccidioidomycosis and this phenomenon has been correlated with a phenotypic marker of infection resistance [30], probably as an efficient manner to eliminate the fungus without tissue damage.

Recently, our group used cDNA microarray to evaluate the early transcriptional response of *P. brasiliensis* to the inner environment of peritoneal murine macrophages at six hours of infection [6]. At the present work we have also analysed the macrophage differential gene expression upon infection with *P. brasiliensis* for six hours, in order to reveal the host molecular response. Induction of pro-inflammatory (Ccl21, Cxcl1 and Ccl22) and phagocytosis-related (Clec1b) genes was observed at six hours, in accordance with an increased internalization of *P. brasiliensis* under those conditions [6]. Thus, considering the analyses of differential gene expression for host and pathogen, at six hours, we propose a model for the *P. brasiliensis*-macrophage interaction (Fig. 2) that proposes the events at molecular level during early infection.

In response to the harsh macrophage microenvironment *P. brasiliensis* up-regulates genes (*sod3* and *hsp60*) related to the detoxification of oxidative radicals and amino acid biosynthesis (*metG*). In addition, genes encoding five enzymes of the glycolytic pathway have been found to be down-regulated, suggesting a glucose-poor environment [6]. Macrophages at the same time point up-regulate genes related to inflammation (chemokines and cytokines) and phagocytosis, probably as an effort to counteract infection by the fungus.

In summary, our results demonstrate that *P. brasiliensis* is a potent inducer of molecules involved in the initial host response to this organism. The kinetic approach used in this microarray study has elucidated a coordinate and temporal
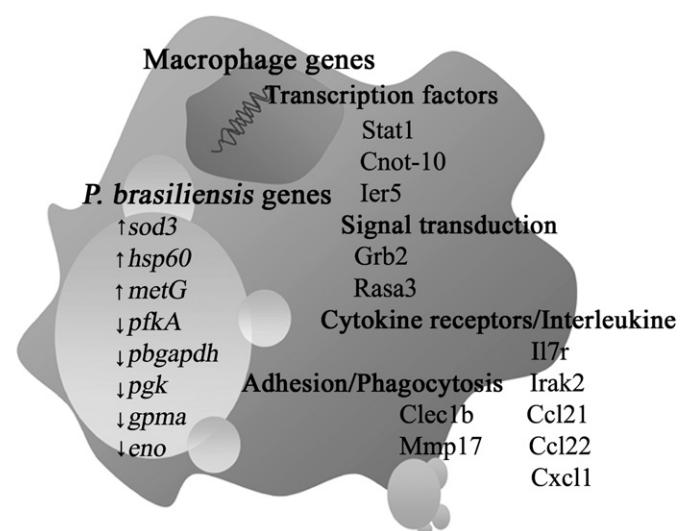
Fig. 2. Proposed model of early differential gene expression at peritoneal macrophage-*P. brasiliensis* interaction. Results for the following genes are showed: *P. brasiliensis*. Arrows indicated gene induction (↑) or gene repression (↓). Differential expression of stress related genes. *sod3* (superoxide dismutase 3), *hsp60* (heat shock protein 60); *pfkA* (phosphofrutokinase A); *metG* (cystathionine β-lyase); *pbgapdh* (glyceraldehyde-3-phosphate dehydrogenase); *pgk* (phosphoglycerate kinase); *gpma* (phosphoglycerate mutase); *eno* (enolase). Macrophage. Gene induction of innate host defense molecules. Cytokine receptors and interleukine: IL7r (Interleukin-7-receptor); Irak2 (Similar to interleukin-1 receptor-associated kinase 2); Ccl21 and Ccl22 (C-C chemokine ligands 21 and 22); Cxcl-1 (C-X-C chemokine ligand 2); Adhesion: Clec1b (C- type lectin like receptor 1b) and Mmp17 (Matrix metalloproteinase 17); Signal transduction: Grb2 (Growth factor receptor bound protein 2); Rasa3 (RAS p21 protein activator 3); Transcription factors: Stat1 (Signal transducer and activator of transcription 1); Cnot10 (CCR4-NOT transcription complex, subunit 10) and Ier5 (Immediate early response 5).

basis of host defense molecules elicited against *P. brasiliensis* infections. The expression data described here should provide a foundation for further studying the pathogenesis of this infectious agent.

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1 **Title: Insights into the pathobiology of *Paracoccidioides brasiliensis* from**

2 **transcriptome analysis – advances and perspectives**

3 **Authors: Simoneide S. Silva[a], Hugo C. Paes[a], Célia M. A. Soares[b] ,Larissa**

4 **Fernandes[a], Maria Sueli S. Felipe[a]**

5 [a] Departamento de Biologia Celular, Universidade de Brasília, 70910-900, Brasília, DF,

6 Brazil.

7 [b] Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Goiás,

8 74001–970, Goiânia, GO, Brazil

9 **Correspondence:** Maria Sueli S. Felipe, Laboratório de Biologia Molecular,

10 Departamento de Biologia Celular, Universidade de Brasília – Brasília, DF, Brazil,

11 70910-900, Tel.: +55 61 3307-2423, Fax: +55 61 3349-8411 E-mail: msueli@unb.br

12 **Key words:** differential gene expression, host-pathogen interaction, macrophage

13 response, *Paracoccidioides brasiliensis,* transcriptome analysis.

14 **Running title:** Insights from *Paracoccidioides brasiliensis* transcriptome

15 **Abstract**

16 *Paracoccidioiddes brasiliensis* is a thermo-dimorphic fungus endemic to Latin

17 America, where it causes the most prevalent systemic mycosis, paracoccidioidomycosis

18 (PCM). DNA microarray technology has been used to identify patterns of gene

19 expression when a microbe is confronted with conditions of interest, such as *in vitro*

20 and/or *ex vivo* interaction with specific cells. *P. brasiliensis* is one organism that has

21 benefited from this approach. Even though its genome has not been sequenced yet,

22 much has been discovered from its transcriptome and DNA array analyses. In this

1

23   review, we will outline the current knowledge in *P. brasiliensis* transcriptome, with

24   focus on differential expression analysis *in vitro* and on the discussion of the genes that

25   are controlled during the host-pathogen interaction *ex vivo* in order to give insights into

26   the pathobiology of this fungus. *In vitro* experiments enabled the delineation of whole

27   metabolic pathways; the description of differential metabolism between mycelium and

28   yeast cells and of the mainly signaling pathways controlling dimorphism, high

29   temperature growth, thermal and oxidative stress and virulence/pathogenicity. Recent *ex*

30   *vivo* experiments provided advances on the comprehension of the plasticity of response

31   and indicate that *P. brasiliensis* is not only able to undergo fast and dramatic expression

32   profile changes but can also discern subtle differences, such as whether it is being

33   attacked by a macrophage or submitted to the conditions of the bloodstream route

34   conditions.

35

36   **Introduction**

37   The fungus *P. brasiliensis* is a thermally-controlled dimorphic pathogen

38   endemic to Latin America. It causes the most prevalent systemic mycosis,

39   paracoccidioidomycosis (PCM) with around ten million people infected, of which about

40   2% will develop the illness [1]. PCM ranges in clinical onset from an acute infection to

41   a chronic, disseminated form that may compromise several organs besides its initial

42   focus (usually the lungs). Infection is thought to be contracted by inhalation of fungal

43   propagules – conidia or mycelial fragments – and is triggered by the dimorphic shift

44   that characterizes this fungus and which consists of its change upon exposure to the

45   body temperature to the yeast form. It is a primary pathogen, infecting

46   immunocompetent hosts, and has a strong bias towards males. The disease is fatal if left

47   untreated and late treatment may result in disabling sequelae.

48   The phylogenetic classification of *P. brasiliensis* is based primarily on sequence

49   analysis. It is placed alongside other pathogenic, free-living fungi such as *Aspergillus*

50   *fumigatus* and *Penicillium marneffei* inside Phylum Ascomycota, Subphylum

51   Pezizomycotina, and Class Eurotiomycetes. The classification puts it with

52   thermodimorphic *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Coccidioides*

53   *immitis* inside Order Onygenales, family Onygenaceae. This classification has been

54   proposed in spite of the fact that no teleomorphic stage has been yet identified for some

55   of them (such a stage is known only for *H. capsulatum* and *B. dermatitidis* among the

56   pathogens). Recent progress on phylogeny of *P. brasiliensis* may be reviewed in detail

57   elsewhere [2]. One of the tasks for the post-genomic era of *P. brasiliensis* will be to

58   establish the cladistic relationships of the fungus with other ascomycetes and trace the

59   history of the interaction of the fungus with animal and human hosts. This will perhaps

60   help us to explain how it evolved to cause paracoccidioidomycosis and the precise

61   determinants of its virulence.

62   *P. brasiliensis* has been shown to be refractory to classical genetic analysis.

63   Recently, however, careful analyses have established it has a genome of 26-35Mb

64   distributed in four or five chromosomes [3-5]. Ploidy is not consensual; some groups

65   have proposed several isolates to be diploid [3, 4], whereas other have used different

66   techniques to propose that most isolates be either haploid or aneuploid [5]. It presents

67   some degree of phenotypic variability; some of them retaining the yeast form even at

68   lower temperatures [6]. Several isolates also differ in their ability to sporulate [7] and

69   cause disease [8]. These presumably reflect a corresponding degree of genetic diversity,

70   which has been supported by recent data [8]. Transcriptome data suggest that the fungus

71   possess sex-related genes [9]. Also, Matute *et al*. [10] presented compelling evidence of

72   intra-specific recombination, although no sexual reproduction has been detected to date.

73   Work on genetic variability of *P. brasiliensis* has been carried out for more than

74   a decade. The early works with random amplified polymorphic DNA analysis [11] had

75   already proved to be able to separate isolates into discrete groups. More recent work

76   combining sequencing and phylogenetic analyses has led researchers to postulate the

77   existence of at least three cryptic species [10]; the same group of researchers has also

78   developed a microsatellite analysis protocol that enables the correct classification of

79   isolates according to each phylogenetic species [12].

80   The genome constitutes the informational core of all biological processes and the

81   study of living organisms depends heavily on our ability to access its contents. For

82   many decades the experimental approach consisted of isolating single genes or

83   regulatory elements and characterizing each one at a time by means of loss-of-function

84   and/or gain-of-function experiments; or by identifying effectors (proteins, cofactors or

85   metabolites) and studying their roles and interactions.

86   In keeping with the informational flow in the cell, genome has been closely

87   followed by transcriptome and proteome. The focus of research has shifted from

88   looking into single genes to understanding global processes and refined controls,

89   including the identification of non-coding RNAs and large-scale phenotypic screening

90   of random mutants [13, 14]. Non-coding sequences have attracted much interest in

91   recent years with the discovery and elucidation of RNA interference (RNAi)

92   mechanisms [15]. RNAi has already been used as an experimental tool to characterize

93   genes by means of loss-of-function experiments in organisms that are refractory to

94   common gene disruption mechanisms [16]. None of these phenomena have been

95   explored in *P. brasiliensis* and thus a large avenue of investigation remains to be

96   opened.

97   The study of pathogens and their interaction with hosts is of special interest.

98   Computer-aided data mining has enabled unambiguous identification of open reading

99   frames, and transcriptional profiling has yielded relevant information concerning

100   differential gene expression [9, 17]. Microarray technology, in conjunction with

101   statistical and experimental validation, has been used to identify patterns of gene

102   expression – selected according to previous genome or transcriptome information –

103   when the microbe is confronted with conditions of interest, such as *ex vivo* interaction

104   with specific cells or exposure to therapeutic agents, signalling molecules or stressors

105   [18, 19].

106   The fungus *P. brasiliensis* is one organism that has benefited from these

107   approaches. Even though its genome has not been sequenced yet, much has been

108   discovered from the transcriptome and DNA array analyses of this fungus. In this

109   review, we will outline the current knowledge from *P. brasiliensis* transcriptome, with a

110   focus on differential expression analysis *in vitro* and how it has helped to advance

111   research in recent years. We have also focused on the discussion of the genes that are

112   controlled during the host-pathogen interaction *ex vivo* in order to give insights into the

113   pathobiology of this fungus.

114   **The transcriptome projects and array studies of *P. brasiliensis***

115   The main global gene analysis in *P. brasiliensis* were performed using the EST

116   (Expressed Sequence Tag) approach, which worked efficiently and seemed to be a

117   useful method to provide the most valuable genetic information of this fungal pathogen.

118   Felipe *et al*. [9, 20] and Goldman *et al*. [21] reported the analysis of 6,022 (from Pb01

119   isolate) and 4,692 (from Pb18 isolate) assembled groups, respectively. Those first

120   results allowed the delineation of whole metabolic pathways, the differential

121   metabolism between mycelium and yeast cells and the mainly signaling pathways

122   controlling dimorphism, high temperature growth, thermal and oxidative stress and

123   virulence/pathogenicity of the fungus. Also, the transcriptome projects highlighted the

124   importance of differential expression genes in both phases – mycelium and yeast, the

125   potentially related virulence factors and possible drug targets.

126   Among the metabolic features generated by the transcriptome, one of the most

127   interesting found, is the differential metabolism between mycelium and yeast cells of

128   *P. brasiliensis*. The mycelium cells appears to have an aerobic metabolism which is

129   suggested by the up-regulation of isocitrate dehydrogenase and succinyl-CoA

130   synthetase enzymes, involved in citrate cycle and also glucokinase, adenylate kinase,

131   uridine kinase and transaldolase. On the other hand, the yeast cells presented induction

132   of genes coding alcohol dehydrogenase I and pyruvate dehydrogenase which evidenced

133   an anaerobic metabolic characteristic favoring fermentation of the pathogenic phase of

134   this fungus [9]. The differential metabolism between mycelium and yeast was

135   reinforced by Nunes *et al*. [22] studies on the transcriptional response of *P. brasiliensis*

136   during the dimorphism, in which they observed an increased expression of alcohol

137   dehydrogenase I and pyruvate decarboxylase genes in the differentiation process from

138   mycelium to yeast, where almost 90% of the cells are already in the yeast form.

139   Marques *et al*. [23] and Andrade *et al*. [24] using the array methodology

140   evaluated the differentially expressed genes in mycelium and yeast cells, which in many

141 cases are important keys to understand the pathobiology of *P. brasiliensis*. Andrade *et*

142 *al.* [24] reported 66 transcriptional modulated genes in mycelium or yeast categorized

143 into two classes, the first group, cell organization includes genes involved in

144 maintenance of cell wall, membrane and cytoskeleton, as the mycelium up-regulated

145 gene hex which encodes a hexagonal peroxisome protein controlling cell integrity, and

146 *bgl* coding 1,3-β-glucosidase involved with cell wall modification during the

147 dimorphism. Among the modulated genes from the yeast phase are *vrp* (verprolin), *cda*

148 (chitin deacetylase), *ags* (α-1, 3-glucan synthase). The α-1,3-glucan synthase also

149 reported in [23] to be positively regulated in the pathogenic form of *P. brasiliensis* can

150 be easily correlated with the biology of this fungus. The α-1,3-glucan is the main

151 component of the yeast cell wall, and it is closed related to the virulence due to its

152 ability to mask the host recognition mechanism of the pathogen, as also reported in *H.*

153 *capsulatum* [25] promoting the fungal escape from the host defenses which contribute

154 to the fungal pathogenesis. The second group of genes consist those involved in

155 metabolism and transport of ions. Genes that regulate the ion metabolism and transport

156 as *isc* (iron-sulphur cluster) and *kpt* which is related to potassium availability were

157 positively regulated in mycelium cells. Those genes appear to be involved in availability

158 of iron and potassium, respectively important for the saprophytic life of *P. brasiliensis*

159 on its ecological niche, the soil. The *pct* gene coding a P-type cation pump is reported to

160 be up-regulated in yeast cells.

161   The observations of Paris *et al.* [26] that yeast cells of *P. brasiliensis* were

162 unable to grow in the presence of inorganic sulphur, were confirmed by the array

163 experiments in which the genes coding sulphur metabolism enzymes such as atp

164 sulphurylase, aps kinase, paps redutase and choline sulphatase showed to be up-

165   regulated in yeast cells of this pathogen, indicating the auxotrophic status for cysteine of

166   the pathogenic phase of this fungus [23, 24, 27].

167   The *in silico* analysis of the transcriptome allowed the identification of almost

168   all components of the conserved signaling pathways already characterized in other

169   eukaryotes as MAP kinases (Mitogen activated protein kinases), PKA/cAMP (Protein

170   kinase A/ cyclic adenosine monophosphate), Ras- GTPases, calcineurin-calmodulin,

171   two component system (histidine kinase), and G proteins coupled receptors [9]. In

172   addition, Nunes *et al.* [22] using microarray reported activation of genes coding for

173   proteins from conserved signaling pathways during the dimorphic transition from

174   mycelium to yeast of *P. brasiliensis*. Among them were G-proteins, Ser/Thr protein

175   kinases, protein kinase A, calmodulin-like protein and calcineurin regulatory subunit.

176   Recently, Bastos *et al.* [28] evaluated the genes regulated on the beginning of cellular

177   transition from mycelium to yeast of *P. brasiliensis*. In accordance, induction of MAP

178   kinases, calcineurin regulatory subunit, serine/threonine kinases and also the histidine

179   kinase and two component sensor kinases was observed [22]. The histidine kinase gene

180   (*drk*) was reported to control the global dimorphism in fungal pathogens [14]. These

181   studies suggest the involvement of those signaling pathways in the cellular

182   differentiation process of *P. brasiliensis.*

183   Fungal pathogens use the conserved mechanism of signaling to promote cell

184   survival under different conditions. The activation of virulence factors is also dependent

185   of the signaling triggered by those cascades, and the main consequence of this activation

186   is the ability of the pathogen to infect and disseminate on the harsh host environment.

187   By a comparison of the *P. brasiliensis* ESTs with *Candida albicans* genes, Felipe *el al.*

188   [9] identified some genes potentially related to fungal virulence. In a more detailed

189   search scan, Tavares *et al*. [29] categorized 30 putative virulence genes into the

8

190   following classes: metabolism, cell wall, detoxification-related, secreted factors, and

191   others. Genes as *icl1* and *mls1* coding respectively, isocitrate lyase and malate synthase

192   of the glyoxylate cycle were potentially correlated with virulence due to their activation

193   on poor carbon growth conditions. As those enzymes are not present in humans, they

194   are also possible drug targets candidates. Other genes related to virulence are identified

195   such   as: *ade2*   (phosphoribosylaminoimidazole   carboxylase),   *nmt1*   (N-

196   myristoyltransferase) and *fas2* (fatty acid synthase α-subunit), *tps1* (trehalose-6-

197   phosphate   synthase)*,*   respectively   involved   in   nucleotide,   lipids   and   glucose

198   metabolism. Among the genes from the second group are those necessary to the cell

199   wall integrity during the vegetative growth and differentiation of *P. brasiliensis*, and

200   includes: *ags1* (α-glucan-synthase gene), *chs3 (*chitin synthase 3), *gna1 (*glucosamine-6-

201   phosphate   acetyltransferase),   *pmt1*   (mannosyl   transferase)   and   *mnt1 (*α-1,2-

202   mannosyltransferase), *phr1* and *phr2* (1,3-β-glucanosyltransferases). The third group

203   comprises the pathogen virulence genes that function on the detoxification of oxidative

204   radicals: as Cu/Zn superoxide dismutase (*sod1*), thiol peroxidase (*tsa1*) alternative

205   oxidase (*aox1*) and catalase (*cat1*). As *P. brasiliensis* is a pathogen that survives

206   intracellularly, the yeast cells have to minimize the toxic substances present in the

207   phagossomes of the macrophage cells, and by this reason the fungus exhibits an

208   antioxidant arsenal of enzymes that are necessary to the survival and consequently

209   virulence of the pathogen. The secreted virulence factors, also reported by Tavares *et al*.

210   [29] include genes encoding proteinases, phospholipases and urease.

211   The *in vitro* transcriptome studies opened a new window on the understanding of

212   *P. brasiliensis* biology. Recently, the genes modulated in the host-pathogen interaction

213   were evaluated trough the transcriptional response of *P. brasiliensis* when yeast cells

214   were internalized into macrophage cells [30]. In addition, Bailão *et al*. [31] also

215   analyzed genes with differential expression when yeast cells were in contact with

216   human blood and rescued from infected mice, to identify the genes required to the *P.*

217   *brasiliensis* adaptation on the host interaction.

218   **Global patterns of gene expression in the host-pathogen interaction**

219   Understanding host-pathogen interactions may provide insights into host

220   defences and the tactics used by pathogens to overcome them. In recent years, several

221   approaches such as cDNA micro arrays, cDNA representational difference analysis

222   (RDA) and serial analysis of gene expression (SAGE) have been developed to identify

223   general profiles of gene expression [32, 33]. Specifically, the cDNA micro array

224   technology has been applied to the analysis of bacterial and fungal interactions with

225   phagocytes, thus identifying key effectors of pathogen virulence and host defence [32,

226   34-36].

227   **Transcriptional profile of *P. brasiliensis* upon infection**

228   The first defence line encountered by *P. brasiliensis* upon infection is pulmonary

229   resident macrophages. Despite being phagocytized, *P. brasiliensis* conidia germinate

230   into the parasitic yeast form, which is equipped to resist the harsh intraphagosomal

231   environment, thus surviving and replicating in non-activated murine and human

232   macrophages. It has been proposed, for PCM and for other systemic mycoses such as

233   histoplasmosis and coccidioidomycosis, that fungal intracellular parasitism is a

234   landmark event for establishment and progression of disease in susceptible hosts, since

235   it enables fungal latency and/or dissemination from the lungs to other organs and tissues

236   [37]. The understanding of this process should reveal key aspects of how these

237   pathogens manage to survive and replicate intracellularly and eventually lead to the

238   development of new antimicrobial drugs. The macrophage phagosome is believed to be

239　a poor source of glucose and amino acids [18, 38]. Nutritional deprivation inside the

240　macrophage induces a similar adaptative response by intracellular bacterial and fungal

241　pathogens [18, 38, 39]. Micro array analysis showed that following phagocytosis, *C.*

242　*albicans* and *Listeria monocytogenes* present a strong reduction in the expression of

243　genes involved in glycolysis an amino acid metabolism [38, 39]. In order to focus the

244　research on the interaction of *P. brasiliensis* with the human host, 1,152 cDNA clones

245　of interest were selected from the transcriptional database, based on previous findings of

246　*P. brasiliensis* transcriptome [9], including putative virulence factors, general

247　metabolism enzymes, heat shock proteins, cell-wall synthetic enzymes and also some of

248　unknown function. In addition, the protocol of RNA extraction from *P. brasiliensis*

249　yeast cells internalized by murine macrophages, without any additional fungal *in vitro*

250　growth was standardized [30]. They observed that early phagocytized *P. brasiliensis*

251　also sense and respond to the phagosomal environment. Genes implicated in glucose

252　and amino acid depletion (*pfkA* - phosphofructokinase*, gapdh* - glyceraldehyde-3-

253　phosphate dehydrogenase*, pgk* - phosphoglycerate kinase*, gpma* - phosphoglycerate

254　mutase*, eno* - enolase*, metG*- cystathionine β-lyase), cell wall metabolism (*fks* - â-

255　glucan synthase) and oxidative stress (*sod3* - Cu,Zn superoxide dismutase and *hsp60* -

256　60 kDa heat shock protein) were differentially expressed by *P. brasiliensis* upon

257　macrophage infection. The data showed a considerable degree of transcriptional

258　plasticity by *P. brasiliensis* in response to the hostile environment of macrophages,

259　which is expected to underlie its adaptability and consequent survival inside that cell

260　[30].

261　　　From the primary site in the lungs, paracoccidioidomycosis may evolve with

262　fungal dissemination via the bloodstream and/or lymphatic system to many organs [40].

263   Recently, using cDNA – RDA technology, Bailão *et al.* [31] reported the differential

264   expression profile of *P. brasiliensis* in conditions that mimic the haematological route

265   of fungal propagation. Under such conditions, several genes, including a transport

266   facilitator (*ctr3*), stress response proteins (30, 70 and 90 kDa heat shock proteins -

267   *hsp30, hsp70* and *hsp90*) and cell-wall remodelling (*sho1* - transmembrane osmosensor,

268   *pas-li*ke - protein with PYP-like sensor domain - PAS domain, *septin-1*), were induced

269   upon blood contact.

270   Nutrient deprivation inside the phagosome induces a similar adaptive response

271   from intracellular bacterial and fungal pathogens and early phagocytized *P. brasiliensis*

272   also sense and respond to the glucose-depleted environment, repressing genes related to

273   glycolysis and amino acid synthesis. In contrast, when *P. brasiliensis* are exposed to

274   blood, genes related to glucose and amino acid metabolism are induced, probably

275   because the milieu is quite rich. Similar results were described upon incubation of *C.*

276   *albicans* in human blood [41].

277   After internalization, macrophages challenge the pathogens releasing a group of

278   toxic antimicrobial molecules, including reactive oxygen and nitrogen intermediates

279   (ROI and RNI, respectively). In response to the oxidative stress generated by the

280   macrophage, the *P. brasiliensis* counter attacks inducing antioxidant gene such as *sod3*

281   [30]. *In silico* analysis showed that the deduced amino acid sequence of the *P.*

282   *brasiliensis sod3* homologue codes a putative membrane GPI-anchored Cu,Zn SOD

283   [42], which would make it more directly accessible to host-derived superoxide anions

284   and thus be more efficient at ROI detoxification. This idea is reinforced by *C. albicans*

285   GPI-anchored Cu,Zn SOD gene, which is induced *in vitro* by oxidative species

286   generators and upon internalization by neutrophils. [41, 43]. Also, genes encoding

287 molecules involved with thermal stress-response as *hsp60* were induced when *P.*

288 *brasiliensis* was ingested by macrophages [30] as well as in exposure to blood as *hsp30*,

289 *hsp70 and hsp90* [31]. These proteins may contribute to the protection of *P. brasiliensis*

290 yeast cells from damage following stress, which occur during infection.

291   Of particular interest for PCM pathogenesis are the genes involved in cell-wall

292 remodelling. The gene for β-glucan synthase (*fks*) is down-regulated in response to the

293 macrophage environment. It has been shown for *P. brasiliensis* and other fungi that the

294 degree of virulence from different isolates in an experimental model of murine infection

295 is related to the relative α-to-β-glucan levels present in their cell wall – avirulent yeast

296 isolates contains less α- and more β-glucan [44, 45]. The mechanism of β-glucan host

297 protection may be related to its capacity to stimulate inflammatory mediators such as

298 tumor necrosis factor-alpha (TNF-α). Since TNF-α has been associated with resistance

299 to *P. brasiliensis* [46, 47], *fks* down-regulation may be an important adaptive

300 mechanism of *P. brasiliensis* to reduce the inflammatory response elicited by the host

301 [30]. In addition, transcripts related to remodelling cell wall and osmotic stress (*gln1 -*

302 glutamine synthetase, *septin-1*, *sho1*, *pas-like*) were also induced in *P. brasiliensis* upon

303 blood contact [31]. The induction of *gln1* and *septin-1* has been speculated to be

304 important in the mediation of chitin deposition. Chitin synthesis has been shown to be

305 essential in the compensatory response to cell-wall stress in fungi, preventing cell death

306 [48]. The *sho1* and *pas-like* genes belong to different classes of osmotic stress sensors.

307 In *C. albicans*, *sho1* is related to fungal morphogenesis by interconnecting two

308 pathways involved in cell wall biogenesis and oxidative stress [49]. Up-regulation of

309 *sho1* and *pas-like* implies their involvement in osmolarity sensing during fungal

310 dissemination through the blood. Fig. 1 shows the proposed model for adaptative

311 changes of *P. brasiliensis* to the host environment, considering the environment of

312 macrophage cells and exposure to blood.

313 **Figure 1**

314 The transcriptional programme of macrophage-ingested cells displays little

315 resemblance to that obtained from *P. brasiliensis* cells exposed to blood. These

316 pioneering works provided advances on the comprehension of the plasticity of response

317 and indicate that *P. brasiliensis* is not only able to undergo fast and dramatic expression

318 profile changes but can also discern even subtle differences, such as whether it is being

319 attacked by a macrophage or submitted to the conditions of the bloodstream route

320 conditions.

321 **Transcriptional response of macrophage cells upon infection with *P. brasiliensis***

322 The host cell response to pathogens is among the best studied examples of

323 cellular reactions to external stimuli. Pathogen-induced phenotypic changes in host cells

324 are often accompanied by marked changes in gene expression. DNA microarrays

325 technology has greatly expanded our ability to monitor changes in the abundance of

326 transcripts in a host upon infection with a microorganism. Macrophages, neutrophils

327 and peripheral blood mononuclear cells (PBMCs) respond to a broad range of microbial

328 stimuli with common transcriptional activation programmes [32, 36, 50]. In this regard,

329 microarray analyses have shown an increase of genes related to innate immunity in

330 macrophages infected with *Mycobacterium tuberculosis*, *C. albicans* and *A. fumigatus*

331 [51, 35, 36]. Recently, for the first time, Silva *et al.* (in revision) [52] used cDNA

332 microarray analysis to identify differential genes of macrophages infected with *P.*

333 *brasiliensis* that are related to several immune processes such as inflammation, cell

334 membrane regulation, transcriptional regulation, signal transduction and apoptosis. This

14

335   last work and the results obtained by Tavares *et al*. [30] studies provided the first data of

336   differential gene expression from host-*P. brasiliensis* interaction, at 6 hours of infection,

337   the same time and experimental conditions. In this sense, we have reported by cDNA

338   microarray analysis that, in response to the harsh macrophage microenvironment, *P.*

339   *brasiliensis* expressed genes primarily associated with glucose and amino acid

340   limitation, cell wall construction, and oxidative stress [30]. In counterpart, macrophages

341   at the same time point up-regulate genes related to inflammation (chemokines and

342   cytokines) and phagocytosis, probably as an effort to avoid host fungal dissemination

343   into different organs and tissues [52].

344   **Concluding remarks**

345   For many decades genetic information of *P. brasiliensis* was not easily and

346   widely accessed at the molecular in order to gain information about the biology,

347   virulence, pathogenicity, interaction with the host and onset of disease. In recent years,

348   the new approach of global gene expression analysis gave us the opportunity to

349   understand the general and differential metabolism in both phases of dimorphic fungi.

350   In addition, it has made possible the identification of potential candidate molecules that

351   contribute to virulence, pathogenicity and others that may be used for drug targeting.

352   Furthermore, it has opened the possibility to access the whole transcriptional response

353   during host-pathogen interaction. Finally, the identification of genes that are

354   differentially expressed under *in vitro* dimorphic transition experiments, *ex vivo*

355   macrophage infection or exposure of *P. brasiliensis* to blood has advanced current

356   knowledge to a better understanding of PCM.

357   As a main consequence of post-transcriptome analysis, the scientific community

358   around *P. brasiliensis* is allocating efforts in order to develop an efficient genetic

359   toolbox to prove and validate the gene function of many of those candidate genes

360   highlighted by the transcriptome data. For example, the generation of random, T-DNA

361   insertional mutants of fungi by means of co-cultivation with *Agrobacterium tumefaciens*

362   has proved to be a powerful technique that has solved at least one major question in the

363   biology of dimorphic fungi [14]. The molecular toolbox to implement this approach in

364   *P. brasiliensis* is still to be developed. Furthermore, these data contribute to a global

365   picture of this systemic illness that may help us to devise comprehensive therapeutic

366   approaches in the near future.

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573

Figure Legend

Figure 1. Model for adaptative changes of *P. brasiliensis* to the host environment. The small arrows ↑ and ↓ indicated induced and repressed genes, respectively. (**a**) *P. brasiliensis* **survival in macrophage phagosome:** oxidative and thermal stress – *sod3*, and *hsp60;* nutritional stress (aminoacid byosynthesis) – *metG*; glycolysis – *pfkA, gapdh, pgk, gpma* and *eno*; cell wall synthesis – *fks* [30]. (**b**) **Induction of *P. brasiliensis* genes during blood exposure:** thermal stress – *hsp30, hsp70* and *hsp90, Ap1-like transcription factor*; nutritional response – *metE1* (s-adenosylmethionine synthetase)*; cell wall/osmolarity sensing – *gln1, pas-like protein., septin, sho1* and *gapdh* [31].
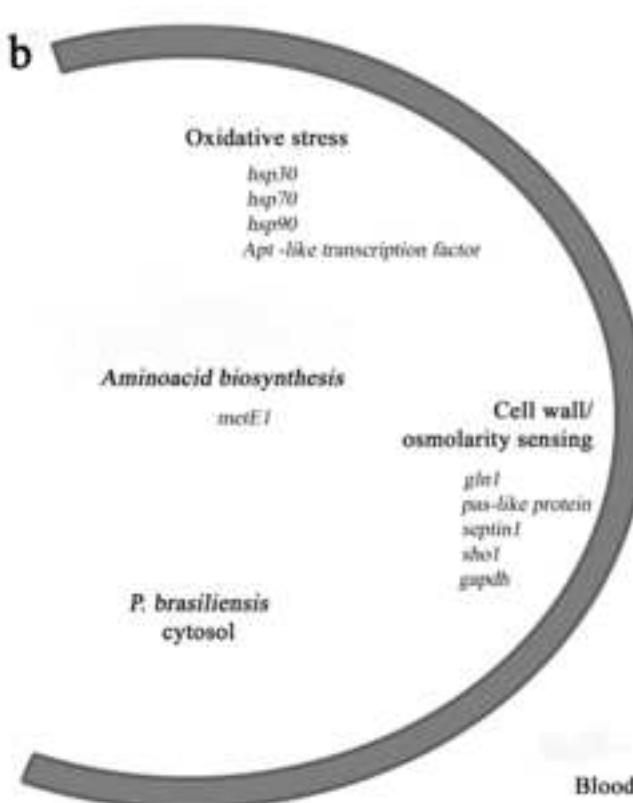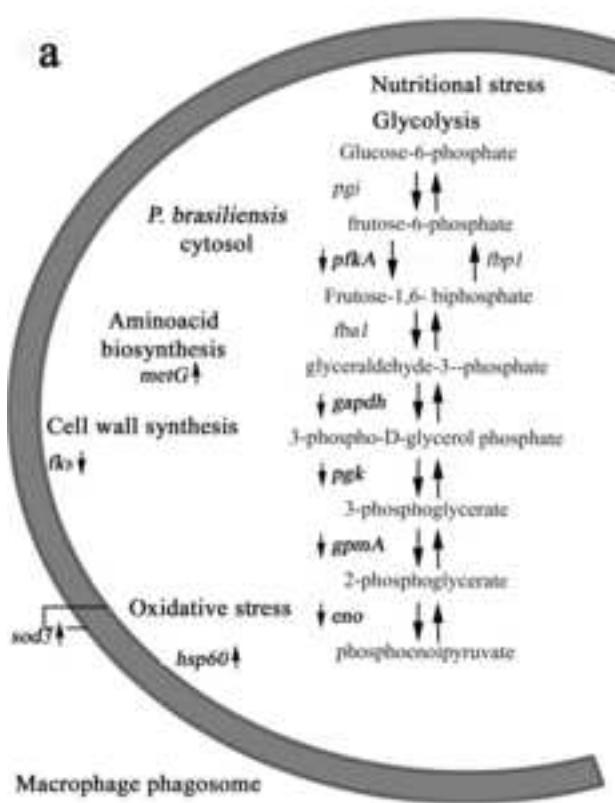
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Transcriptional Profiles of the Human Pathogenic Fungus *Paracoccidioides brasiliensis* in Mycelium and Yeast Cells*[S]

Maria Sueli S. Felipe,[a,b] Rosângela V. Andrade,[a,c] Fabrício B. M. Arraes,[a,c] André M. Nicola,[a,c]
Andréa Q. Maranhão,[a] Fernando A. G. Torres,[a] Ildinete Silva-Pereira,[a]
Márcio J. Poças-Fonseca,[a] Élida G. Campos,[a] Lídia M. P. Moraes,[a] Patrícia A. Andrade,[a]
Aldo H. F. P. Tavares,[a] Simoneide S. Silva,[a] Cynthia M. Kyaw,[a] Diorge P. Souza,[a]
PbGenome Network,[d] Maristela Pereira,[a] Rosália S. A. Jesuíno,[e] Edmar V. Andrade,[e]
Juliana A. Parente,[e] Gisele S. Oliveira,[e] Mônica S. Barbosa,[e] Natália F. Martins,[f] Ana L. Fachin,[g]
Renato S. Cardoso,[g] Geraldo A. S. Passos,[g,h] Nalvo F. Almeida,[i] Maria Emília M. T. Walter,[j]
Célia M. A. Soares,[e] Maria José A. Carvalho,[a,c] and Marcelo M. Brígido[a,c]

From the [a]Departamento de Biologia Celular, Universidade de Brasília, 70910-900, Brasília, DF, Brazil, [j]Departamento de Ciência da Computação, Universidade de Brasília, 70910-900, Brasília, DF, Brazil, [f]Embrapa-Recursos Genéticos e Biotecnologia, W5 Norte, 70770-900, Brasília, DF, Brazil, [e]Departamento de Bioquímica, Universidade Federal de Goiás, 74001-970, Goiânia, GO, Brazil, [i]Departamento de Computação e Estatística, Universidade Federal de Mato Grosso do Sul, 79070-900, Campo Grande, Mississippi, Brazil, [g]Departamento de Genética, Universidade de São Paulo, 14040-900, Ribeirão Preto, SP, Brazil, and [h]Faculdade de Odontologia, Universidade de São Paulo, 14040-900, Ribeirão Preto, SP, Brazil

Paracoccidioides brasiliensis **is the causative agent of paracoccidioidomycosis, a disease that affects 10 million individuals in Latin America. This report depicts the results of the analysis of 6,022 assembled groups from mycelium and yeast phase expressed sequence tags, covering about 80% of the estimated genome of this dimorphic, thermo-regulated fungus. The data provide a comprehensive view of the fungal metabolism, including overexpressed transcripts, stage-specific genes, and also those that are up- or down-regulated as assessed by *in silico* electronic subtraction and cDNA microarrays. Also, a significant differential expression pattern in mycelium and yeast cells was detected, which was confirmed by Northern blot analysis, providing insights into differential metabolic adaptations. The overall transcriptome analysis provided information about sequences related to the cell cycle, stress response, drug resistance, and signal transduction pathways of the pathogen. Novel *P. brasiliensis* genes have been identified, probably corresponding to proteins that should be addressed as virulence factor candidates and potential new drug targets.**

The dimorphic human pathogenic fungus *Paracoccidioides brasiliensis* is the etiological agent of paracoccidioidomycosis (PCM)[1] (1), a major health problem in Latin America. High positive skin tests (75%) in the adult population reinforce the importance of the mycosis in endemic rural areas, where it has been estimated to affect around 10 million individuals, 2% of whom will develop the fatal acute or chronic disease (2). The acute form of PCM chiefly compromises the reticuloendothelial system; the chronic form mainly affects adult males with a high frequency of pulmonary and/or mucocutaneous involvement (1). Chronic severe multifocal PCM may also cause granulomatous lesions in the central nervous system (3). Regardless of the affected organ, PCM usually evolves to the formation of fibrotic sequelae, permanently hindering the patient's health.

P. brasiliensis Undergoes a Dimorphic Process in Vivo—It is assumed that the fungus exists as a soil saprophyte, producing propagules that can infect humans and produce disease after transition to the pathogenic yeast form (4). Pathogenicity has been intimately associated with this process, since *P. brasiliensis* strains unable to differentiate into the yeast form are avirulent (5). Mammalian estrogens inhibit dimorphism, explaining the lower incidence of disease in females (6). The mycelium-to-yeast transition in *P. brasiliensis* is governed by the rise in temperature that occurs upon contact of mycelia or conidia with the human host. *In vitro*, it can be reversibly reproduced by shifting the growth temperature between 22 and 36 °C. Molecular events related to genes that control signal transduction, cell wall synthesis, and integrity are likely to be involved in this dimorphic transition.

[1] The abbreviations used are: PCM, paracoccidioidomycosis; contig, group of overlapping clones; EST, expressed sequence tag; PbAEST, *P. brasiliensis* assembled EST sequence; MAPK, mitogen-activated protein kinase.

P. brasiliensis genome size was estimated to be ~30 Mb (7). A study of *P. brasiliensis* gene density suggests that this fungus contains between 7,500 and 9,000 genes,[2] which is in agreement with the estimated gene number for ascomycete fungi genomes.

Here are presented the results of an effort to achieve a comprehensive metabolic view of the *P. brasiliensis* dimorphic life cycle based on analysis of 6,022 groups generated from both mycelium and yeast phases. This view arises from both a general metabolism perspective and the identification of the precise metabolic points that distinguish both morphological phases. Overexpressed genes and those that are up- or down-regulated in both stages were identified. Expression levels were assessed by cDNA microarrays and some were confirmed by Northern blot. Drug targets and genes related to virulence were also detected in several metabolic pathways. Finally, the majority of genes involved in signal transduction pathways (cAMP/protein kinase A, Ca^{2+}/calmodulin, and MAPKs) possibly participating in cell differentiation and infection were annotated, and now we are able to describe the corresponding signaling systems in *P. brasiliensis.*

MATERIALS AND METHODS

Fungus—P. brasiliensis isolate Pb01 (ATCC MYA-826) was grown at either 22 °C in the mycelium form (14 days) or 36 °C as yeast (7 days) in semisolid Fava Neto's medium. Following incubation, cells were collected for immediate RNA extraction with Trizol reagent (Invitrogen).

Construction of cDNA Libraries and Sequencing—Poly(A)$^+$ mRNA was isolated from total mycelium and yeast RNA through oligo(dT)-cellulose columns (Stratagene). Unidirectional cDNA libraries were constructed in λZAPII following supplier's instructions (Stratagene). Phagemids containing fungal cDNA were then mass-excised and replicated in XL-1 Blue MRF′ cells. In order to generate ESTs, single pass 5′-end sequencing of cDNAs was performed by standard fluorescence labeling dye terminator protocols with T7 flanking vector primer. Samples were loaded onto a MegaBACE 1000 DNA sequencer (Amersham Biosciences) for automated sequence analysis.

EST Processing Pipeline and Annotation—PHRED quality assessment and computational analysis were carried out as previously described (8). EST assembly was performed using the software package CAP3 (9) plus a homemade scaffolding program. Sequences of at least 100 nucleotides, with PHRED ≥20, were considered for clustering. A total of 20,271 ESTs were selected by these exclusion criteria. Contaminant and rRNA sequences were then removed to generate a set of 19,718 ESTs, which was submitted to CAP3 clustering, generating 2,655 contigs and leaving 3,367 ESTs as singlets. Contigs plus singlets comprise the base set of 6,022 *P. brasiliensis* assembled EST sequences (PbAESTs) that underwent further analysis. Annotation was carried out using a system that essentially compared these assemblies with sequences available in public databases. The BLASTX program (10) was used for annotation along with GenBank™ nonredundant (nr), cluster of orthologous groups (COG), and gene ontology (GO) data bases. The GO data base was also used to assign EC numbers to assemblies. Additionally, we used the FASTA program (11) to compare assemblies with *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* predicted polypeptides. The INTERPROSCAN program (12) was used to obtain domain and family classification of the assemblies. Metabolic pathways were analyzed using maps obtained in the KEGG Web site (13) with annotated EC numbers, and this information was used to help in assigning function to PbAESTs.

Differential Expression Analysis in Silico by Electronic Subtraction—To assign a differential expression character, the contigs formed with mycelium and yeast ESTs were statistically evaluated using a test previously described (14) with a confidence of 95%.

cDNA Microarrays and Data Analysis—A set of two microarrays containing a total of 1,152 clones in the form of PCR products was spotted in duplicate on 2.5 × 7.5-cm Hybond N$^+$ nylon membranes (Amersham Biosciences). Arrays were prepared using a Generation III Array Spotter (Amersham Biosciences). Complementary DNA inserts of both *P. brasiliensis* libraries were amplified in 96-well plates using

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[2] C. Reinoso, G. Niño-Vega, G. San-Blas, and A. Dominguez (2003) IV Congreso Virtual de Micologia, personal communication.

vector-PCR amplification with T3 forward and T7 reverse universal primers. Membranes were first hybridized against the T3 [α-^{33}P]dCTP-labeled oligonucleotide. The amount of DNA deposited in each spot was estimated by the quantification of the obtained signals. After stripping, membranes were used for hybridization against α-^{33}P-labeled cDNA complex probes. The latter were prepared by reverse transcription of 10 μg of filamentous or yeast *P. brasiliensis* total RNA using oligo(dT)$_{12-18}$ primer. One hundred microliters of [α-^{33}P]cDNA complex probe (30–50 million cpm) was hybridized against nylon microarrays. Imaging plates were scanned by a phosphor imager (Cyclone; Packard Instruments) to capture the hybridization signals. BZScan software was employed to quantify the signals with background subtraction. Spots were matched with a template grid. The ratio between vector and cDNA complex probe hybridization values for each spot was used as the reference normalization value. Total intensity normalization using the median expression value was adopted as previously described (15). Gene expression data analyzed here were obtained from three independent determinations for each phase (filamentous or yeast). We used the significance analysis of microarrays method (16) to assess the significant variations in gene expression between both mycelium and yeast. Briefly, this method is based on *t* test statistics, specially modified to high throughput analysis. A global error chance, the false discovery rate, and a gene error chance (*q* value) are calculated by the software.

Northern Blot Analysis—Total RNA (15 μg) was separated in a 1.5% denaturing formaldehyde agarose gel and transferred to a Hybond-N nylon membrane (GE Healthcare). Probes were radiolabeled with the random primers DNA labeling system (Invitrogen) using [α-^{32}P]dATP. Membranes were incubated with the probes in hybridization buffer (50% formamide, 4× SSPE, 5× Denhardt's solution, 0,1% SDS, 100 μg/ml herring sperm DNA) at 42 °C overnight and then washed twice (2× SSC, 1% SDS) at 65 °C for 1 h. Signal bands were visualized using a Typhoon 9210 phosphor imager (GE Healthcare).

URLs—Details of the results and raw data are available for download from the World Wide Web: Pbgenome project Web site (www.biomol.unb.br/Pb); Gene Ontology Consortium (www.geneontology.org); Cluster of Ortologous Genes (www.ncbi.nlm.nih.gov/COG); INTER-PROSCAN (www.ebi.ac.uk/interpro/); National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/); Kyoto Encyclopedia of Genes and Genomes (www.genome.ad.jp/kegg); BZScan Software (tagc.univ-mrs.fr); Audic and Claverie statistical test (telethon.bio.unipd.it/bioinfo/IDEG6_form/); Significance Analysis of Microarrays method (www-stat.stanford.edu/~tibs/SAM/); *Candida albicans* data base (genolist.pasteur.fr/CandidaDB/); genomes from *Aspergillus nidulans* and *Neurospora crassa* (www.broad.mit.edu/annotation/fungi/aspergillus/).

RESULTS

Transcriptome Features—In sequencing the *P. brasiliensis* transcriptome, EST data were generated from nonnormalized cDNA libraries of mycelium and yeast cells. The size range of the cDNA inserts ranged from 0.5 to 2.5 kb. Single pass 5′ sequencing was performed on 25,598 cDNA clones, randomly selected from both libraries. Upon removal of bacterial and rRNA contaminant sequences, a total of 19,718 high quality ESTs underwent CAP3 assembly, yielding 2,655 contigs and 3,367 singlets, which constitute the so-called 6,022 *P. brasiliensis* Assembled EST (PbAEST) data base. Contigs presented an average size of 901 bp, and the number of ESTs assembled into contigs varied from 2 to 657 in the largest one (PbAEST 1068), which corresponds to M51, a previously reported *P. brasiliensis* mycelium-specific transcript (17). Of the 6,022 PbAESTs, 4,198 (69.4%) showed a probable homologue in GenBank™, and 4,130 (68.3%) showed a fungus homologue (Fig. 1*A* and Supplemental Table I). We had used MIPS functional categories to classify 2,931 PbAESTs into 12 major groups. *P. brasiliensis* showed a slightly higher percentage of PbAESTs (4%) related to cellular communication and signal transduction (Fig. 1*B*) compared with *S. cerevisiae* functional categorization (3.4%).

Highly and Differentially Expressed Genes—The 27 highly transcribed genes found in the *P. brasiliensis* transcriptome, using a cut-off of 50 reads, are shown in Supplemental Table II. Some of them were previously reported (8). Also, up- and down-regulated genes in mycelium and yeast cells were detected by statistical comparison of the number of sequences in corre-
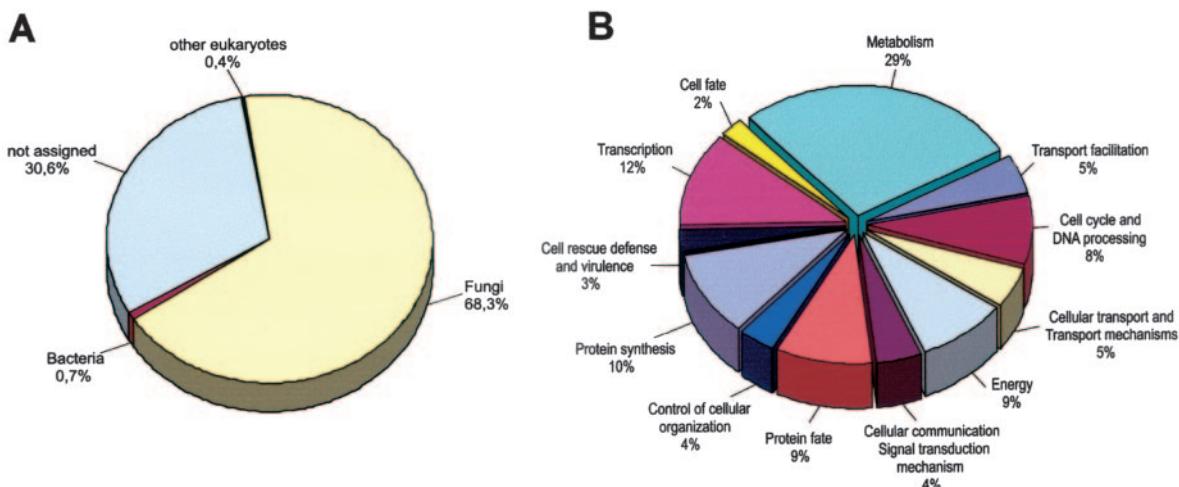
FIG. 1. ***P. brasiliensis* transcriptome characterization.** *A*, distribution of blast best hit among organisms. Each PbAEST was tested against the GenBank™ nr data base, and the best hit organism was computed. A PbAEST was considered as not assigned when the best hit exceed an *E* value of 10^{-10}. *B*, functional categorization of the PbAESTs using MIPS classification. We included 2931 curator-reviewed annotations in this analysis.

sponding PbAESTs (Table I). In order to support the electronic subtraction data, cDNAs from each phase were used to probe cDNA microarrays membranes containing 1,152 clones, which were selected based on the following criteria: (i) ESTs exclusive for a particular morphotype; (ii) ESTs corresponding to genes more expressed in mycelium or yeast cells; and (iii) some ESTs equally expressed in both cell types. From the 1,152 clones, 328 genes were up-regulated during the dimorphic transition: 58 in mycelium and 270 in yeast (data not shown).

The cDNA microarray experiment confirmed most of the electronic subtraction data and also points out to new differentially expressed genes. Among them, a subclass of about 40 up-regulated genes in mycelium and yeast are described in Table I, which includes M51, M32, hydrophobin 1/2, the highly expressed yeast PbY20 protein, and some other genes that have previously been described as differentially expressed in *P. brasiliensis* by different approaches (17–20). Other key up-regulated genes related to the metabolism of *P. brasiliensis* (Table I) are described and discussed elsewhere in this work. Interestingly, we have found a yeast phase preferentially expressed gene that possibly encodes a previously characterized *P. brasiliensis* estradiol-binding protein (21), also described in *C. albicans* and in other fungi (22). It is speculated that the interaction of the 17-β-estradiol hormone with a cytoplasmic protein inhibits the mycelium-to-yeast transition, explaining the lower incidence of PCM in females.

Metabolic Overview—*P. brasiliensis* seems to be capable of producing ATP from the classical pathways of glycolysis, alcohol fermentation, and oxidative phosphorylation, since alcohol dehydrogenase, cytochrome genes, ATP synthase subunits, and pyrophosphatase genes were annotated. All genes encoding glycolytic enzymes were identified in both mycelium and yeast. Genes corresponding to the citrate cycle enzymes and to the components of complexes I, II, III, and IV were found, reflecting the ability of the fungus to perform complete aerobic pyruvate degradation and oxidative phosphorylation. Its putative capacity to also grow in anaerobiosis was evidenced by the alternative conversion of pyruvate to ethanol. Last, it may be able to utilize two-carbon sources in the form of acetate and ethanol through the glyoxylate cycle and obtain sulfite and nitrite from the environment.

In order to validate the carbon source utilization profile predicted by the transcriptome data, two *P. brasiliensis* isolates (Pb01 and Pb18) were grown in McVeigh-Morton minimum

medium supplemented with different carbon sources and growth patterns were qualitatively evaluated (Supplemental Table III). We observed that, in accordance to the transcriptome analysis prediction, several mono- and disaccharides, such as D-glucose, D-fructose, D-galactose, D-mannose, D-sorbitol, α-trehalose, maltose, and sucrose were indeed utilized. On the other hand, the predicted assimilation of D-inositol was not confirmed. Transcripts related to the consumption of L-sorbose and L-lactose were not detected; in fact, *P. brasiliensis* was unable to grown in L-sorbose as the sole carbon source. We consider that the unpredicted fungal growth in L-lactose can be explained by the fact that the *P. brasiliensis* cDNA libraries were not constructed under induction conditions. The observation that fructose, galactose, and glycerol were only utilized by Pb01 and not by Pb18 isolate may simply reflect strain biological variability as previously observed (7). A detailed description of *P. brasiliensis* metabolism, including a list of PbAESTs, is shown in Supplemental Table IV.

Differential Metabolism between Mycelium and Yeast—The up-regulated genes encoding enzymes in mycelium and yeast cells listed in Table I are highlighted in Fig. 2. The differential expression pattern of these genes (with the exception of glucokinase from mycelium cells) was confirmed by Northern blot analysis (Fig. 3). In general, the gene overexpression pattern suggests that mycelium saprophytic cells possess an aerobic metabolism, in contrast with yeast cells. Actually, mycelium up-regulated genes correspond to the main regulatory points of the citrate cycle, such as the genes coding for isocitrate dehydrogenase and succinyl-CoA synthetase; this strongly suggests a metabolic shunt to oxidative phosphorylation. Also, glucokinase is induced, producing glucose 6-phosphate, which is possibly converted through the oxidative pentose phosphate pathway to ribose 5-phosphate, and then to salvage pathways of purine and pyrimidine biosynthesis. In fact, this correlates well with the overexpression of adenylate kinase and uridine kinase genes. The excess of ribose 5-phosphate is probably converted to fructose 6-phosphate and glyceraldehyde 3-phosphate by the nonoxidative pentose phosphate pathway catalyzed by the overexpressed transaldolase. Those sugars are converted to pyruvate and acetyl-CoA for the citrate cycle in aerobic conditions.

In contrast, *P. brasiliensis* yeast cells overexpress the genes encoding alcohol dehydrogenase I and pyruvate dehydrogenase E1 subunit (Table I and Fig. 3); the latter can be detected in high levels in cultures of *S. cerevisiae* grown both anaerobically

TABLE I

Differentially expressed genes in mycelium and yeast cells detected by electronic subtraction and cDNA microarray analysis

The PbAESTs were analyzed as to their differential expression by two methods: a statistical analysis of the number of mycelium and yeast ESTs clustered in each PbAEST (14) and a cDNA microarray analysis of 1,152 PbAESTs, chosen according to the electronic subtraction criteria. A differential pattern of genes encoding enzymes was used in the analysis of the differential metabolism.

PbAEST	EC number	Annotated function	Number of reads[a]		p value[b]	-Fold change[c]	Accession number/Best hit organism/ E value
			M	Y			
Mycelium up-regulated genes							
1068		M51[d,e]	653	4	0.000000	41666.0	BE758605/*P. brasiliensis* /0.0
2274	4.4.1.5	Lactoylglutathione lyase[e]	75	0	0.000000	7.0	NP_105614.1/*Mesorhizobium loti*/ 1e-11
2521		Hydrophobin 1[d,f]	56	0	0.000000		AAM88289.1/*P. brasiliensis*/2e-51
1789		HSP90 co-chaperone[f]	19	10	0.018169		CAD21185.1/*N. crassa*/4e-48
2509	1.15.1.1	Copper-zinc superoxide dismutase[f]	14	5	0.010801		Q9Y8D9/*A. fumigatus*/1e-68
2458		Unknown[f]	13	6	0.025336		
2478		Hydrophobin 2[d,f]	9	0	0.000951		AAR11449.1/*P. brasiliensis*/2e-70
1287	1.13.11.32	2-nitropropane dioxygenase[f]	8	1	0.008606		CAB91335.2/*N. crassa*/e-133
1318		Amino acid permease[e]	8	0	0.001907	50.4	CAD21063.1/*N. crassa*/0.0
1470		Unknown[e]	8	2	0.021572	20.1	
2269	2.7.4.3	Adenylate kinase[f]	5	1	0.046263		NP_011097.1/*S. cerevisiae*/1e-42
2364		Unknown[e]	5	1	0.046263	3.6	
379		Unknown[e]	5	1	0.046263	4.9	
1092	4.2.1.22	Cystathionine β-synthase[f]	4	0	0.030842		AAL09565.1/*Pichia pastoris*/4e-96
2356	2.2.1.2	Transaldolase[f]	4	0	0.030842		NP_013458.1/*S. cerevisiae*/e-108
2476	3.1.2.22	Palmitoyl-protein thioesterase[f]	4	0	0.030842		I58097/*H. sapiens*/8e-42
4135	1.1.1.41	Isocitrate dehydrogenase[g]	1	0	0.248690	3.1	O13302/*Acetobacter capsulatum*/6e-31
5530	6.2.1.5	β-Succinyl CoA synthetase[g]	1	0	0.248690	2.7	T49777/*N. crassa*/9e-73
4749	2.7.1.2	Glucokinase[g]	1	0	0.248690	1.7	Q92407/*Aspergillus niger*/2e-50
4246	2.7.1.48	Uridine-kinase[g]	1	0	0.248690	2.7	T41020/*S. pombe*/3e-28
Yeast up-regulated genes							
2536		Y20 protein[e,d]	27	88	0.000000	8.7	AAL50803.1/*P. brasiliensis*/e-106
2431	1.1.1.1	Alcohol dehydrogenase I[f]	2	45	0.000000		P41747/*Aspergillus flavus*/e-129
737	3.5.1.41	Xylanase/chitin deacetylase[e]	8	33	0.000023	2.8	NP_223015.1/*Helicobacter pylori*/ e-113
201		Putative membrane protein Nce2[e]	0	27	0.000000	25.2	NP_015475.1/*S. cerevisiae*/5e-08
797	3.1.6.6	Choline sulfatase[e]	3	15	0.001602	4.8	NP_248721.1/*P. aeruginosa*/e-104
814		Glyoxylate pathway regulator[e]	0	15	0.000016	17.7	NP_009936.1/*S. cerevisiae*/4e-37
1704		60S ribosomal protein L19[f]	0	14	0.000032		NP_596715.1/*S. pombe*/6e-49
1585	1.8.4.8	PAPS reductase[e]	1	12	0.000815	5.1	AAG24520.1/*Penicillium chrysogenum*/e-121
63		Putative methyltransferase[e]	3	11	0.011314	2.5	CAD21381.1/*N. crassa*/2e-46
778		Putative estradiol-binding protein[e]	3	11	0.011314	29.5	NP_012049.1/*S. cerevisiae*/1e-31
136		Unknown[d,f]	4	10	0.030950	3.9	
767		Unknown[e]	3	10	0.017732	2.2	
701	1.2.4.1	Pyruvate dehydrogenase[f]	1	9	0.004973		Q10489/ *S. pombe*/1e-72
1724		Putative sterol transporter[e]	0	6	0.007915	29.3	NP_013748.1/*S. cerevisiae*/4e-12
171	2.6.1.42	Branched-chain aminotransferase[f]	0	5	0.015790		NP_012078.1/*S. cerevisiae*/7e-87
1983	1.6.5.3	NADH dehydrogenase (ubiquinone reductase)[f]	0	4	0.031496		S47150/*N. crassa*/1e-19
244	1.1.1.69	Gluconate dehydrogenase[f]	0	4	0.031496		NP_471610.1/*Listeria innocua*/ 1e-09
258	3.3.2.1	Isochorismatase[f]	0	4	0.031496		NP_436193.1/*Sinorhizobium meliloti*/1e-20
279	2.5.1.15	Dihydropteroate synthase[f]	0	4	0.031496		T49535/*N. crassa*/1e-38
314	2.6.1.1	Aspartate aminotransferase[f]	0	4	0.031496		NP_509047.1/*Caenorhabditis elegans*/4e-96
555	6.2.1.3	Acyl-CoA synthetase[f]	0	4	0.031496		NP_275799.1/*Methanothermobacter thermautotrophicus*/9e-89
756	6.3.5.7	Glutamyl-tRNA amidotransferase[f]	0	4	0.031496		Q33446/*A. nidulans*/1e-15
865	4.1.3.1	Isocitrate lyase[f]	0	4	0.031496		AAK72548.2/*Coccidioides immitis*/e-119
963	2.6.1.9	Histidinol-phosphate aminotransferase[f]	0	4	0.031496		P36605/*S. pombe*/4e-87
980	3.5.1.4	Acetamidase[f]	0	4	0.031496		AAK31195.1/*Aspergillus terreus*/2e-09
3073	1.14.13.3	Phenylacetate hydroxylase[g]	0	1	0.249998	2.3	AAF21760.1/*P. chrysogenum*/2e-48

[a] Number of mycelium (M)- and yeast (Y)-derived ESTs in the PbAEST.

[b] p value for the Audic and Claverie test.

[c] -Fold change found for the microarray experiments.

[d] Previously shown to be differential by Northern blot or proteome analysis.

[e] Electronic subtraction and cDNA microarray analysis; differential pattern in both analyses.

[f] Electronic subtraction differential pattern and not assayed in cDNA microarray analysis.

[g] Singlets that are differential in cDNA microarray analysis.

and aerobically in the presence of ethanol (23). The carbohydrate metabolism is probably shifted toward ethanol production, reflecting the anaerobic behavior of the yeast form as previously reported (24). Several pathways that provide substrates for the glyoxylate cycle are up-regulated in the yeast cells (Table I and Fig. 3). First, isocitrate lyase redirects the metabolic flow using ethanol and acetate as two-carbon sources and generating oxaloacetate, which can be reconverted to glu-

FIG. 2. **Comparison of the expression pattern of genes encoding for enzymes in mycelium-to-yeast cell differentiation of *P. brasiliensis.*** For the detailed metabolic comparison between mycelium and yeast metabolism, see Supplemental Table IV, since we have presented in this figure only the central pathways for carbohydrate metabolism and citrate cycle. Genes that are overexpressed are *boxed*, either in mycelium or yeast cells, according to the criteria described in Table I.
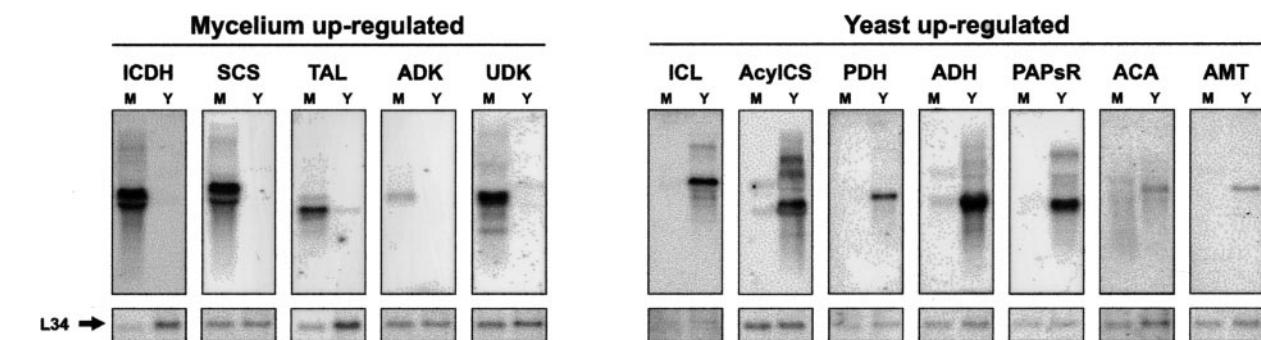
FIG. 3. **Northern blot analysis of mycelium and yeast up-regulated genes of *P. brasiliensis.*** Total RNA samples from both mycelium (*M*) and yeast (*Y*) were blotted onto nylon membranes and hybridized against gene-specific radiolabeled probes. *ICDH*, isocitrate dehydrogenase; *SCS*, β-succinyl-CoA-synthetase, *TAL*, transaldolase; *ADK*, adenylate kinase; *UDK*, uridylate kinase; *ICL*, isocitrate lyase; *acyl-CS*, acyl-CoA synthetase; *PDH*, pyruvate dehydrogenase; *ADH*, alcohol dehydrogenase; *PAPsR*, phosphoadenylyl sulfate reductase; *ACA*, acetamidase; *AMT*, aminotransferase. The constitutive 60 S ribosomal protein L34 was used as a loading control.

cose. In addition, the branched-chain aminotransferase gene is also overexpressed (as are other aminotransferase genes, such as those of aspartate and histidinol-P) and converts valine, leucine, and isoleucine to acetyl-CoA, which is then fed to the cycle. The yeast differential acetamidase also contributes to this pathway by deriving acetate from acetamide. Furthermore, the up-regulated acyl-CoA synthetase generates acetyl-CoA in the first step of β-oxidation, which may also be taken up by the cycle. Finally, the generation of sulfite by phosphoadenylyl sulfate reductase provides acetate for the glyoxylate cycle as mentioned above. The overall analysis suggests that ATP production through alcohol fermentation and the respiratory chain occurs in a biased pattern, the former being preferential in the yeast form and the latter in mycelium.

Yeast cells are rich in chitin; the high expression of chitin deacetylase reveals its possible involvement in cell wall loosening, reorganization, and synthesis of newly components during cell growth and budding of yeast cells. This enzyme is not present in humans and thus represents a possible drug target. In mycelium, overexpression of cystathionine β-synthase and nitroalkane oxidase strongly suggests that mycelium cells take up sulfite and nitrogen, respectively, from the environment for metabolic processing. Finally, the probable role of the remaining overexpressed gene encoding palmitoylthioesterase remains unclear. In contrast, the enzymes isochorismatase and ubiquinone-reductase are greatly up-regulated in the yeast form, strongly suggesting a high production of ubiquinone by *P. brasiliensis*, which could be involved in cellular oxidative stress under anaerobic conditions. The high yeast expression of dihydropteroate synthase produces, as a consequence, high levels of tetrahydrofolate, which probably will increase the metabolic flow toward purine biosynthesis. The meaning of the high expression in yeast of choline sulfatase, gluconate dehydrogenase, glutamyl-tRNA amidotransferase, and phenylacetate hydroxylase also remains unclear.

Cell Cycle and Genetic Information—The main genes in-

volved in cell cycle and in the basic genetic information flow machinery (DNA replication, repair, recombination, transcription, RNA processing, translation, and post-translational modifications) are well conserved in comparison with their counterparts from *S. cerevisiae*. Also, sequences related to mitochondrial replication, budding, sporulation, and mating were also annotated (Supplemental Table V).

From the cell cycle-related orthologues identified in *P. brasiliensis*, those related to the structure and assembly of the cytoskeleton, chromatin structure, chromosome segregation, cyclins, and cell cycle control genes were highlighted. Genes related to the major DNA repair mechanisms found in yeast (mismatch, base excision, and recombination systems) were identified in *P. brasiliensis*, although not every component was represented, since cells were not subjected to DNA-damaging conditions. The *RAD52* gene, which plays an essential role in *S. cerevisiae* recombination, is also present in the *P. brasiliensis* transcriptome.

Among the identified transcription factors, the orthologues for *MAT*, *MCM1*, and *NsdD* are of relevance, since they are implicated in ascomycete sexual reproduction. These genes represent a strong evidence for mating in *P. brasiliensis*, so far not yet described, which is reinforced by the detection of six transcripts involved in meiotic recombination.

Stress Responses—Cell differentiation in *P. brasiliensis* requires a temperature shift, which might be associated with a stress response. We have found 48 sequences encoding molecular chaperones and their associated co-chaperones in *P. brasiliensis* transcriptome (Supplemental Table VI). These sequences were divided into nine groups: small chaperones (four genes), HSP40 (9), HSP60 (10), HSP70 (7), HSP90 (4), HSP100 (4), 14-3-3 (2), calnexin (1), and immunophilins (7). Eight of these are differentially expressed: calnexin, *cct7* (cytoplasmic hsp60) and *sba1* (HSP90/70 co-chaperone) for the mycelium form and *cpr1* (HSP90/70 co-chaperone), *hsp42*, *hsp60*, *ssc1* (HSP70), and *hsp90* for the yeast form. From these, *hsp60* and *hsp70* had been previously characterized as differentially expressed in yeast (25, 26). cDNA microarray analysis confirmed the differential expression pattern of *sba1*. Furthermore, the number of chaperone and co-chaperone ESTs is 38% larger in the yeast cDNA library than in the mycelium library. These data represent an evidence of an altogether higher expression of HSPs in yeast cells, compatible with growth at 37 °C.

Oxidative agents may cause stress and damage to *P. brasiliensis* cells. They may originate from the activity of host macrophages or from intracellular oxidative species. *P. brasiliensis* contains several genes encoding enzymes with known or putative antioxidant properties, such as superoxide dismutases, catalases (two isoenzymes), peroxiredoxins, and a novel cytochrome *c* peroxidase (Supplemental Table VII). Homologues to genes encoding secondary antioxidant enzymes belonging to the glutathione *S*-transferase family were also found. Several transcription factors may be involved in the induction of antioxidant defenses in *P. brasiliensis*. Homologues to *YAP1*, *HAP3*, and *SKN7* from *S. cerevisiae* (27) were discovered in the transcriptome, showing that the oxidative stress regulators from *P. brasiliensis* and baker's yeast might be conserved.

Signal Transduction Pathways—Transcriptome analysis and reverse annotation revealed several putative components of the biosignaling pathways in *P. brasiliensis* (Supplemental Table VIII), such as (i) MAPK signaling for cell integrity, cell wall construction, pheromone/mating, and osmotic regulation; (ii) cAMP/protein kinase A, regulating fungal development and virulence, and (iii) calcium-calmodulin-calcineurin, controlling growth at high temperature. Furthermore, a *ras* homologue sequence was detected raising the possibility of cross-talk

among the distinct signal transduction pathways (Fig. 4).

In budding yeast, the MAPK cascade responsible for cell integrity mediates cell cycle regulation and cell wall synthesis, responding to different signals including temperature, changes in external osmolarity, and mating pheromone. Components of this pathway identified in *P. brasiliensis* encompass the most classical steps, with the exception of a cell surface tyrosine kinase-like receptor that was not found in the transcriptome so far analyzed. Rho1p is a small GTP-binding protein of the *Rho* subfamily required for cell growth and coordinated regulation of cell wall construction (28) through the synthesis of β-1,3-glucan. It also activates Pkc1p, which in turn regulates the MAPK pathway.

Transcripts related to the pathway for activation by mating pheromone were identified in the *P. brasiliensis* transcriptome. The intermediary components appear to be constitutively expressed in both mycelium and yeast forms. Intriguingly, mating has not yet been described in *P. brasiliensis*. Conversely, the Hog1 MAPK cascade is activated when there is an increase in the environment osmolarity. One of its targets, Glo1p, which controls genes required for cell adaptation and survival upon osmotic stress in *S. cerevisiae* (29), was also detected in *P. brasiliensis*.

The cAMP/protein kinase A is a cascade known to regulate fungal differentiation and virulence. From the genes identified in *P. brasiliensis*, we highlight a homologue to several fungal adenylate cyclases; the low affinity cAMP phosphodiesterase, encoded by the gene Pde1; homologues to both the regulatory and the catalytic subunits of protein kinase A, which is involved in the regulation of the cell surface flocculin Flo11p/Muc1p (30). In *P. brasiliensis* exogenous cAMP is known to inhibit the process of filamentation (31). Both the catalytic (CnaA) and the Ca^{+2}-binding regulatory B (CnaB) subunits of calcineurin were found in *P. brasiliensis*. In dimorphic fungi, cAMP- and calcineurin-dependent pathways seem to be involved in differentiation. As in the pathogenic fungus *Cryptococcus neoformans* (32), calcineurin might also play a role in mating of *P. brasiliensis*. In several pathogenic and nonpathogenic fungi, *RAS* is involved in filamentation, pseudohyphal/hyphal growth, and mating (33). A *RAS*-related transcript was identified in *P. brasiliensis*, but further studies are required to elucidate its function in mycelium-to-yeast transition and in the mechanism of pathogenicity.

Virulence Genes, Drug Targets and Resistance—In order to identify genes that could be related to *P. brasiliensis* virulence, its transcriptome has been searched for orthologues assigned as virulence factors in human pathogenic fungi, as defined by Falkow's postulate (34). Table II lists 28 *P. brasiliensis* sequences, which were previously experimentally established as virulence or essential genes in *C. albicans*, *C. neoformans*, and *Aspergillus fumigatus*. They were subdivided into four classes: metabolism-, cell wall-, and signal transduction-related and others. Some of these genes has been considered for antifungal therapy and are also listed in Table III as potential drug targets.

MAPK-related sequences, whose orthologues in *C. albicans* were experimentally correlated to hyphal formation and virulence, were also detected. The extrapolation to the *P. brasiliensis* model is not direct, since yeast, not hyphae, is the pathogenic cell type, but several MAPK homologues are found in species exhibiting diverse morphology and infection habits (35). A *cavps34* orthologue, identified in *P. brasiliensis* transcriptome (*vps34*), is implicated in the protein/lipid transport from the Golgi apparatus/endosome to the vacuole and has been proved to be important to *C. albicans* virulence (36).

Noteworthy is the finding of glyoxylate cycle genes in
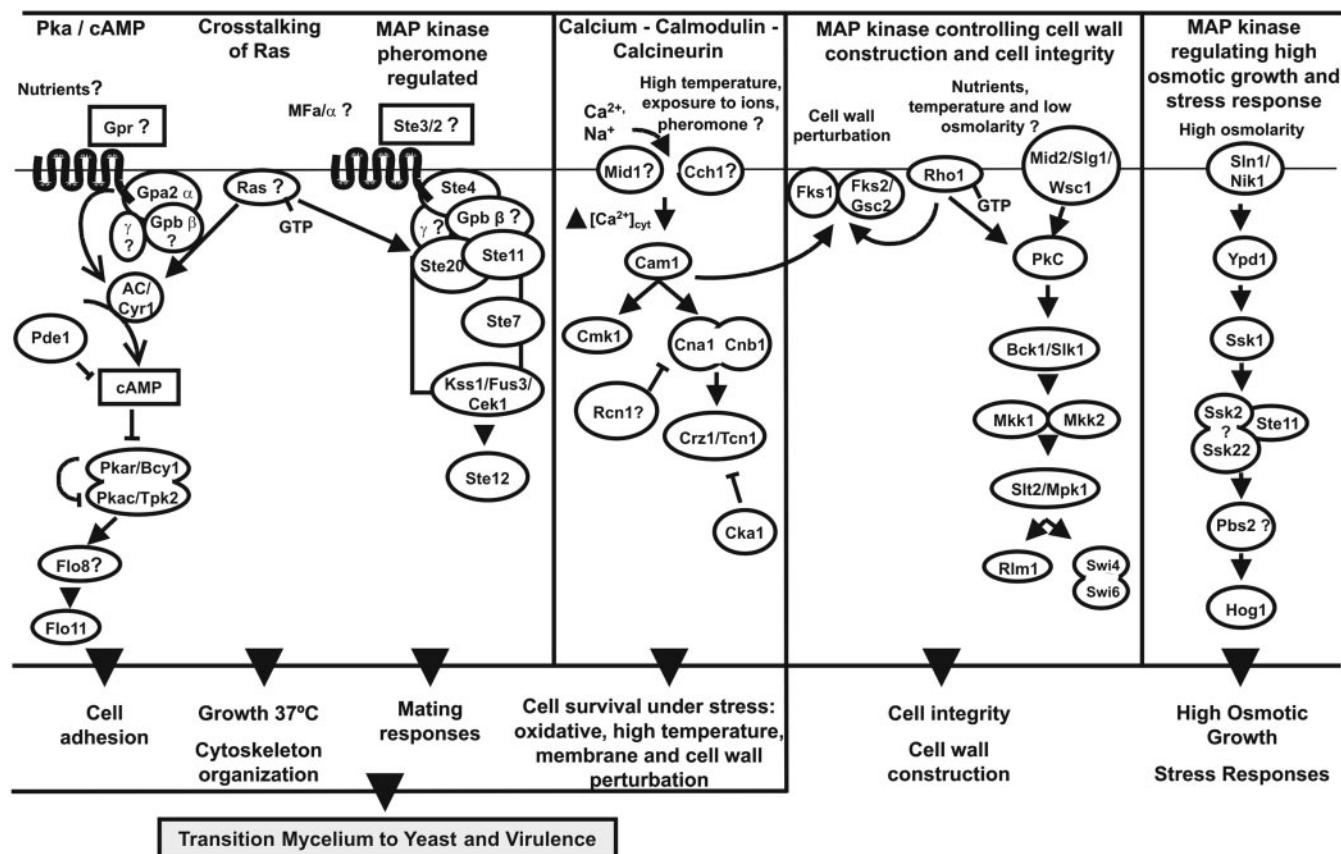
FIG. 4. **Signaling pathways in *P. brasiliensis*.** Shown are cAMP/protein kinase A regulating fungal development and virulence; MAPK signaling for cell integrity, cell wall construction, pheromone/mating, and osmoregulation; calcium-calmodulin-calcineurin controlling cell survival under stress conditions; and Ras allowing cross-talk of extracellular signals. For abbreviations of gene names see Supplemental Table VIII.

P. brasiliensis, since its activity has been reported as a fungal virulence requirement (37). The activity of the key enzymes malate synthase and isocitrate lyase was reported to be up-regulated in *C. albicans* upon phagocytosis (38). Both enzymes were detected in the *P. brasiliensis* transcriptome, with isocitrate lyase being overexpressed in the yeast phase, as confirmed by Northern blot analysis (Fig. 3).

The cell wall, as the most obvious difference between human and fungal cells, represents a prime target for antifungals. Genes involved in its biogenesis and assembly can act as virulence factors and therefore are putative drug targets. We have identified orthologues to chitin synthase 3 (*chs3*), glucosamine-6-phosphate acetyltransferase (*gna1*), mannosyltransferase (*pmt1*), and α1,2-mannosyltransferase (*mnt1*) genes and glycosidases Phr1p and Phr2p. The expression of the two last genes in *C. albicans* is responsive to the product of *prr2* (39), a pH-related transcription factor also present in the *P. brasiliensis* transcriptome. The detection of chitin deacetylase, as an overexpressed yeast gene confirmed by cDNA microarray and Northern blot (data not shown), points out to a novel target for drug research in *P. brasiliensis*.

Microbe resistance to reactive oxygen and nitrogen intermediates plays an important role in virulence (40). We were able to identify sequences that are oxidative stress response orthologues, including an alternative oxidase (*aox1*), a copper/zinc superoxide dismutase (*sod1*), and two different catalase orthologues, one of them a peroxisomal *cat1*, as recently described (41).

The urate oxidase gene detected in the *P. brasiliensis* transcriptome, but not in *S. cerevisiae*, *C. albicans*, and *Homo sapiens* genomes, suggests that uric acid could be degraded to allantoin. In addition, the presence of a *C. neoformans* urease

orthologue also probably reflects the degradation of urea to ammonia and carbamate. A role in virulence and sporulation has been assigned for both genes (42). The production of urea has been involved in an improved *in vitro* survival for those microorganisms exposed to an acidic environment. In this view, it could be related to the survival of the fungus in the host cells.

The development of new drugs is crucial, considering the problem of emerging drug resistance and toxicity (37). Novel drug targets have been found through the analysis of genome sequences. The genes listed in Table III have no homologues in the human genome and therefore could be considered for the development of new antifungal drugs. Most therapies designed to treat fungal infections target the ergosterol biosynthetic pathway (43). The orthologue of C-24 sterol methyltransferase (*ERG6*) is present in *P. brasiliensis*. In addition, modulation of sphingolipid metabolism exerts a deep impact on cell viability. The synthesis of inositol-phosphoryl-ceramide from phytoceramide catalyzed by the product of the *aur1* gene, present in *P. brasiliensis*, corresponds to the first specific step of this pathway (44). Translation elongation factors have also been pointed out as drug targets (37). In the *P. brasiliensis* transcriptome, we have found an elongation factor-3 sequence that is absent in human genome (45) and thus can be addressed for pharmaceutical purposes.

Twenty PbAESTs annotated as related to multiple drug resistance genes were identified (Supplemental Table IX). They include 12 *S. cerevisiae* orthologues, 10 of which are related to the ABC transporter and two to major facilitator superfamilies (46). One of them corresponds to Pfr1, a gene recently described in *P. brasiliensis* (47), and another is related to the *CDR1* gene from *C. albicans*, which is up-regulated in the presence of human steroid hormones (48). It has been speculated that

TABLE II

Putative virulence or essential genes found in P. brasiliensis transcriptome related to the experimentally confirmed orthologues of C. albicans, C. neoformans, and/or A. fumigatus

PBAEST	Orthologue name	AC number/Organism	E value[a]	Remarks
Metabolic genes				
2403	*ura3*[b]	DCCKA (*C. albicans*)	3e-41	
		O13410 (*A. fumigatus*)	2e-83	
0670	*nmt*[b]	AAA34351 (*C. albicans*)	8e-60	Lipid synthesis
		AAA17547 (*C. neoformans*)	1e-60	
3750	*fas2*[b]	JC4086 (*C. albicans*)	7e-33	
1224	*hem3*	094048 (*C. albicans*)	1e-58	Hemosynthesis
3819	*tps1*[b]	CAA69223 (*C. albicans*)	1e-36	Glucose metabolism
1693	*icl1*	AAF34690 (*C. albicans*)	1e-112	Glyoxylate cycle
0831	*mls1*	AAF34695 (*C. albicans*)	1e-122	Glyoxylate cycle
1735	*pabaA*[b]	AAD31929 (*A. fumigatus*)	1e-12	Purine synthesis
Cell wall genes				
4346	*chs3*	P30573 (*C. albicans*)	7e-22	Potential drug targets
4968	*gna1*[b]	BAA36496 (*C. albicans*)	4e-16	
1067	*mnt1*	CAA67930 (*C. albicans*)	9e-49	
2980	*pmt1*	AAC31119 (*C. albicans*)	4e-46	
2382	*phr1*	AAF73430 (*C. albicans*)	2e-40*	
1375	*phr2*	AAB80716 (*C. albicans*)	1e-114	
Signal transduction				
4452	*cek1*	A47211 (*C. albicans*)	3e-30	Hyphal formation
1110	*cpp1*	P43078 (*C. albicans*)	6e-16	
267	*cst20*	AAB38875 (*C. albicans*)	6e-48	
358	*hog1*[b]	Q92207 (*C. albicans*)	2e-59	Osmoregulation
988	*nik1*[b]	AAC72284 (*C. albicans*)	7e-37	Hyphal development
Other fungal virulence determinant genes				
623	*cat1*[b]	CAA07164 (*C. albicans*)	1e-172	Peroxisomal catalase
3553	*mdr1*[b]	CAA76194 (*C. albicans*)	2e-27	
3306	*plb1*[b]	AAF08980 (*C. albicans*)	2e-38	Important in host cell penetration
4267	*top1*[b]	Q00313 (*C. albicans*)	4e-56	
5012	*vps34*[b]	CAA70254 (*C. albicans*)	2e-29	Vesicle trafficking
2516	*sod1*[b]	AAK01665 (*C. neoformans*)	4e-51	Nitric oxide detoxification
2463	*ure1*[b]	AAC62257 (*C. neoformans*)	6e-76	
1102	*aox1*[b]	AAM22475 (*C. neoformans*)	2e-48	Resistance to oxidative stress

[a] All *P. brasiliensis* assembled ESTs are BBH with *C. albicans* ortologues, except *phr1* (marked with an asterisk).
[b] Putatively novel *P. brasiliensis* virulence genes.

TABLE III

Potential drug targets genes found in P. brasiliensis transcriptome with no homologues in the human genome

PbAEST	Annotated function	Orthologue accession numbers	E-value	Remarks
Cell wall				
5198	β-1,3-glucan synthase	AAD37783	2e-108	Preferentially expressed in mycelium
4988	α-1,3-glucan synthase	AAL18964	2e-70	Preferentially expressed in yeast
0265	Rho	AAK08118	2e-92	Signal transduction
1147	Chitin synthase I	AAF82801	2e-81	
1927	Chitin synthase II	Q92444	3e-66	
4346	Chitin synthase IV	AF107624	2e-65	
3958	Chitin synthase asmA	JC5546	1e-64	
0737	Xylanase/Chitin deacetylase	ZP_00126582	1e-12	Up-regulated in *P. brasiliensis* yeast cells
5473	Bud neck involved	NP_014166	1e-12	Required to link CHS3p and CHS4p to the septins
1063	α-1,2-Mannosyltransferase	NP_009764	1e-20	Involved in protein glycosylation
Glyoxylate cycle				
2402	Malate synthase	P28344	1e-37	
1688	Isocitrate lyase	AAK72548.2	1e-144	Up-regulated in *P.brasiliensis* yeast cells
Other targets				
1959	$\Delta(24)$-Sterol C-methyltransferase	T50969	4e-44	Ergosterol biosynthesis
0200	Aureobasidin resistance protein	AAD22750	1e-43	Sphingolipid synthesis
0845	Elongation factor 3	BAA33893	1e-142	Unique and essentially required for fungal translational machinery
4129	Urate oxidase	P33282	6e-77	Sporulation and pathogenesis
2456	Urease	AAC49868	3e-94	Sporulation and pathogenesis

steroid hormones are involved in morphological changes as well as in pathogenicity in *P. brasiliensis* and also in drug resistance in *C. albicans*. Interestingly, the process of infection of *P. brasiliensis* is strongly biased toward males, albeit the role of steroid hormones in the expression of ABC transporters in this organism remains to be investigated.

DISCUSSION

The *P. brasiliensis* transcriptome described here is represented by 6,022 EST clusters that may cover about 80% of the fungal total genome, whose gene number has been estimated to be ~8,000 genes.[3] This number greatly exceeds the previous EST studies in this fungus (8, 49). The analysis compares the two fungal cell types as well as their metabolic behavior. The results obtained probably reflect the adaptations associated with the mycelium (soil) and yeast (human host) environments. Most importantly, they provide new insights with respect to

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[3] G. San-Blas, personal communication.

signal transduction pathways, virulence genes, and drug targets for this pathogen.

The transcription profile of the mycelium infective phase suggests the shunting of pyruvate into aerobic metabolism, since the expression of the ESTs encoding enzymes of the trichloroacetic acid cycle are up-regulated in this fungal phase. In contrast, the yeast transcription profile evidenced the deviation of pyruvate from the glycolytic pathway into anaerobic metabolism; this observation is consistent with a lower oxygen level in infected tissues. Its putative ability to produce ethanol suggests a potential anaerobic pathway for *P. brasiliensis*, which is dependent on the metabolic state of the cell. It seems that the main regulatory effector on the shunting of the end product of glycolysis into aerobic or anaerobic metabolism is temperature; therefore, it can be hypothesized that this physical factor is the central trigger of all of these molecular events, since it was the only parameter changed in the *in vitro* cultivation of yeast and mycelium of *P. brasiliensis*. Experiments are currently being carried out in order to confirm the *in vivo* expression profile of the differentially expressed genes in macrophages and human pulmonary epithelial cells infected by *P. brasiliensis*.

Since *P. brasiliensis* is a medical problem in Latin America, the prediction of new drug targets from sequence information is of great importance. Chitin deacetylase, which is absent in humans and highly expressed in the parasitic yeast, could be a specific drug target for PCM therapy if it is shown to play a key role in the fungal metabolism during human infection. Functional analysis of the *P. brasiliensis* genes described in this work will lead to important information on cellular differentiation, pathogenicity, and/or virulence. These issues can only be addressed when molecular tools are developed for this organism. In conclusion, the knowledge of the transcribed sequences of *P. brasiliensis* will most likely facilitate the development of new therapeutics to PCM and other medically relevant mycosis.

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BMC Genomics

Cell organisation, sulphur metabolism and ion transport-related genes are differentially expressed in *Paracoccidioides brasiliensis* mycelium and yeast cells

Rosângela V Andrade[1], Hugo C Paes[1], André M Nicola[1], Maria José A de Carvalho[1], Ana Lúcia Fachin[2], Renato S Cardoso[2], Simoneide S Silva[1], Larissa Fernandes[1], Silvana P Silva[3], Eduardo A Donadi[2], Elza T Sakamoto-Hojo[2], Geraldo AS Passos[2], Célia MA Soares[3], Marcelo M Brígido[1] and Maria Sueli S Felipe*[1]

Address: [1]Depto. de Biologia Celular, Universidade de Brasília, 70910–900. Brasília-DF, Brazil, [2]Depto de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 14040–900, Ribeirão Preto, SP, Brazil and [3]Depto de Bioquímica e Biologia Molecular, Universidade Federal de Goiás, 74001–970, Goiânia, GO, Brazil

Email: Rosângela V Andrade - rvieira@unb.br; Hugo C Paes - sorumbatico@gmail.com; André M Nicola - a0058343@aluno.unb.br; Maria José A de Carvalho - mariajac@gmail.com; Ana Lúcia Fachin - analuf@rge.fmrp.usp.br; Renato S Cardoso - rscardoso@hotmail.com; Simoneide S Silva - simoneidesilva@yahoo.com.br; Larissa Fernandes - larissaf@unb.br; Silvana P Silva - petrofez@uol.com.br; Eduardo A Donadi - eadonadi@fmrp.usp.br; Elza T Sakamoto-Hojo - etshojo@usp.br; Geraldo AS Passos - passos@rge.fmrp.usp.br; Célia MA Soares - celia@icb.ufg.br; Marcelo M Brígido - brigido@unb.br; Maria Sueli S Felipe* - msueli@unb.br

* Corresponding author

Abstract

Background: Mycelium-to-yeast transition in the human host is essential for pathogenicity by the fungus *Paracoccidioides brasiliensis* and both cell types are therefore critical to the establishment of paracoccidioidomycosis (PCM), a systemic mycosis endemic to Latin America. The infected population is of about 10 million individuals, 2% of whom will eventually develop the disease. Previously, transcriptome analysis of mycelium and yeast cells resulted in the assembly of 6,022 sequence groups. Gene expression analysis, using both *in silico* EST subtraction and cDNA microarray, revealed genes that were differential to yeast or mycelium, and we discussed those involved in sugar metabolism. To advance our understanding of molecular mechanisms of dimorphic transition, we performed an extended analysis of gene expression profiles using the methods mentioned above.

Results: In this work, continuous data mining revealed 66 new differentially expressed sequences that were MIPS(Munich Information Center for Protein Sequences)-categorised according to the cellular process in which they are presumably involved. Two well represented classes were chosen for further analysis: (i) control of cell organisation – cell wall, membrane and cytoskeleton, whose representatives were *hex* (encoding for a hexagonal peroxisome protein), *bgl* (encoding for a 1,3-β-glucosidase) in mycelium cells; and *ags* (an α-1,3-glucan synthase), *cda* (a chitin deacetylase) and *vrp* (a verprolin) in yeast cells; (ii) ion metabolism and transport – two genes putatively implicated in ion transport were confirmed to be highly expressed in mycelium cells – *isc* and *ktp*, respectively an iron-sulphur cluster-like protein and a cation transporter; and a putative P-type cation pump (*pct*) in yeast. Also, several enzymes from the cysteine *de novo* biosynthesis pathway were shown to be up regulated in the yeast form, including ATP sulphurylase, APS kinase and also PAPS reductase.

Conclusion: Taken together, these data show that several genes involved in cell organisation and ion metabolism/ transport are expressed differentially along dimorphic transition. Hyper expression in yeast of the enzymes of sulphur metabolism reinforced that this metabolic pathway could be important for this process. Understanding these changes by functional analysis of such genes may lead to a better understanding of the infective process, thus providing new targets and strategies to control PCM.

Background

The availability of great amounts of raw genomic and transcriptome data collected from several organisms has prompted the development of large-scale gene expression analysis which will ultimately help to unravel the function of many genes in diverse biological contexts. Different approaches such as cDNA microarrays [1-3], *in silico* ESTs subtraction [4,5] and serial analysis of gene expression – SAGE [6,7] are widely employed to assess differential gene expression patterns leading to the discovery of a great number of genes that are over or under expressed in each physiological context. The successful use of the cDNA microarray approach in fungal pathogens such as *Candida albicans* [8-13], *Histoplasma capsulatum* [14] and *Cryptococcus neoformans* [15] has resulted in the identification of genes involved in cell viability and opened new experimental perspectives to understand host-parasite interactions and thus develop new therapeutic approaches to systemic mycoses [8,11].

Paracoccidioidomycosis (PCM) is a human illness endemic to Latin America [16]; its area of incidence spreads non-uniformly from Mexico to Argentina [17], being higher in Brazil, Venezuela, Colombia and Argentina [18,19,16]. An estimation for Brazil points to an incidence rate between 1 and 3 and a mortality rate of 1.4 per million [20]. McEwen *et al*. [21] reported an overall infected population of 10 million individuals in Latin America, 2% of whom will eventually develop the disease. In nature, another important mammalian host is the armadillo *Dasypus novemcinctus* [22]. PCM affects the skin, lymph nodes and various internal organs, including the lungs – where it causes granulomatous processes – and the central nervous system [19,23]. Its clinical presentations range from a localised and benign disease to a progressive and potentially lethal systemic infection [24]. The disease is more frequent in adult males, who account for up to 90% of all cases. Healthy rural workers are the main targets, but PCM affects immunosuppressed individuals as well [25,26], including as much as 30% of AIDS patients [27]. All patients from whom the fungus is isolated must be treated and, in spite of new antifungal drugs, pulmonary fibrosis is still the most frequent sequel. The outcome of infection depends on several factors, including host responses and the virulence of the infecting isolate.

The causative agent of PCM, the thermo-regulated dimorphic fungus *P. brasiliensis*, is believed to be a free-living mycelium saprobe that undergoes transition to the yeast pathogenic form upon temperature change from the environmental 24–26°C to the mammalian body temperature of 37°C. This switch is necessary and sufficient to trigger morphotype interconversion *in vitro*, which makes this fungus an interesting model to study fungal cell differentiation at the molecular level. The biochemical events regulating dimorphic transition in *P. brasiliensis* are yet poorly defined, although relevant molecular-level information on this process has been partially described in the transcriptome analyses of two different *P. brasiliensis* isolates [28-30].

The exact ecological niche of this pathogen is still unknown [17], but *P. brasiliensis* can be retrieved from the soil. The fungus *Penicillium marneffei* is greatly similar in that it is a human opportunistic pathogen that also undergoes thermally-controlled dimorphic transition upon infection, can also infect a wild mammal (the bamboo rat) and has an yet unknown natural reservoir. Genomic data provided evidence that, in the case of *P. marneffei*, the fungus may have a sexual stage as a free-living organism [31].

Phylogenetic analysis of members of the order Onygenales demonstrated a close relationship of *P. brasiliensis* with the pathogenic fungi *Blastomyces dermatitidis*, *Emmonsia parva* and *Histoplasma capsulatum* [32]. *P. brasiliensis* can be fitted with *B. dermatitidis* and *E. parva* in the family Onygenacea [33]. Recently it was reported that *P. brasiliensis* is in fact a complex of at least three closely correlated phylogenetic species [34]. So far, the sexual phase of the ascomycete *P. brasiliensis* was not reported limiting our knowledge about the mechanisms that contribute to its dimorphism, pathogenicity, and virulence. *P. brasiliensis* isolates shows chromosomal polymorphism; it contains 4–5 chromosomal DNA molecules with molecular sizes ranging from 2–10 Mb [35,36]. The genome size was estimated to be around 30 Mb [37] and DNA sequencing of ~ 50 Kb revealed a density of one gene per 3.5–4.5 Kb, suggesting a total of 7,500–9,000 genes [38].

Recently, our group analysed the transcriptome of the Pb01 isolate, represented by a set of 6,022 clusters. The 16 genes that were then found to be differentially expressed

by both methods used – *in silico* EST subtraction and cDNA microarray – were categorised by function. We chose to discuss in that work those that were involved in core metabolic pathways such as sugar metabolism [28]. Now, continued overlap analysis from raw data revealed 66 new genes that are differentially expressed in one or other morphotype. Upon categorisation by known databases we have selected two MIPS [39] classes, which were chosen to be confirmed by northern blotting. Here we present the result of this extended analysis, and discuss the putative roles the differential genes – related to cell organisation and ion metabolism and transport – play in the corresponding morphotype of this pathogen. One of the discussed pathways – *de novo* cysteine synthesis from inorganic sulphate, a branch of sulphur metabolism – was almost entirely up-regulated in the yeast form. The importance of sulphur metabolism to the life cycle of pathogenic fungi has been extensively reviewed elsewhere [40,41] and recently new data from microarray experiments have arisen from work in *H. capsulatum* that support a role of organic sulphate in the maintenance of the yeast phase [14]. In a previous report [42], the importance

of organic sulphates to the growth and differentiation of *P. brasiliensis* was assessed. This phenomenon demanded further investigation and prompted us to assess up- and downregulation of sulphur metabolism genes in mycelium and yeast cells and also dimorphic transition in both directions without inorganic sulphate as a sulphur source. We have thus found that this compound is unnecessary for the process.

Results and discussion
Differentially expressed genes identified by in silico EST subtraction and cDNA microarray
Comparative gene expression profiling in dimorphic fungi can reveal key proteins involved in commitment to differentiation and gene regulation. From the 66 new PbAESTs (*P. brasiliensis* assembled expressed sequence tags) identified in this work, thirteen of which correspond to up-regulated genes in mycelium and fifty four which are differential for yeast cells (Tables 1 and 2). This set complements the one generated previously [28], which included 16 genes that were differential by the same overlap analysis and also 30 genes that were differential

Table 1: Mycelium up-regulated genes identified by *in silico* ESTs subtraction and cDNA microarray.

PbAEST	Acession Numbers (GenBank)	Annotated function	Number of reads		P-value[a]	Fold change	Accession Number/Best-hit organism/E-value	Functional categories
			M	Y				
202	CA582032	1,3-beta-glucosidase*	7	2	0.036942	12.3	AAL09828.1/*C. immitis*/1.0E-132	Control of cell organization: Cell wall and membrane
2155	CA582352	Peroxisomal membrane protein PEX16 (peroxin-16)	7	0	0.004174	1.4	EAL88469.1/*A. fumigatus*/3.0E-64	
186	CA583085	HEX*	13	8	0.049272	3.4	EAL91716.1/*A. fumigatus*/3.0E-66	
2496	CA583518	Iron-sulphur cluster nifU-like protein*	5	1	0.048854	1.7	EAL90111.1/*A. fumigatus*/8.0E-58	Ion transport
4179	CN245816	Potassium transporter protein*	0	1	-[b]	5.2	CAA08814.1/*N. crassa*/4.0E-22	
1420	CN247275	U1 small nuclear ribonucleoprotein	9	1	0.00526	1.6	EAL91268.1/*A. fumigatus*/1.0E-60	Transcription
1029	CA582332	Methyltransferase	32	1	0.000000	2.1	EAL84975.1/*A. fumigatus*/1.0E-56	Others
2096	CA581148	Unkown	20	1	0.000006	5.6	-	
514	CA583322	Unkown	15	1	0.000138	23.4	-	
1045	CA581951	Unkown	13	2	0.001769	24	-	
1178	CN247241	Unkown	10	0	0.000535	8.5	-	
1664	CN247289	Unkown	10	3	0.018648	2.5	-	

[a] FDR = 4,8% and Q-value < 5%.
[b] Not significant by Audic-Claverie's method.
* Up-regulated genes confirmed by northern blotting.
** Not assayed by cDNA microarray but confirmed as up-regulated in mycelium by northern blotting.

Table 2: Yeast up-regulated genes identified by *in silico* ESTs subtraction and cDNA microarray.

PbAEST	Acession Numbers (GenBank)	Annotated function	Number of reads M	Number of reads Y	P-value[a]	Fold change	Accession Number/Best-hit organism/E-value	Functional categories
1422	CA581980	Alpha-1,2-mannosyltransferase (Alg11)	4	11	0.019803	2.0	EAL88400.1/*A. fumigatus*/1.0E-130	Control of cellular organization: Cell wall and membrane
4988	CN253911	Alpha 1,3-glucan synthase*	-	1	-	5.7	AAV52833.1/*P brasiliensis*/4.0E-93	
2162	CN238153	Putative WW domain protein (probable membrane protein)	4	12	0.013092	3.6	EAL85876.1/*A. fumigatus*/6.0E-17	
136	CA582283	Involved in cytoskeletal organization and cellular growth (verprolin)*	4	10	0.029289	4.0	NP_013441.1/*S. cerevisiae*/2.3	
667	CA583397	Adenylylsulphate kinase	3	8	0.038949	2.1	EAL90409.1/*A. fumigatus*/1.0E-88	Ion transport and metabolism
48	CA582091	ATP-sulphurylase	10	18	0.023038	4.8	EAL92915.1/*A. fumigatus*/0.0	
2031	CA581274	Outer mitochondrial membrane protein porin	1	14	0.000207	1.3	XP_323644.1/*N. crassa*/1.0E-108	
2724	CA581633	P-type Cu(2+) transporting ATPase*	0	1	-[b]	3.8	NP_009854.1/*S. cerevisiae*/1.7E-20	
635	CN247312	ATP citrate lyase	1	7	0.014984	1.9	EAL88915.1/*A. fumigatus*/0.0	Energy
2016	CN242578	ATPase inhibitor; Inh1	2	14	0.000835	2.7	NP_010100.1/*S. cerevisiae*/4.0E-08	
563	CA583982	Cytochrome c oxidase subunit VII	11	43	0.000002	2.1	AAT77147.1/*P. brasiliensis*/3.0E-26	
2398	CN240705	Disulfide isomerase	3	8	0.038949	2.1	EAL91387.1/*A.fumigatus*/3.0E-61	
540	CN240558	Cytochrome C oxidase biogenesis protein	0	5	0.015111	1.8	XP_214182.2/*R. norvegicus*/1.0E-06	
578	CA582837	Pyruvate dehydrogenase e1 component beta subunit	2	7	0.033994	1.6	EAL86696.1/*A. fumigatus*/2.0E-99	
407	CA583387	Succinyl-CoA synthetase alpha subunit	7	19	0.004468	2.6	EAL91981.1/*A. fumigatus*/1.0E-155	
284	CN239025	Ubiquinol-cytochrome C reductase complex ubiquinonE-binding protein QP-C	0	4	0.030475	1.5	EAL90680.1/*A. fumigatus*/7.0E-29	
378	CA580847	Argininosuccinate synthase	0	6	0.007492	1.7	NP_229577.1/*T. maritime*/4.0E-77	Amino acid metabolism and transport
1618	CA583639	Aromatic-L-amino-acid decarboxylase	1	33	0.00000	17.2	EAL86509.1/*A. fumigatus*/0.0	
125	CA583825	Glycine cleavage system h protein	4	9	0.042192	1.4	EAL90537.1/*A. fumigatus*/6.0E-36	
1674	CA583874	Aldolase	5	14	0.010368	37.9	AAL34519.2/*P. brasiliensis*/0.0	C-compound and carbohydrate metabolism
42	CA581699	Phosphoglycerate kinase	1	10	0.002512	2.6	EAL90363.1/*A. fumigatus*/0.0	
9	CA581893	Beta-ketoacyl synthase (Cem 1)	1	5	0.045709	2.2	EAL87667.1/*A. fumigatus*/1.0E-88	Lipid, fatty-acid and isoprenoid metabolism
780	CA581145	GPR/FUN34 family protein	0	11	0.000225	14.9	EAL87502.1/*A. fumigatus*/6.0E-67	
1989	CA581550	Acetyl-coenzyme A synthetase (AcetatE – CoA ligase) (Acyl-activating enzyme)	1	9	0.004605	2.0	EAL89682.1/*A. fumigatus*/0.0	
1550	CA582818	NADH-cytochrome b5 reductase	0	6	0.007492	5.4	EAL88164.1/*A. fumigatus*/1.0E-86	

Table 2: Yeast up-regulated genes identified by *in silico* ESTs subtraction and cDNA microarray. *(Continued)*

300	CA581937	Nucleoside diphosphate kinase	6	58	0.00000	1.6	AAP85295.1/*A. fumigatus*/2.0E-67	Nucleotide metabolism
547	CA583473	6,7-dimethyl-8-ribityllumazine synthase	0	6	0.007492	1.4	AAD55372.1/*A. fumigatus*/9.0E-56	Metabolism of vitamins, cofactors, and prosthetic groups
924	CN240624	Coproporphyrinogen III oxidase	2	7	0.033994	2.7	EAL88456.1/*A. fumigatus*/0.0	
867	CA580742	NADH pyrophosphatase	1	5	0.045709	5.7	EAL85969.1/*A. fumigatus*/1.0E-159	
1490	CA583063	Pyridoxamine 5'-phosphate oxidase	0	10	0.000453	3.5	AAC28862.1/*S. commune*/2.0E-32	
447	CA580589	NADH:ubiquinone oxidoreductase B18 subunit	1	10	0.002512	1.4	EAL92195.1/*A. fumigatus*/9.0E-33	
488	CA582788	Exonuclease II	1	5	0.045709	1.9	EAL85993.1/*A. fumigatus*/1.0E-138	Transcription, translation and ribosome structure
165	CN241393	RNP domain protein	3	13	0.003962	1.8	EAL89070.1/*A. fumigatus*/5.0E-81	
2436	CA580512	Splicing factor u2af 35 kd subunit	2	7	0.033994	2.5	EAL86523.1/*A. fumigatus*/1.0E-103	
253	CN240426	Zinc finger, C3HC4 type	0	5	0.015111	1.4	NP_593329.1/*S. cerevisiae*/3.0E-10	
551	CN239696	Ribosomal protein L35**	5	10	0.044755	-	AAL08563.1/*P. brasiliensis*/1.0E-63	
979	CA582579	60S ribosomal protein L7/L12 precursor	1	8	0.008358	1.3	EAL89813.1/*A. fumigatus*/4.0E-49	
175	CA581863	Complex I intermediatE-associated protein CIA30 precursor	4	15	0.003399	5.6	EAL92946.1/*A. fumigatus*/1.0E-114	Protein fate and Secretion
832	CN242383	Glutathione S transferase	1	7	0.014984	2.0	NP_588171.1/*S. pombe*/7.0E-42	
2387	CA584103	Non-classical export protein (Nce1)	1	7	0.014984	55.6	EAL87256.1/*A. fumigatus*/1.0E-29	
1823	CA583903	Profilin	1	5	0.045709	1.3	NP_014765.1/*S. cerevisiae*/8.0E-14	
4188	CN245872	Mating type protein (MAT1–2)*	1	0	-	8.0	EAL89707.1/*A. fumigatus*/2.0E-36	Mating Type
50	CA581392	Cu-Zn superoxide dismutasE-related*	0	8	0.001842	2.1	CAB97297.1/*N. crassa*/3.0E-30	Virulence and oxidative stress
2059	CN241260	Ribosome associated protein (Stm1)	6	31	0.000007	1.7	EAL92489.1/*A. fumigatus*/2.0E-32	Others
2005	CA580764	Signal peptide protein	1	6	0.026442	2.3	EAL93249.1/*A. fumigatus*/7.0E-68	
39	CA581046	Unknown	0	6	0.007492	2.2	-	
33	CA582496	Unknown	0	8	0.001842	3.1	-	
1442	CA581846	Unknown	3	16	0.000836	4.5	-	
2399	CA581839	Unknown	1	5	0.045709	2.5	-	
512	CA583749	Unknown	0	6	0.007492	4.3	-	
639	CA581506	Unknown	0	7	0.003715	1.7	-	
718	CN247671	Unknown	0	6	0.007492	1.8	-	
765	CA581478	Unknown	0	10	0.000453	3.9	-	
529	CA580398	Unknown	1	5	0.045709	18.8	-	

[a] FDR = 4,8% and Q-value < 5%.

[b] Not significant by Audic-Claverie's method.

* Up-regulated genes confirmed by northern blotting.

** Not assayed by cDNA microarray but confirmed as up-regulated in yeast by northern blotting.

according to *in silico* EST subtraction alone. MIPS functional categories [43] were used to classify the 66 PbAESTs into 14 major groups (data not shown). Gene categorisation revealed some that are involved in energy production (11%) – this was expected considering the adaptation process that is required for the mycelium-to-yeast transition; control of cell wall organisation (10%); ion metabolism and transport (8%); transcription, translation and ribosome structure (8%); virulence and oxidative stress (4%). Manual annotation under stringent criteria of sequence alignment with other dimorphic fungi gene sets allowed us to ascribe a putative biological function to many of those genes. The genes that belonged in two categories – cell wall organisation and ion metabolism and transport – were selected for confirmation by northern blotting.

Mycelium and yeast up-regulated genes involved in cell organisation

The *hex* and *bgl* genes, which code for the hexagonal peroxisome protein and 1,3 β-glucosidase, respectively, were up-regulated in mycelium cells and are categorised as involved in cell wall, membrane and cytoskeleton organisation (Figure 1a). The hexagonal peroxisome protein has been identified in different filamentous ascomycetes such as the plant pathogen *Magnaporthe grisea* [44] and in *Neurospora crassa* [45], being the major protein of the Woronin body, a septal pore-associated organelle [46,47]. HEX1p has been shown to seal septal pores in response to cellular damage and is strongly implicated in cell integrity maintenance [45]. In *M. grisea*, *hex1* mutants present delayed host penetration and subsequent disruption of invasive hyphal growth in plants. Inability of these mutants to survive under nitrogen starvation *in vitro* has also been observed [44]. Deletion of *hex1* in *N. crassa* eliminates Woronin bodies from the cytoplasm and results in hyphae that exhibit a cytoplasmic-bleeding morphotype in response to cell lysis [45]. It was thus proposed that the Woronin body represents a new category of peroxisome acting in the maintenance of cellular integrity and virulence in filamentous fungi [45]. We hypothesise that these highly specialised vesicles are involved in the protection of *P. brasiliensis* against cellular damage as well as its survival during invasive growth and host colonisation in the process of infection. Future investigations are required to elucidate the role of Woronin bodies/HEX1 protein in *P. brasiliensis*.

Another mycelium up-regulated gene codes for β-1,3-glucosidase, an enzyme that hydrolyses the O-glycosidic linkages of β-glucan. This polysaccharide is an important cell wall constituent in *P. brasiliensis* mycelium cells in contrast with α-glucans, which predominate in the yeast cell wall [48]. A hypothesis formulated by Kanetsuna *et al.* [49] and modified by San Blas and San Blas [50] explains
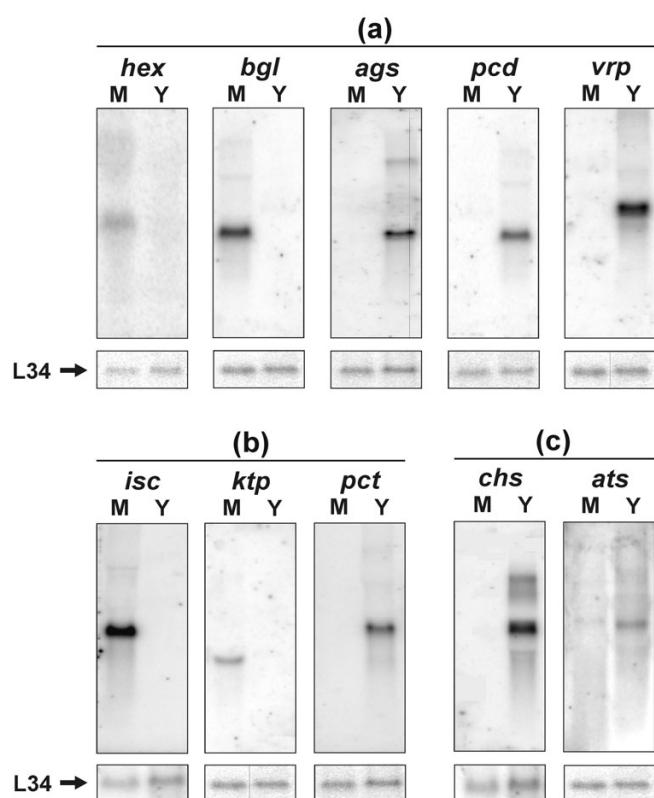
Figure 1
Northern blot analysis of mycelium and yeast up-regulated genes of *P. brasiliensis*. Total RNA samples from both mycelium (M) and yeast (Y) were blotted onto nylon membranes and hybridised against gene-specific radiolabelled probes: **(a)** Control of cell organisation: *hex* – Hexagonal peroxisome protein, *bgl* –1,3 beta-glucosidase, *ags* – alpha 1,3-glucan synthase, *cda* – Chitin deacetylase, *vrp* – Verprolin; **(b)** Ion transporters: *isc* –Iron-sulphur cluster-like protein, *ktp* – Potassium transporter, *pct* – Putative P-type Cu(2+) transporting ATPase; **(c)** Sulphur metabolism: *chs* – Choline sulphatase, *ats* – ATP sulphurylase. The constitutive 60S ribosomal protein L34 was used as a loading control.

the differentiation from mycelium to yeast and vice-versa based on a change on cell wall composition. At 37°C, there is an increased synthesis of chitin and α-glucan, and low levels of β-glucan, which results in the yeast form. In contrast, at 22°C, α-glucan synthesis occurs at low rates and long β-glucan fibrils are formed in the budding spots. In keeping with these morphological and biochemical events, 1,3-β-glucosidase increased levels are correlated to the shift to the mycelium phase.

Other three genes coding for proteins from the same category were confirmed to be up-regulated in yeast cells: *ags* (α-1,3-glucan synthase), *cda* (chitin deacetylase) and *vrp*-verprolin (Fig. 1a). The *P. brasiliensis* 1,3-α-glucan synthase gene was first described by Pereira *et al.* [51].

Recently, it was demonstrated that it is strongly up-regulated in yeast cells [28,52], which was confirmed in this work by northern blotting analysis. Rappleye *et al.* [53] silenced the 1,3-α-glucan synthase gene in *H. capsulatum* and demonstrated that α-(1,3)-glucan is an important virulence factor and affects the ability of *H. capsulatum* to kill macrophages and colonise murine lungs. In *C. neoformans*, mutants for 1,3-α-glucan synthase failed to assemble the capsule, which is an important virulence factor of this pathogen [54]. Morphogenetic transition is the essence of *P. brasiliensis* life cycle: for instance, low levels of α-1,3-glucan in the cell wall of the yeast form have been correlated with low virulence [55]. Virulent cultures of *P. brasiliensis* isolates grown i *n vitro* for long periods have thinner cell walls, low α-1,3-glucan levels and are consequently less virulent [56]. Our results suggest that α-glucan synthase is involved in the dimorphic transition of *P. brasiliensis* and possibly in its virulence. The cell wall is an essential and dynamic fungal structure that has been implicated in several pathogenic processes. Being absent in mammalian cells, it may be a relevant target to drug therapies. In this context, the gene that encodes α-1,3-glucan synthase was demonstrated to be a virulence factor using RNAi approaches in *Cryptococcus neoformans* [54] and *H. capsulatum* [53], and seems to be an ideal target for new antifungal drugs. In *P. brasiliensis* glucan polymers constitute 95% of yeast cell wall [49] and thus any interference in cell wall synthesis through glucan synthases is likely to affect virulence directly.

Chitin deacetylase enzyme (CDA) catalyses the conversion of chitin to chitosan by deacetylation of N-acetyl-D-glucosamine residues. Chitosan is a flexible, soluble polymer that integrates the cell wall of some fungi, such as *S. cerevisiae* [57] and *C. neoformans* [58]. In *S. cerevisiae*, chitosan is only found during sporulation [59]. The molecular characterisation of two sporulation-specific chitin deacetylase genes, *CDA1* and *CDA2*, both of which contribute to spore wall rigidity, was described previously [59]. In *S. cerevisiae*, *cda1* mutants present a more diffuse chitosan layer, while their surface layer remains intact. In *cda2* mutant cells, by comparison, the chitosan layer is not detected at all. In the spore walls of *cda1* and *cda2* mutants both outer layers are missing due to defects on wall maturation. However, in *C. neoformans*, a study reported that chitin is present in the yeast cell wall and most of it is continually deacetylated to chitosan. Mutants for chitin deacetylase show suppression of growth due to the lack of chitosan and therefore have a reduced infection capability [58]. The same study hypothesized that this constant remodelling of the cell wall contributed to cellular integrity in this fungus. In *P. brasiliensis*, we identified a highly expressed *cda* gene in yeast cells that presents similarity to the *C. neoformans*. If the *C. neoformans* model is closer to what is found in *P. brasiliensis*, then chitin synthase and chitin deacetylase may be potential targets to antifungal therapy.

Verprolin is required for a fully polarised distribution of cortical actin patches and viability at high temperature. This is the first time that verprolin is described in *P. brasiliensis*, a pathogen that has as an intrinsic characteristic the ability to grow at the human body temperature, 37 °C. The inability of *vrp-1* mutants to grow at 37 °C was reported by Naqvi *et al.* [60] in the non-pathogenic yeast *S. cerevisiae*. Likewise, we hypothesise that verprolin is involved in the ability of *P. brasiliensis* to grow at 37°C and in cell cytoskeleton organisation since this gene is over expressed in yeast cells. Considering that the actin cytoskeleton plays a crucial role on fundamental processes such as cell growth, differentiation and migration, localised membrane growth, endocytosis, and cell division [61], this protein is likely to play a key role in cell maintenance and viability of *P. brasiliensis* inside the host cell.

Mycelium and yeast up-regulated genes involved in ion metabolism and transport

Two genes putatively implicated in ion transport were confirmed to be highly expressed in mycelium cells: *isc* and *ktp*, an iron-sulphur cluster protein and a cation transporter, respectively. In contrast, a putative P-type cation pump (*pct*) was up-regulated in the yeast form (Figure 1b).

It has been reported that the ISC protein is responsible for mitochondrial uptake of iron and seems to monitor the cytoplasmic levels of this ion. In *S. cerevisiae*, the double knock-out of the homologues *ISU1* and *ISU2* is lethal. Defective mutants are distinguished by iron accumulation in the mitochondrial matrix and its respective decrease in the cytosol [62]. In *C. neoformans*, complementation, cloning and sequencing of such genes has recently been accomplished [63]. It has long been hypothesised that iron is a limiting factor for infectivity during cryptococcosis as well as in other systemic mycoses, in that the host normally provides only limited amounts of this compound. Arango and Restrepo [64]demonstrated iron availability to be essential for growth of mycelium and yeast of *P. brasiliensis*; but especially for mycelium, whose growth was totally prevented by the addition of the iron chelator phenanthroline to the medium, an effect observed only to a lesser extent in yeast. The effect of phenanthroline was reversed partially in mycelium and totally in yeast by addition of excess iron. This is in good agreement with the overexpression of the ISC protein in the mycelial phase. In *P. brasiliensis* it could be involved in monitoring the amount of iron in the environment and in providing a means of storage of this metal.

The *ktp* sequence from *P. brasiliensis* aligned best with potassium transporter proteins of the HAK family, which are mainly implicated in the resistance to potassium starvation. In *N. crassa*, the closest homolog of *P. brasiliensis*, KTP coexists with another potassium transporter of the TRK family [65]. It has been hypothesised that soil organisms are universally equipped with a powerful K+-concentrating apparatus, as these organisms are faced with a very diluted and variable environment, thus being forced to pump potassium in against a steep gradient [65]. This is likely to be the case of *P. brasiliensis*, whose ecological niche for the mycelium form is thought to be the soil.

Another yeast up-regulated gene is *pct*, a putative member of the E1-E2 (P-type) family of ATPases. These are ATP-dependent proteins which regulate transmembrane flow of all relevant cations, including Na+, H+, Mg2+, Ca2+, Cd2+, Cu2+ and K+ [66]. In *C. albicans*, the E1-E2 ATPase gene, *CDR1*, confers resistance to both copper and silver, the latter being used as an antimicrobial agent [67]. A similar function could be attributed to the *P. brasiliensis pct* gene, although alignment data are insufficient to identify which cation this protein transports.

Sulphur metabolism

Several enzymes from the cysteine *de novo* biosynthesis pathway (Figure 2) were shown to be up-regulated in the

yeast form of *P. brasiliensis*. Our previous analysis [28] had already confirmed over expression of paps reductase (the third in the pathway). *In silico* EST subtraction and cDNA microarray showed yeast up-regulation for atp sulphurylase and aps kinase; the former was confirmed by northern blotting (Figure 1c). Thus, we can strongly suggest that the yeast form synthesises cysteine actively from inorganic sulphate.

In order to reinforce these data, we have evaluated the importance of inorganic sulphate to growth and differentiation. Auxotrophy of *P. brasiliensis* yeast for several sources of organic sulphate – including cysteine itself and sulphydrylic compounds – has been reported before [42]. It was concluded then that organic sulphate deprivation suppressed growth in the yeast phase and prevented myc-elium-to-yeast differentiation, whereas the mycelial phase is able to grow on either inorganic or organic sulphur [68]. Also, the saprophytic, mycelial form of *H. capsulatum* is prototrophic while the pathogenic yeast form requires cysteine [69]. It has been reported that exogenous cysteine is required for both yeast phase growth and morphological transition from mycelium-to-yeast of *H. capsulatum* [41,70]. In this work, both mycelium and yeast cells of *P. brasiliensis* were incubated in modified MVM medium without inorganic sulphate, apart from the negligible amounts present in the trace elements solution. Dimor-
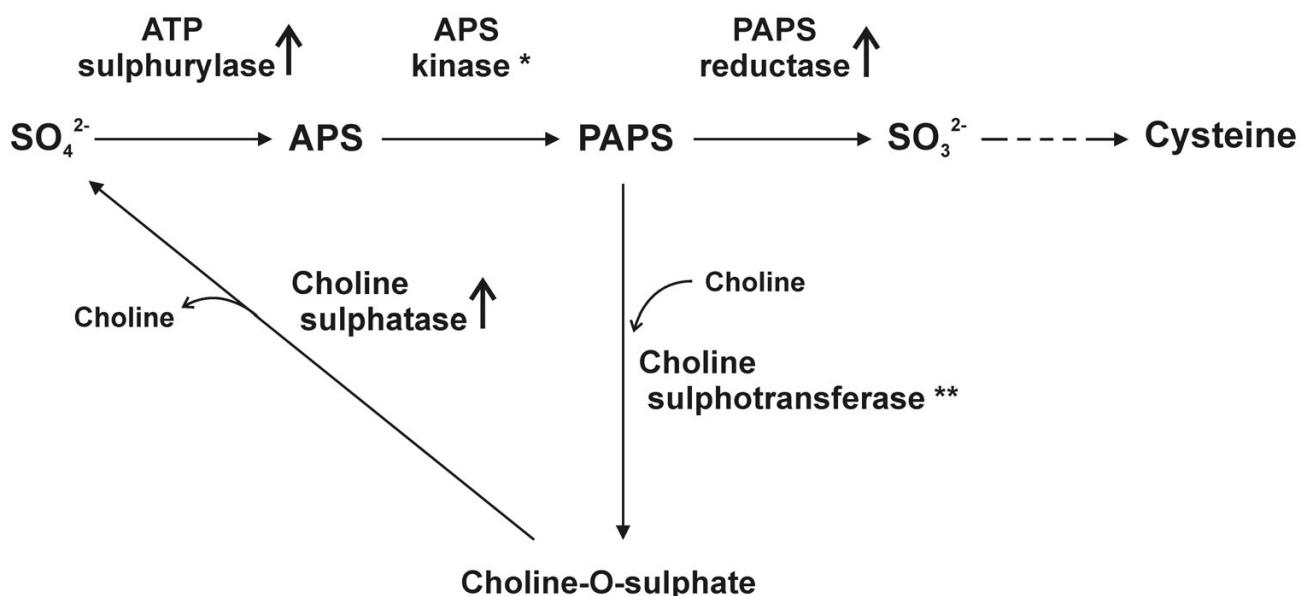
Figure 2
Up-regulated genes encoding enzymes from the cysteine *de novo* biosynthesis pathway. Arrows indicate enzymes identified as up-regulated both by *in silico* subtraction, cDNA microarray and confirmed by northern blotting experiments. (*) enzyme identified as up-regulated by both *in silico* subtraction and cDNAs microarray but not assayed by northern blotting. (**) indicates an enzyme not found in the transcriptome of *P. brasiliensis*.

phic transition was assessed in the mycelium to yeast direction and in the opposite way. Sustained growth was observed for both morphotypes (data not shown) and, upon the corresponding temperature shifts, differentiation was successfully triggered in both directions (Figure 3). Thus, inorganic sulphate seems to be unnecessary for the transition, quite contrarily to organic sulphate. In this context, it is interesting to consider a branch of the cysteine biosynthetic pathway (Fig. 2). In fungi and plants a fraction of PAPS, which is toxic to fungi if it reaches high cytosolic levels, is used by choline sulphotransferase to produce choline-O-sulphate [40], which serves as an osmoprotectant and cytosolic sulphur store in these organisms. We have not found a homologue of choline sulphotranferase in *P. brasiliensis* to date, but the enzyme choline sulphatase, which degrades its product to choline and sulphate, is also over expressed in the yeast morphotype, as confirmed here (Figure 1c) and previously reported [52]. The *C. neoformans met3* mutant, which lacks ATP sulphurylase activity, had a substantial defect in melanin formation, significantly reduced growth rate, and greatly increased thermotolerance. In the murine inhalation infection model, the *met3* mutant was avirulent and was deficient in its ability to survive in mice [71]. In this context, disrupting the genes encoding choline sulphatase or ATP sulphurylase in *P. brasiliensis* should reveal its role

in the growth, maintenance of yeast cells and pathogenicity of this fungus. It is interesting that another intracellular pathogen of humans, the bacterium *Mycobacterium tuberculosis*, depends on sulphur compounds for expression of its full virulence, drug resistance and overall survival inside the macrophage. It has developed a very efficient sulphate activation pathway (SAC) that ensures constant synthesis of PAPS at high rates, from which sulphate may be distributed to other synthetic pathways [72]. The SAC includes the bacterial counterparts of ATP sulphurylase and APS kinase, the latter of which performs PAPS synthesis by coupling it with GTP hydrolysis by a GTPase that is also present in SAC. Whether similar mechanisms are present in pathogenic fungi such as *P. brasiliensis* remains to be investigated.

Conclusion

Taken together, these data show that several genes involved in cell organisation and ion metabolism/transport are differential in their expression along dimorphic transition, which is in accordance with the proposed model for this process in Figure 4. While α-glucan is synthesised during yeast phase due to high expression of 1,3 α-glucan synthase, β-glucan is degraded by the action of 1,3 β-glucosidase during hyphal growth. The *cda* gene is probably involved in the cell wall synthesis of yeast cells,
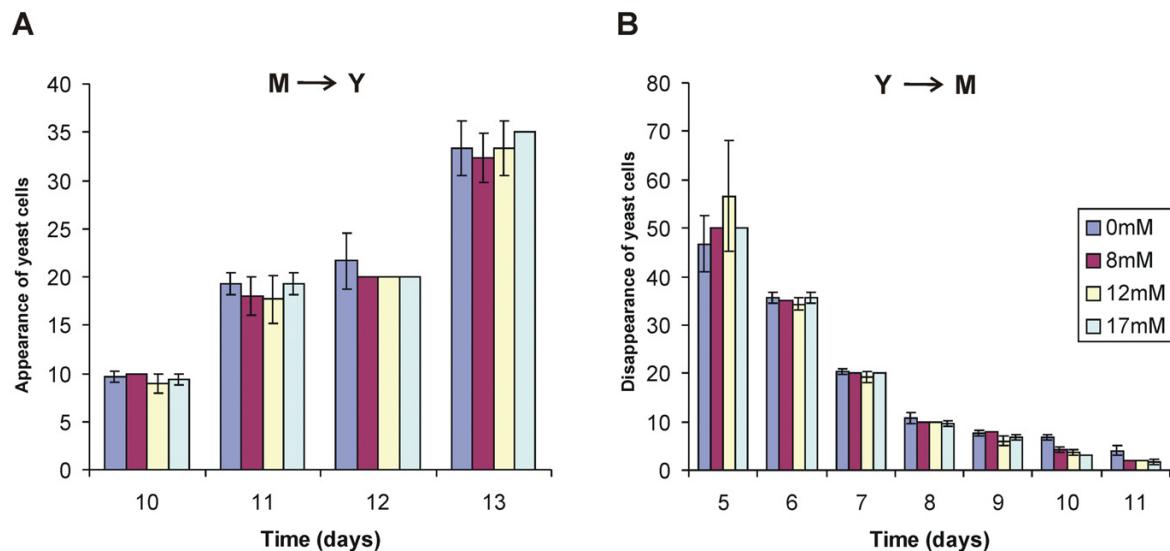
Figure 3
Cell differentiation of *P. brasiliensis* in modified MVM medium without inorganic sulphate. The fungus was grown in four different concentrations of sulphate salts (0, 8, 12 and 17 mM; the latter is the original concentration of MVM medium). (A) The appearance of yeast cells was verified daily in the transition from mycelium to yeast after temperature shift to 37°C, (B) The disappearance of yeast cells was verified daily in the transition from yeast to mycelium after temperature shift to 22°C. Triple samples were counted for each time point. The coloured boxes indicate the average of the three samples and bars represent the standard deviation of the mean. As observed, the presence or absence of inorganic sulphate did not affect transition in either direction.
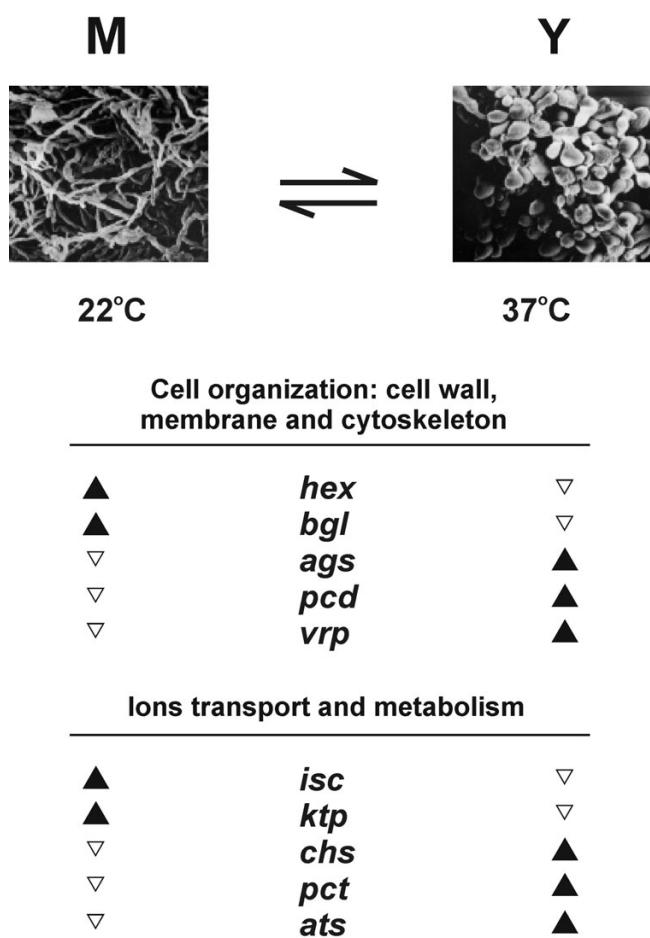
Figure 4
Genes involved in cell organisation (cell wall, membrane and cytoskeleton), sulphur metabolism and ion transport. Genes that were identified as up-regulated in mycelium (22°C) or yeast (36°C) cells of *P. brasiliensis* are represented by black arrowheads. Electron microscopy was performed by Silva *et al.* [78].

since it is over expressed in this phase. In addition, genes related to septal sealing and cytoskeleton organisation (*hex* and *vpr*) are also probably implicated in the stabilisation and maintenance of mycelium and yeast cells in the environment and at 37°C in the human host. Also, the differential expression pattern of genes that control metabolism and transport of potassium, iron, copper and sulphur ions suggests that they may influence directly the pathogen adaptation to the host environment. Specifically, in spite of the undisturbed growth and differentiation on depletion of inorganic sulphate, the over expression of enzymes from *de novo* cysteine synthesis lends support to previous findings about the importance of this pathway to yeast metabolism. Understanding these changes by functional analysis of such genes may lead to

a better understanding of the infective process, thus providing new targets and strategies to control PCM.

Methods
Strains and cultures
P. brasiliensis clinical isolate Pb01 (ATCC-MYA-826) was used in this study. Cells from both mycelium and yeast forms were grown on semi-solid Fava Neto's medium [73] for 7 to 10 days at 22°C or 37°C, respectively.

Overlap analysis – in silico EST subtraction and cDNA microarrays
This work was based on the output of previous large-scale expression analysis experiments, as outlined in reference 28. Briefly, we have constructed a λZAP II® (Invitrogen) cDNA library from mycelium and yeast mRNA fractions and 5'-sequenced the mass-excised cloned fragments with the T7 vector primer. Raw sequence data were quality-assessed by PHRED and assembled by CAP3, thus generating a set of 6,022 PbAESTs (singlets and contigs). For functional annotation of sequences we used the nr (NCBI) database. *In silico* electronic subtraction was performed according to the Audic and Claverie [74] statistical approach, with a 95% confidence rate. For cDNA microarray 1,152 clones were selected and PCR-amplified for spotting onto nylon-membranes in triple experiments. Hybridisation against [α-^{33}P] dCTP-labeled total RNA from mycelium or yeast and phosphor imager signal capture were performed as in [28]. After signal quantification and background subtraction [75], statistical analysis was carried out with the SAM (Significance Analysis of Microarrays) method [76]. Data from both experiments were overlapped to identify differential genes, thus generating the set of 66 sequences we used in this work.

Northern blot analysis
Total RNA (15μg) from mycelium and yeast cells of *P. brasiliensis* was separated on denaturing 1,5 % agarose gel and blotted onto a Hybond-N membrane (GE Healthcare). Probes were radiolabeled using [α-P^{32}]dATP by random priming according to supplier's instructions (Invitrogen), purified and used in overnight hybridisation (50% formamide, 4X SSPE, 5X Denhardt's solution, 0,1% SDS, 100μg/ml herring sperm DNA) at 42°C. The membranes were then washed under stringency conditions of 2X SSPE-0.1% SDS at 65°C for 1h. Signal bands were visualised using the Typhoon 9210 Phosphor Imager (GE HealthCare).

Dimorphic transition without inorganic sulphate
We incubated both mycelium and yeast cells on modified versions of McVeigh and Morton's medium – MVM [77] where ammonium and magnesium sulphate salts were gradually replaced by their chloride counterparts, in the following chloride concentration set points: 0, 8, 12 and

17 mM, where the first corresponds to the original recipe and the last, to virtual absence of inorganic sulphate, apart from negligible amounts in the trace elements solution (~ 8 μM). Molar concentrations of both magnesium and ammonium were thus conserved. We have also evaluated whether dimorphic transition occurred normally in the medium without inorganic sulphur. To achieve this, five flasks containing 100 ml of modified MVM were inoculated with comparable amounts of mycelium (100 mg wet mass) and yeast (2.5 × 10⁷ cells) previously grown on standard MVM. Samples were incubated in rotating shakers (120 rpm) at 36 and 22°C, respectively, thus triggering dimorphic transition. Fungal viability and progress of transition were assessed by serial 100 μl sampling every 24 hours (three independent samples). Each sample was coloured with Janus Green and the number of yeast cells was counted in a light microscope with the aid of a Neubauer counting chamber.

Accession numbers
The accession numbers of the EST sequences analysed in this work are shown in the Tables 1 and 2.

Abbreviations
ags alpha 1,3-glucan synthase

aps adenosine 5'-phosphosulphate

ats ATP sulphurylase

bgl 1,3 beta-glucosidase,

BLAST basic local alignment search tool

cda chitin deacetylase

cDNA complementary DNA

chs choline sulphatase

COG clusters of orthologous groups

e-value extreme value distributionESTs

ESTs expressed sequence tags

GO gene ontology

hex hexagonal peroxisome protein

isc iron-sulphur cluster-like protein

ktp potassium transporter

MIPS Munich information center for proteins sequences

PAPS phosphoadenylyl-sulfate reductase

PbAETs *P. brasiliensis* assembled EST sequences

PCM paracoccidioidomycosis

pct putative P-type Cu(2+) transporting ATPase

SAGE serial analysis of gene expression

SAM significance analysis of microarrays

vrp verprolin

Authors' contributions
RA and MF planned and designed the study, developed the experiments and the data analysis, wrote the main draft of the paper and support the preparation of the figures and tables. HP supports the discussion of the results and revised the manuscript. AN participated in the *in silico* ESTs subtraction analysis of the raw data generated by the transcriptome project. MC analysed the results of the microarray experiments, helped in the manuscript edition, and prepared the figures. AL executed the microarray experiments. MC, RC and MB participated in the normalization process of the microarray raw data and helped to make the statistical analyses. SS participated of the differentiation experiment involved of the inorganic sulphur, and of the preparation of the RNA of *P. brasiliensis* to make the microarray experiments. LF participated of the analysis of the cell wall organization. SP helped in the ESTs amplification and on the analysis of the sulphur metabolism. GP, ES, ED designed the microarray experiments. CS participated on the Pb ESTs annotation. All authors read and approved the final manuscript.

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Upregulation of glyoxylate cycle genes upon Paracoccidioides brasiliensis internalization by murine macrophages and in vitro nutritional stress condition.

Upregulation of glyoxylate cycle genes upon *Paracoccidioides brasiliensis* internalization by murine macrophages and *in vitro* nutritional stress condition

Short title: *Paracoccidioides brasiliensis* glyoxylate cycle genes expression.

L.S. DERENGOWSKI[1], A.H. TAVARES[1], S. SILVA[1], L.S. PROCÓPIO[1], M.S.S. FELIPE[1] & I. SILVA-PEREIRA[1]*

[1]Lab. de Biologia Molecular, CEL/IB, Universidade de Brasília – Brasília-DF, Brasil.

*Corresponding author: Campus Universitário Darcy Ribeiro, Universidade de Brasília, Instituto de Biologia, Departamento de Biologia Celular, Laboratório de Biologia Molecular, 70910-900, Brasília, DF, Brasil. Fax: +55 61 349 8411. *E-mail*: xocolau@unb.br

Summary

Paracoccidioides brasiliensis, the etiologic agent of paracoccidioidomycosis, is a facultative intracellular human pathogen that can persist within macrophages's phagolysosome, revealing that this fungus has evolved defense mechanisms in order to survive under nutritionally poor environments. The analysis of *P. brasiliensis* transcriptome revealed several virulence factors orthologs of other microorganisms, including the glyoxylate cycle genes. This cycle allows the utilization of C2 compounds as carbon source to gluconeogenesis. Semiquantitative RT-PCR analyses revealed that these genes were upregulated when *P. brasiliensis* was recovered from murine macrophages, without any additional *in vitro* growth. The induction of this cycle, in response to macrophages microenvironment, was shown to be coordinated with the upregulation of the gluconeogenic phosphoenolpyruvate carboxykinase gene. In addition, assays employing RNA extracted from *P. brasiliensis* grown in a medium with acetate instead of glucose, also showed increased levels of glyoxylate cycle transcripts. Our main results suggest that *P. brasiliensis* uses the glyoxylate cycle as an important adaptive metabolic pathway.

Key words: Glyoxylate cycle, *Paracoccidioides brasiliensis*, virulence genes, semiquantitative RT-PCR, murine macrophages infection.

Introduction

The dimorphic fungus *Paracoccidioides brasiliensis* is the etiologic agent of Paracoccidioidomycosis (PCM), the most prevalent human systemic mycosis in Latin America [1, 2]. Although the ecology of this microorganism still remains unclear, it is believed that *P. brasiliensis* is found in nature in a mycelial saprophytic form. Infection is probably acquired by inhalation of airborne mycelial structures which, into the lungs, can differentiate to yeast form [3]. The shift from environmental temperature (26 °C) to host body temperature (37 °C) triggers the transition from mycelium to yeast and seems to be essential to the establishment of the infective process [4, 5]. The different outcomes of this interaction can be expressed as: i) pathogen eradication; ii) its encasement in a granulome; or iii) its dissemination throughout the body [6, 7].

The establishment of infection is associated with the virulence of the pathogen and the host immunological response. The main host defense mechanism against *P. brasiliensis* infection is the cell-mediated immune response, characterized by the production of TNF-α, IL-12 and IFN-γ that activate macrophages [8 - 10]. However, in the absence of such cytokines, or in susceptible hosts, this fungus is able to survive and replicate within the phagolysosome of nonactivated murine and human macrophages [11, 12]. Thus, the facultative intracellular life style of *P. brasiliensis* must be compatible with the inhospitable microenvironment imposed by phagocytic cells. Although little is known about the factors required for intracellular persistence of this fungus, the analysis of the *P. brasiliensis* transcriptome project [13, 14] allowed Tavares et al. [15] to identify in *P. brasiliensis* orthologs of genes described in other microorganisms as virulence factors important to

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survival inside host cells. Among these, ortholog genes of the glyoxylate cycle isocitrate

lyase (*icl*) and malate synthase (*mls*), which encode the key enzymes of this cycle [16, 17],

were identified.

The role of glyoxylate cycle in pathogenesis has been demonstrated for several

microorganisms, especially bacteria and fungi. The activity of this cycle allows C2

compounds to be used as carbon sources to gluconeogenesis. In fact, several evidences

indicate that intracellular pathogens may find, preferentially, two-carbon (C2) compounds,

as products of fatty-acid degradation, for energy production [18, 19]. In this sense, upon

phagocytosis, *Mycobacterium tuberculosis* and *Candida albicans*, as other microorganisms,

respond inducing glyoxylate cycle genes, suggesting that its activity is required for

persistence and survival within macrophages [20 - 23]. This shift from fermentative to non-

fermentative metabolism during the infective process probably includes a co-activation of

glyoxylate cycle and gluconeogenic genes, and a downregulation of glycolytic genes.

However, the glyoxylate cycle genes expression is not always associated with

gluconeogenesis, as observed in *C. albicans*, following macrophages infection [23].

Alternatively, the glyoxylate anaplerotic reactions can generate intermediates of other

metabolic pathways.

Here we investigate the expression of glyoxylate cycle genes after co-culture of *P.

brasiliensis* yeast cells with murine macrophages and upon *in vitro* cultivation in a

nutritional stress condition. The analyses were done using semiquantitative reverse

transcription PCR (sqRT-RCR) and the amplified DNA fragments were quantified by

densitometry. The results showed that *P. brasiliensis icl* and *mls* transcripts levels were

increased either following phagocytosis as well as in response to *in vitro* cultivation under

glucose deprivation. Furthermore, in order to evaluate the transcriptional status of genes

related to other central carbon metabolism, we have also analyzed the expression of pyruvate kinase (*pyk*) and phosphoenolpyruvate carboxykinase (*pck*) genes, which encode key enzymes of the glycolytic and gluconeogenesis pathways, respectively.

Material and Methods

Paracoccidioides brasiliensis **strain and growth conditions**

The clinical isolate of *P. brasiliensis*, strain *Pb*01 (ATCC-MYA-826), was maintained as yeast cells at 36 ℃ on the semi-solid Fava Neto´s medium constituted of: 0.3 % protease peptone, 1 % peptone, 0.5 % beef extract, 0.5 % yeast extract, 4 % glucose, 0.5 % NaCl, 1.6 % agar, pH 7.2 [24]. For *ex vivo* assays, the yeast form of *P. brasiliensis* was grown in Fava-Neto semi-solid medium for 7 days before infection of murine macrophages or being used as a control [25]. For *in vitro* assays, employing different carbon sources, 2,5 x 10^7 *P. brasiliensis* yeast cells were first inoculated in complex medium YPD (yeast-peptone-dextrose) and grown up to exponential phase (36 ℃ / 130 rpm). The glucose level, as determined by DNS assay (dinitrosalicylic acid), was monitored in order to assure that the fungal cells were not submitted to a nutritional stress. Following 6 days, cells were harvested by centrifugation, washed and incubated for different time intervals (0,5 h; 1 h; 5 h) in defined McVeigh Morton (MVM) medium [26] supplemented with 2 % glucose or 2 % acetate as sole carbon source. The cell viability was observed after all treatments using vital Janus green stain [27]. These treatments were carried out in triplicate in order to perform a statistical analysis.

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icl **gene sequence and deduced protein analysis**

The *P. brasiliensis icl* complete coding sequence was obtained by the sequencing of cDNA clones from mycelium and yeast cDNA libraries of *P. brasiliensis* [13, 14]. The nucleotide sequence was submitted to the pipeline of analysis as described by Felipe et al. [13]. Alignments were performed using BLAST (http://www.ncbi.nlm.nih.gov) and Clustal (http://www.ebi.ax.uk/clustaw) programs. The deduced protein sequence was analyzed using the ExPASy proteomics server (http://www.expasy.org).

Southern blot analysis

About 15 µg of *P. brasiliensis* genomic DNA were digested with *Bam*HI, *Bgl*II, *EcoR*I, *Xba*I and *Xho*I restriction endonucleases, separated on 1 % agarose gel and transferred to a Hybond N$^+$ membrane, as specified by the manufacturer (Amersham Biosciences). The *P. brasiliensis icl*-related cDNA fragments were labeled using (α-^{32}P)-dATP by random priming procedure (MegaPrime DNA labeling System, Amersham Biosciences), purified and used in overnight hybridization, at 42 °C [28]. The membranes were then washed to a final stringency of 0.1 X SSPE - 0.1 % SDS at 65 ºC for 20 min, and exposed either to an X-ray film at –80 ºC for 48 hours or to a Phosphor Screen (Molecular Dynamics – Amersham Biosciences) for 2 hours.

Total RNA extraction of *P. brasiliensis*

The *P. brasiliensis* total RNA was extracted using Trizol reagent (Gibco-BRL), according to suppliers' recommendations, as previously described [29, 30]. To remove any

genomic DNA contamination, RNA was treated with RNase-free-DNaseI (Promega), followed by enzyme inactivation (EDTA 2,5 mM; 65° C / 10 min) and ethanol precipitation.

Infection of J774 macrophage cell line with *P. brasiliensis* yeast cells

The macrophage-like cell line J774.1 (ATCC TIB-67) were cultured as an adherent monolayer in RPMI 1640 (Gibco) medium supplemented with 10 % heat-inactivated fetal calf serum (Gibco), 3.024 g l^{-1} NaHCO$_3$ and 2mM L-glutamine. *In vitro* cultured yeast cells of *P. brasiliensis* were suspended in RPMI medium containing 20% fresh BALB/c mouse serum at 37 °C for 30 min in order to opsonization takes place. Macrophage monolayers (2 $\times$ 10^7 cells/150 cm^2 culture flask) were then infected with 2 $\times$ 10^6 opsonized yeast cells, which gave a yeast-to-macrophages ratio of 1:5, and incubated for 9 h at 37 °C in an atmosphere containing 5 % CO$_2$ [25].

Total RNA extraction of *P. brasiliensis* yeast cells internalized by J774 macrophage cell line

At 9 h after infection, extracellular fungi were removed by exhaustive washing with RPMI pre-warmed to 37 ºC. Macrophages were then lysed with GTC solution (guanidine thiocyanate 4 M, sodium N-lauryl sarcosine 0.5 %, tri-sodium citrate 50 mM and 2-mercaptoethanol 0.1 M) and intact fungi were harvested by centrifugation (8000 $\times$ *g* for 10 min) followed by RNA extraction and amplification using Trizol (Gibco) and MessageAmp aRNA kit (Ambion), respectively, according to the manufacturer's instructions. Due to the limited quantity of total RNA extracted in a single macrophage infection experiment (~ 3 µg), gene expression analyzed here was obtained from three independent sets of pooled

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RNA. Each pool consisted of 3-4 macrophage infection experiments [25], assuring adequate experimental biological repetitions.

Total RNA from *P. brasiliensis* yeast cells cultured in Fava-Neto's medium for 7 days was extracted and amplified exactly as described above, and used as control.

Semiquantitative RT-PCR

The transcriptional analysis of *P. brasiliensis* cultured *in vitro* in response to different carbon sources was carried out employing sqRT-PCR methodology. The cDNA first strand was synthesized from 2 µg of DNAse-treated total RNA in a 25 µL reaction containing: 0,5 µg primer dT_{12-18}; first strand buffer 1X; 8 mM DTT; 0,4 mM dNTPs; 200 U SuperScript II Reverse Transcriptase (Invitrogen).

The cDNA synthesis using RNA obtained from *P. brasiliensis* yeast cells recovered from macrophages was done as described below. One µL of RNA amplification reaction (equivalent to 250 ng of mRNA) was employed to cDNA first strand synthesis in a reaction exactly as described above, except by replacing the primer dT_{12-18} by random primers. All cDNA synthesis reactions were carried out at 42 °C for 1 h, followed by 20 minutes at 70°C to inactivate the enzyme, according to suppliers' recommendations. In all RT-PCR experiments we always added as a negative control reaction, a system where Reverse transcriptase was omitted.

Five µL of cDNA reaction were amplified in a final reaction volume of 25 µL containing: *Taq* DNA Polymerase buffer 1X; 1,5 mM $MgCl_2$; 0,2 mM dNTPs; 0,2 µM specific primers (*icl*, *mls*, *pyk* or *pck*) and 0,2 µM α-tubulin primers (used as internal control); 2U of *Taq* DNA Polymerase (Cenbiot-RS/Brasil). After a first step of denaturation

(94ºC for 2 min), the amplification was done for 24 cycles consisting of: 94 ºC for 1 min, 60 ºC for 1 min and 72 ºC for 1 min; followed by a final extension at 72 ºC for 10 min. The conditions were chosen so that all amplifications were in the exponential phase, as previously described previously by Marone et al. [31]. The appropriated number of cycles, in which the amplification is on the exponential range, was defined testing a number of cycles ranging from 24 to 36. The optimal number of cycles should be in the same range for the specific gene of interest and for the housekeeping α-tubulin (*α-tub*) gene, used as an internal control [32] in all experiments carried out in this work. Since the intensity of specific amplification products reached a plateau at around 30 cycles (data not show), all semiquantitative analysis employed a number of 25 cycles. The RT-PCR products were analyzed by 1.5 % agarose gel electrophoresis. Each set of reaction always included a negative control containing RNA instead of cDNA, to rule out genomic DNA contamination.

The primers used in sqRT-PCR experiments were determined using the software Primer 3, available on-line (http://www-genome.wi.mit.edu). Primers were always chosen according to the parameters: optimal length 20-22 bases; optimal Tm of 60 °C; length of amplification product between 200 and 600 bp, as described bellow in table 1.

Quantitative analysis

The quantitative analysis of gene expression levels was performed by densitometry employing the Scion Image software, available on-line (http://www.scioncorp.com). Amplified product intensity was expressed as relative absorbance units (AU). The ratio between the relative AU determined for the amplified

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gene of interest and the internal control α-tubulin was calculated to normalize variations in

sample concentration and as a control for reaction efficiency. Mean and standard error of

all performed experiments were calculated after this normalization in relation to α-tubulin

gene amplification.

The statistical analyses were performed using the software "Mynova", version 1.3

(S. Brooks, Copyright 1993). The statistical test applied was Student´s *t* test. A *P* value ≤

0.05 was considered significant.

Nucleotide sequence accession number

The GeneBank accession numbers of the nucleotide sequence(s) used in this work

are cited on table 1.

Results

The *P. brasiliensis* glyoxylate cycle related genes

The analysis of *P. brasiliensis* transcriptome revealed orthologs of glyoxylate cycle

genes (*icl* and *mls*) from other human facultative intracellular pathogenic fungi [14, 15].

The BLAST comparative analyses of the sequences described in the *P. brasiliensis*

transcriptome project [13, 14], enabled us to identify two putative *P. brasiliensis* isocitrate

lyase genes, with 46 % of identity at the amino acid level. Southern blot analysis confirmed

that, these sequences indeed correspond to two different *icl*-related genes (figure 1). In

addition, subsequent analyses revealed that the expression pattern of one of these genes,

was not significantly altered when this fungus was cultivated in media containing glucose

or acetate as the sole carbon source (data not show). Altogether, these observations allowed us to suggest that these genes probably correspond to isocitrate lyase (*icl*) and methylisocitrate lyase (*mcl*), which encode key enzymes of glyoxylate and methylcitrate cycles, respectively. Although these enzymes catalyze analogous reactions, the methylcitrate and glyoxylate cycles differ with respect to the preferential utilization of C3 instead of C2 compounds, respectively [33, 34]. In the present work, we describe the regulation pattern of *icl* and *mls* genes, which encode the major regulatory enzymes of the glyoxylate cycle.

The entire coding sequence of *P. brasiliensis icl* gene was determined, revealing a deduced primary sequence of 537 amino acid residues with a predicted molecular mass of 60 kDa, p*I* of 6.79 and a conserved isocitrate lyase signature motif (K - [KR] - C - G - H - [LMQR]) (data not shown).

Analysis of glyoxylate cycle, gluconeogenic and glycolytic genes expression following *P. brasiliensis* yeast cells internalization by murine macrophages

In order to evaluate *P. brasiliensis* transcriptional response following yeast cells co-culture with murine macrophages, the expression analysis of the glyoxylate cycle *icl* and *mls* genes as well as the *pck* and *pyk* genes was undertaken employing sqRT-PCR methodology.

The RT-PCR experiments were performed using RNA extracted from *P. brasiliensis* yeast cells recovered from murine J774 macrophages after 9 hours of co-culture, without any additional *in vitro* growth after host cells infection (figure 2). These experiments were carried out employing specific primers directed to the internal control (α-

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tub), and to the experimental genes (*icl*, *mls, pck and pyk*). Following the RT-PCR product

quantification by densitometry, appropriate statistical analysis were performed, revealing

that after macrophage internalization both glyoxylate cycle genes (*icl* and *mls*) showed

higher expression levels, when compared to cells grown *in vitro* in a conventional medium,

as control. Thus, the significant induction of *icl* and *mls* observed following phagocytosis

suggests a role of this metabolic pathway in *P. brasiliensis* adaptation inside macrophages,

strongly reinforcing a nutritional deprivation inside the phagosome. In addition to the

induction of glyoxylate cycle genes in the macrophage inhospitable environment, the up-

regulation of *pck* gene expression was also observed (figure 2). The co-regulation of

glyoxylate cycle and gluconeogenesis is consistent with the importance of these pathways

in providing energy from C2 compounds and fatty acids metabolism, as may occur inside

macrophages phagolysosome [21, 23, 35]. In contrast, following phagocytosis, no

significant repression of the glycolytic gene *pyk* was observed in our sqRT-PCR analyses

(figure 2).

**Upregulation of *P. brasiliensis* glyoxylate cycle genes under *in vitro* nutritional stress
condition**

Aiming to investigate the expression profile of the genes analyzed upon fungal

internalization by macrophages, we evaluated the transcriptional response of *P. brasiliensis*

yeast cells to an *in vitro* nutritional condition thought to simulate a feature of the

phagosome. The gene expression analysis was carried out by sqRT-PCR, as described

above. At first, *P. brasiliensis* yeast cells were grown in a complex medium for 6 days, a

time at which the fungal cells were not submitted to a nutritional stress, as shown by the

determination of reducing sugars by DNS assay (data not show). After 6 days, the cells

were harvested, washed and incubated for three different periods of time (0,5 h; 1 h and 5

h) in defined media containing glucose 2 % (MVM-glucose) or acetate 2 % (MVM-

acetate), as the sole carbon source. Following all treatments, carried out in triplicates, we

observed no differences between the viability of cells cultivated in MVM-glucose or

MVM-acetate as accessed by vital Janus green staining (data not show). The fungal cells

were harvested by centrifugation and total RNA was extracted and used in sqRT-PCR

experiments as described above. Figure 3B shows the levels of the target amplification

product relative to the internal control (*α-tub*) when *P. brasiliensis* was grown in the

presence of glucose (G) or acetate (A) as the sole carbon source, for different periods of

time. Following appropriate statistical analysis, we observed a significant increase in the

expression levels of *icl* and *mls* genes when the cells were grown in a medium containing

acetate as the sole carbon source. This result strongly suggests that the expression levels of

these genes are dependent on the primary carbon source, as observed in other pathogenic

fungi, like *C. albicans* [23] and *Cryptococcus neoformans* [35]. However, differently from

other fungi, a considerable level of these glyoxylate cycle transcripts was consistently

detected when *P. brasiliensis* was grown in 2 % of glucose (figure 3).

**Analysis of gluconeogenic and glycolytic genes expression when *P. brasiliensis* was
cultivated under nutritional stress *in vitro***

According to the results described above, the growth of *P. brasiliensis* yeast cells on

C2 compounds as the sole carbon source activates the glyoxylate cycle in order to produce

biosynthetic metabolic precursors. In conjunction with the phosphoenolpyruvate

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carboxykinase (PCK) or malic enzyme, the glyoxylate cycle can also provide phosphoenolpyruvate (PEP) and pyruvate, respectively, required to direct the carbon flux into the gluconeogenic pathway. The involvement of PCK in the modulation of the carbon flux in response to nutrient availability suggests that this metabolic gene expression may be also regulated by different carbon sources. In this context, the *pck* mRNA level of *P. brasiliensis* yeast cells following *in vitro* treatment in defined media, supplemented with only glucose or acetate, was quantified by sqRT-PCR assay, as described previously. In contrast with the glyoxylate cycle genes expression results, the *pck* gene was expressed at equivalent levels during the growth of *P. brasiliensis* in the presence of glucose as well as acetate (figure 3). In addition, the pyruvate kinase (*pyk*) mRNA level, that encodes a enzyme of glycolysis, was also quantified when *P. brasiliensis* yeast cells were cultivated in glucose or acetate as carbon source. Similar to *pck* gene expression data, we observed that *P. brasiliensis pyk* gene was expressed at roughly equivalent levels during growth for 5 hours, both in the presence of glucose or acetate (figure 3).

Discussion

The ability of *P. brasiliensis* to survive and successfully adapt within phagocytic cells depends on its mechanisms to respond to the metabolic constraints imposed by macrophages. Since it is believed that the microenvironment inside host cells is a hostile habitat, differential regulation of specific genes is probably involved in the establishment and adaptation of this pathogen in the context of the host. Accordingly to this scenario, the analysis of *P. brasiliensis* transcriptome by Tavares et al. [15] revealed, several putative

orthologs to virulence genes of other human facultative intracellular pathogens, such as metabolism-, cell wall-, detoxification-related genes and secreted factors. Among these virulence factors, we identified other fungi *icl* and *mls* ortholog genes, which encode isocitrate lyase and malate synthase respectively.

It is often assumed that the modulation of metabolic-related genes can be associated to the microorganism response to a changing environment. In this context, we verified the carbon source influence in *P. brasiliensis* glyoxylate cycle genes expression. As expected, our studies showed that *P. brasiliensis icl* and *mls* genes are upregulated in response to acetate as the sole carbon source (figure 3). However, a basal level of glyoxylate cycle genes expression was observed when glucose is the sole carbon source, suggesting some carbon fluxes through this pathway in that condition. This result is consistent with *icl* regulation in *M. tuberculosis* [36, 37]. On the other hand, in the pathogenic fungus *C. neoformans*, northern blot analyses showed a repression of *icl* gene expression by as little as 0.2% of glucose [35]. However, previous results from our group, also using northern blot assay, revealed a relatively high level of *icl* transcripts when *P. brasiliensis* yeast cells were grown in a rich medium [14]. In the present work, using RT-PCR methodology, we confirm these data, strongly suggesting that differently from other fungi, *high glucose levels do not repress P. brasiliensis glyoxylate cycle genes*. This basal expression could be responsible for the apparently weak induction in the experiments described in this manuscript. In accordance with our results, Cánovas and Andrianopoulos [38] recently described in *Penicillium marneffei*, also a thermally dimorphic opportunistic human pathogen, that the *acuD* gene, encoding ICL enzyme, is regulated by both, carbon source and temperature. Northern blot analyses revealed that the *acuD* gene was strongly induced at 37°C, with

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relatively small differences in the transcript levels when the fungus was incubated at 37°C either in glucose or acetate as carbon sources [38].

The transcriptional modulation of the glyoxylate cycle genes in response to nutrient availability is probably connected with the regulation of other metabolic pathways. In fact, a constitutive carbon source-responsive promoter element necessary for activation of glyoxylate cycle genes was also identified in upstream regions of genes involved in gluconeogenesis in *S. cerevisiae* [39-41]. Accordingly, it was verified in other microorganisms, like *C. albicans* and *M. bovis*, that the gluconeogenic gene *pck* is induced after growth on non-fermentative media [23, 42]. However, differences in the control of gluconeogenesis in *Aspergillus nidulans* were found, where the transcriptional activator FacB, which mediates acetate induction of *icl* gene, is not directly involved in *pck* induction [43]. In *P. brasiliensis*, we verified that the regulation of gluconeogenic and glyoxylate cycle genes was not completely linked, since *pck* gene was expressed at the same levels when this fungus was cultured for 5 h either in acetate or glucose (figure 3). Interestingly, Barelle et al. [23] also described that upon *C. albicans* macrophages and neutrophils internalization the glyoxylate anaplerotic reactions can produce intermediates to other metabolic pathways. In this sense, the analysis of *P. brasiliensis icl*, *mls* and *pck* promoters, in progress by our group, should be essential to better elucidate the role of the transcriptional regulation of these genes in the central carbon metabolism.

On the other hand, in a nutritional stress condition, the *pyk* and *pfk* genes are supposed to be repressed, as observed in *C. albicans* and *S. cerevisiae* yeast cells [44, 23]. However, in *P. brasiliensis*, we observed an equivalent expression level of *pyk* gene during growth for 5 h in glucose or acetate (figure 3). It is possible that, during this initial growth of *P. brasiliensis* yeast cells on acetate, part of the phosphoenolpyruvate generated from

oxaloacetate could be metabolized in gluconeogenesis, while a fraction is converted by

pyruvate kinase in the glycolytic direction, to sustain proper pyruvate availability for

biomass synthesis [45]. At this incubation period, the growth rate is similar when *P.*

brasiliensis is cultivated in both carbon sources. However, an overnight culture shows a

significant reduction of cells growth in MVM-acetate when compared to MVM-glucose

medium (data not show). This result may be also explained by the pre-culture conditions

since even when fungal cells were grown, in YPD for 6 days more than 1.5 % of reducing

sugars was still observed (data not shown). Under this condition, *P. brasiliensis* cells may

have synthesized reserve carbohydrates, as glycogen, which may be used in carbon

metabolism. Finally, it is probable that the central carbon metabolism is regulated to a large

extent by post-transcriptional mechanisms, mainly in the early response to environmental

changes [46].

The induction of glyoxylate cycle genes in media containing C2 compounds can

play an important role in *P. brasiliensis* persistence during infection. Since this fungus is a

facultative intracellular pathogen and glycolytic substrates are supposed to be absent or

sparse in the phagolysosome environment [22], glyoxylate cycle may be required for the

utilization of C2 compounds derived from fatty acids in energy production. The role of this

pathway in microorganisms' survival within macrophages has been shown in pathogens,

such as *M. tuberculosis* and *C. albicans* [20 - 23, 37]. In *P. brasiliensis*, glyoxylate cycle

genes were also induced following phagocytosis (figure 2), suggesting that this pathway

could be used by this intracellular pathogen to subsist on C2 (acetyl-CoA) compounds

within host cells. The switches to this anaplerotic pathway, in conjunction with the

sequential action of PCK enzyme, are required for the production of phosphoenolpyruvate

(PEP), an essential biosynthetic precursor of glucose synthesis. Here, we show that *pck*

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gene is also upregulated after phagocytosis (figure 2) indicating that the respective gene product, probably displays a role in the successfully *P. brasiliensis* adaptation within macrophages. Although the *in vitro* results when *P. brasiliensis* was incubated for 5 hours under acetate as carbon source do not show a significantly increase of *pck* transcript (figure 3), it is important to be emphasized that the phagolysosome corresponds to a complex habitat, with probably other factors being also required for *pck* gene regulation. While the nutritional composition of this organelle is essentially unknown, it is recognized that the phagolysosome milieu is replete of antimicrobial compounds and has an acidic pH [19]. In fact, consistent with our proposition, studies showed that *Agrobacterium tumefaciens pck* gene is induced by acidic pH [47].

The activation of gluconeogenic pathway is normally coordinated with the repression of the glycolytic pathway. Although our analysis shows that the expression levels of *P. brasiliensis pyk* gene was not significantly altered after phagocytosis (figure 2), DNA microarray assays performed by our group, showed downregulation of the glycolytic phosphofrutokinase gene (*pfk*), when *P. brasiliensis* infected murine peritoneal macrophages [25]. The *pfk* gene encodes another controlling protein of the glycolytic pathway that also catalyzes an essential irreversible reaction. We believe that the apparently conflicting results found in glycolytic genes expression upon *P. brasiliensis* yeast cells internalization by macrophages could be explained by the observation that a small percent of cells are non-phagocytosed at the end of our time course experiment. A similar result was previously described by Fradin et al. [48], comparing the transcriptional response of *C. albicans* in human blood and in a mouse infection model. Furthermore, since the sqRT-PCR methodology is less sensitive when the gene expression levels among samples show

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small differences, the presence of an heterogeneous cell population would also influence this apparently conflicting result.

In conclusion, we observed that the transcriptional regulation of *P. brasiliensis* glyoxylate cycle genes is carbon source dependent, as demonstrated by *in vitro* assay. The upregulation of these glyoxylate cycle genes, coordinated with the increase of the gluconeogenic transcript levels, was also verified following co-culture of *P. brasiliensis* with murine macrophages, indicating a shift to a non-fermentative metabolism when *P. brasiliensis* is phagocytosed by host cells. The differential expression of *icl*, *mls* and *pck* genes suggests a role of glyoxylate cycle and gluconeogenic pathways in the adaptation of this fungus to the internal harsh milieu of macrophages, as well as to other hostile environments. Although the real importance of these pathways in the disease progression needs to be confirmed by virulence assays, our results reinforce the importance of the central carbon metabolism regulation to pathogen survival and adaptation inside host cells.

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Figure 1. Two different *P. brasiliensis* putative *icl*-related genes. Equal amounts of *P. brasiliensis* genomic DNA (10µg) were digested with several restriction enzymes and separated onto 1% agarose gel (lane 1 - *Bgl*II, lane 2 - *Hin*dIII, lane 3 - *Pst*I, lane 4 - *Xho*I and lane 5 - *Bam*HI). The results after gel blotting to a nylon membrane and hybridization with the two radiolabeled cDNA probes corresponding to PbAEST 1688 (P1 – *pbicl*) and PbAEST 1381 (P2 – *pbicl*-related) are shown in panels **A** and **B**, respectively. The size of some restriction DNA fragments, λ-DNA/*Eco*RI/*Hin*dIII (A) or λDNA/*Hin*dIII (B), used as molecular markers, are shown.

Figure 2. Expression analysis of *P. brasiliensis* genes encoding key enzymes of the glyoxylate cycle, gluconeogenesis and glycolysis, in response to fungal internalization by murine macrophages. After co-culture of *P. brasiliensis* yeast cells with murine macrophage cells J774 for 9h, the fungal cells were harvested by centrifugation, after macrophages lysis. The *P. brasiliensis* total RNA was extracted and used in RT-PCR experiments, as described in material and methods section. As a control condition, total RNA from *P. brasiliensis* yeast cells cultivated *in vitro,* in conventional Fava Neto's medium for 7 days, was employed. Panel **A** shows the results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from the control and macrophage internalization experiments are shown in lines C and M, respectively. Panel **B** shows the semiquantitative analysis, performed exactly as described in figure 2, of *P. brasiliensis icl*, *mls*, *pck* and *pyk* genes expression, when *P. brasiliensis* were cultivated *in vitro* (■) or upon murine macrophages internalization (□). Bars represent standard errors of RT-PCR duplicate experiments, *$P < 0,05$.

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Figure 3. Expression analysis of *P. brasiliensis* genes encoding key enzymes of the central C-metabolism, in response to fungal *in vitro* incubation in the presence of glucose or acetate as the sole carbon source. After *P. brasiliensis* incubation in defined media containing 2 % glucose (MVM-glucose) or 2 % acetate (MVM-acetate) for different periods of time, total RNA was extracted and used in RT-PCR experiments, employing specific primers directed to the internal control (*α-tub*) and the experimental (*icl*, *mls*, *pck* or *pyk*) genes. Panel **A** presents the results of one of the triplicate RT-PCR experiments, showing the amplified *P. brasiliensis* cDNAs obtained in response to fungal cultivation in MVM-glucose (line G) or MVM-acetate (line A) medium, for 0,5 h, 1 h or 5 h. Panel **B** shows the semiquantitative analysis of *P. brasiliensis icl*, *mls*, *pck* and *pyk* genes expression, performed by densitometry, employing the Scion Image software (http://www.scioncorp.com). The *icl*, *mls*, *pck* or *pyk* transcript levels were calculated in relation to the internal control *α-tub* for each experimental condition, glucose (■) or acetate (□), as described in panel A. Bars represent standard errors of triplicate experiments, *$P <$ 0,05.

Table 1. Oligonucleotides employed in the semiquantitative RT-PCR analysis.

Primer	Gene and Accession Number[*]	Sequence	Length of amplification product (bp)
5'TUB	α-tubulin	5'-TTCGTTGATCTGGACCCTTC -3'	199
3'TUB	CN242437	5'-GGAGGGACGAGCAGTTATCA-3'	
5'MLS	malate synthase	5'-TTCAATTTCCCTCCTGACGA-3'	414
3'MLS	CN253867	5'-AGCTGTGGCAATGGGTTAAG-3'	
5'ICL	isocitrate lyase	5'-GCTCACCCAGATGGTCAAAT-3'	265
3'ICL	EF032483	5'-AGTATCCGCATCCGCAATAA-3'	
5'ICL-rel	isocitrate lyase-related	5'-TTGCAGCAGACGTATCCAAG-3'	304
3'ICL-rel	CN246564	5'-TTCCTTCTCGAATCCCATTG-3'	
5'PCK	phosphoenolpyruvate carboxykinase CA580673	5'-GCGTTACAGAATCAGCGTCA-3'	311
3'PCK		5'-GGCTGACAGGGTAGTCTTGC-3'	
5'PYK	pyruvate kinase	5'-ATGGCTTCATCTCCTCCAAA-3'	391
3'PYK	CN253237	5'-CGCAGGGATTTCGATACCTA-3'	

[*] These nucleotide sequences were submitted to the GenBank[TM]/EBI Data Bank.
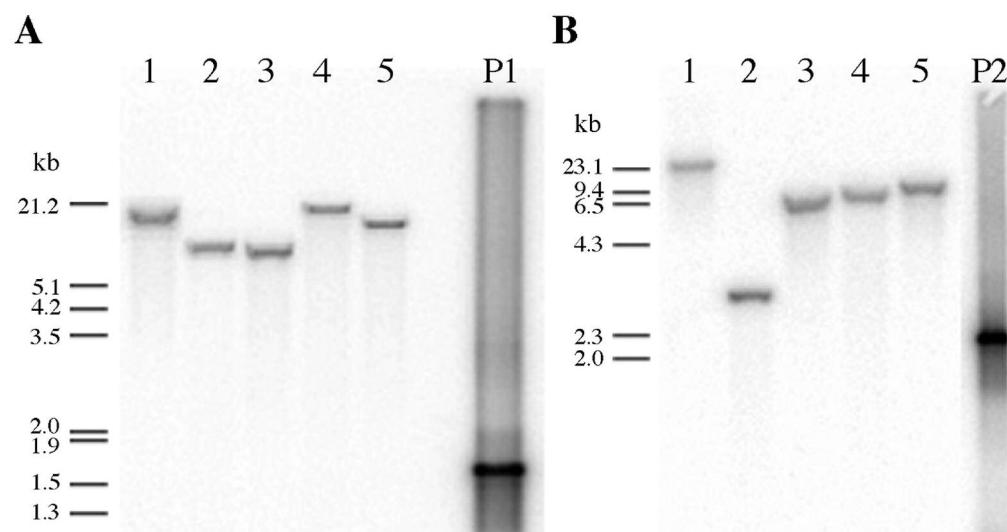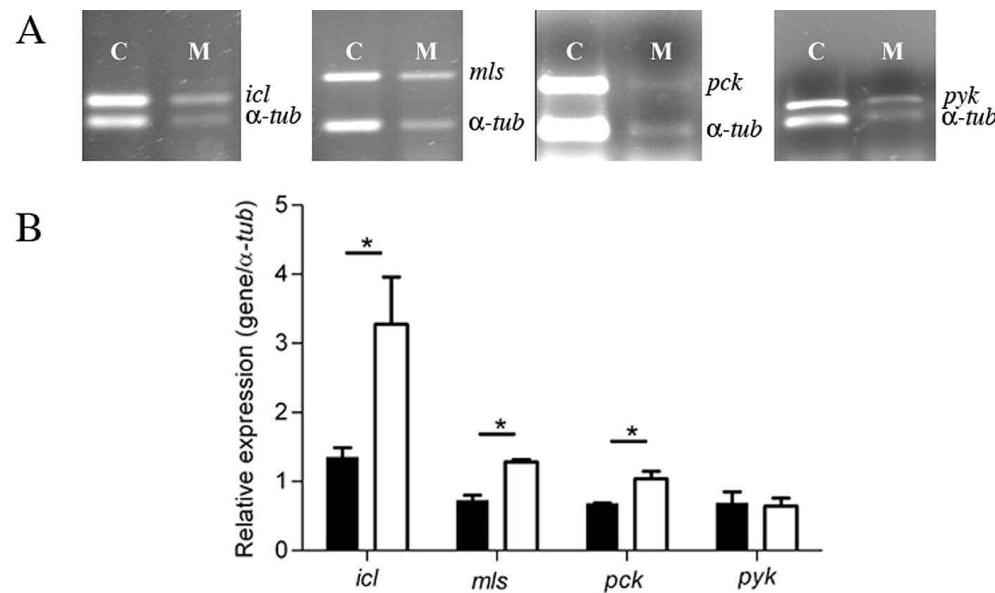
119x69mm (300 x 300 DPI)
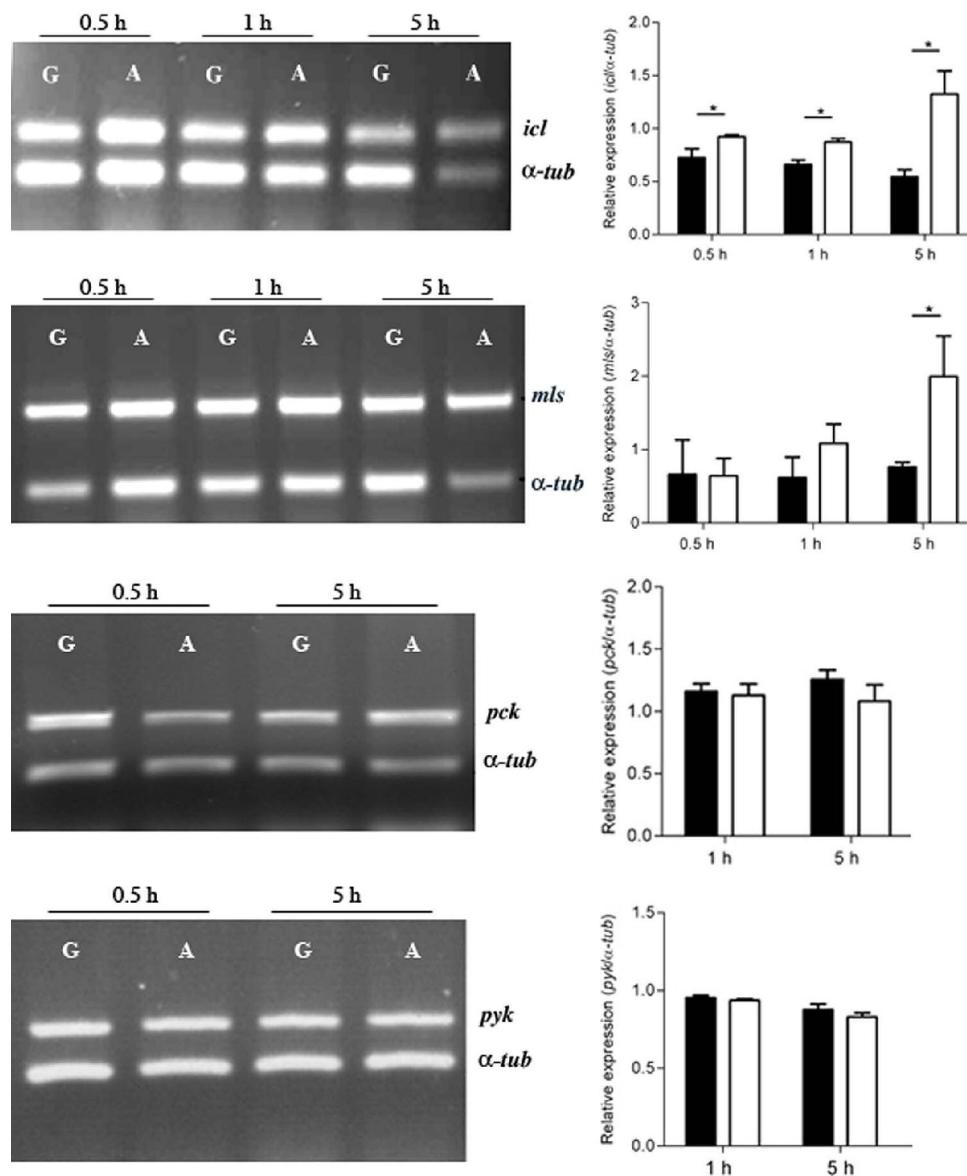
152x182mm (300 x 300 DPI)

Transcriptional profile of *ras1* and *ras2* and the potential role of farnesylation in the dimorphism of the human pathogen *Paracoccidioides brasiliensis*

Larissa Fernandes[1], Hugo C. Paes[1], Aldo H. Tavares[1], Simoneide S. Silva[1], Alessandra Dantas[1], Célia M.A. Soares[2], Fernando A.G. Torres[1] & Maria Sueli S. Felipe[1]

[1]Departamento de Biologia Celular, Universidade de Brasília, Brasília, DF, Brazil; and [2]Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Goiás, Goiânia, GO, Brazil

Correspondence: Maria Sueli S. Felipe, Laboratório de Biologia Molecular, Departamento de Biologia Celular, Universidade de Brasília, Brasília, DF, Brazil, 70910-900. Tel.: +55 61 3307 2423; fax: +55 61 3349 8411; e-mail: msueli@unb.br

Abstract

Paracoccidioides brasiliensis is a thermo-dimorphic fungus that causes a human systemic mycosis with high incidence in Latin America. Owing to their participation in the control of pathogen morphogenesis, differentiation and virulence, it was decided to characterize *ras* genes in *P. brasiliensis*. *ras1* and *ras2* were identified to be coding for two different proteins with high identity. The *ras* transcriptional pattern was investigated by reverse transcription PCR (RT-PCR) during mycelium-to-yeast (M → Y) transition, heat shock at 42 °C and after internalization of yeast cells by murine macrophages. Both genes were downregulated inside macrophages and *ras1*, at 42 °C. In contrast, *ras* genes did not show any transcriptional variation during the M → Y transition. The fact that Ras proteins are attached to the membrane via farnesylation prompted the use of a farnesyltransferase inhibitor to investigate the importance of this process for vegetative growth and dimorphic transition. Farnesylation blockage interfered with the vegetative growth of yeast cells and stimulated germinative tube production even at 37 °C. During Y → M transition, the inhibitor increased filamentation in a dose-dependent manner, indicating that impaired farnesylation favours the mycelium form of *P. brasiliensis*. The results suggest that *ras* genes might have a role in dimorphism, heat shock response and in host–pathogen interaction.

Introduction

Paracoccidioides brasiliensis (Pb) is the aetiological agent of the paracoccidioidomycoses (PCM), one of the human systemic mycosis with a high prevalence in Latin America (Brummer *et al.*, 1993). About 10 million people are estimated to be infected with this pathogen (Restrepo *et al.*, 2001). Despite the high incidence, little is known about how the infective form enters its host. The main hypothesis is that the disease is acquired through inhalation of propagules, because the ecological niche of this fungus is probably the soil (De Albornoz, 1971; Ferreira *et al.*, 1990; Silva-Vergara *et al.*, 1998).

Paracoccidioides brasiliensis is a thermo-dimorphic fungus, appearing as a mycelium at 22 °C and as yeast at 37 °C. Both forms are multinucleated (San-Blas, 1993). No teleomorphic (sexual) state has been determined as of now; the

same is also true for the exact ploidy, all of which makes classical genetic analysis unfeasible. Genome size ranges from 25.8 to 75.6 Mb, while the estimated ploidy and number of nuclei per cell varies with the isolate (Feitosa *et al.*, 2003). Some strains that are unable to switch from mycelium to yeast are avirulent, which implies that the pathogenicity of *P. brasiliensis* appears to be related to the ability to differentiate (San-Blas & Nino-Vega, 2001). The components of the signalling pathways of *P. brasiliensis* such as cAMP/PKA, Ca^{2+}/calmodulin–calcineurin (de Carvalho *et al.*, 2003) and MAP-kinases were found in *P. brasiliensis* through transcriptome analysis and reverse annotation (Felipe *et al.*, 2005; Fernandes *et al.*, 2005).

The *ras* genes are conserved from fungi to humans (Barbacid, 1987). Some mutations that cause dominant activation of RAS are highly associated with tumours in mammalian cells (Barbacid, 1987). The Ras proteins belong

to a small GTP-binding proteins family with GTPase activity. The conversion of Ras from an inactive GDP-bound form to an active GTP-bound one occurs in response to a variety of extracellular stimuli (Wiesmuller & Wittinghofer, 1994). For Ras proteins to work properly, they must be attached to the membrane by the addition of a farnesyl group, a lipid intermediate from ergosterol biosynthesis in fungi, to their carboxy terminal region, a step catalysed by the farnesyltransferase enzyme. Ras genes have been identified as key components in signalling cascades in diverse fungi species such as *Saccharomyces cerevisiae* (DeFeo-Jones *et al.*, 1983), *Cryptococcus neoformans* (Alspaugh *et al.*, 2000; Waugh *et al.*, 2002), *Candida albicans* (Feng *et al.*, 1999; Leberer *et al.*, 2001), *Aspergillus fumigatus* (Fortwendel *et al.*, 2004, 2005) *Penicillium marneffei* (Boyce *et al.*, 2005), and others. In these organisms, Ras proteins act as sensors of environmental conditions to control diverse cellular processes including cell cycle progression, cAMP synthesis, cell differentiation and morphogenesis, cytoskeleton organization and expression of virulence genes in pathogens. In the budding yeast *S. cerevisiae*, Ras proteins are involved in the life span, response to stress including heat shock and also control of the nutritional state of the cell (Breviario *et al.*, 1986; Engelberg *et al.*, 1994; Wang *et al.*, 2004).

In the present work, two *ras* genes have been identified – *ras1* and *ras2* – and Southern blot analysis showed one copy of each in the genome of the human pathogen *P. brasiliensis*. As these genes are involved in cell morphogenesis and differentiation, response to heat shock and nutrient deprivation, it was decided to assess the transcriptional response of *ras1* and *ras2* during the temperature-dependent dimorphic switch from mycelium to yeast, during heat shock at 42 °C and during *in vivo* macrophage infection. To evaluate the potential role of Ras in *P. brasiliensis*, fungal cells were treated with a farnesyltransferase (FPT) inhibitor on vegetative growth of mycelium and yeast and during the dimorphic switch.

Materials and methods

Paracoccidioides brasiliensis strain and growth conditions

The clinical isolate of *P. brasiliensis* strain Pb01 (ATCC-MYA-826) was maintained as yeast cells at 37 °C in Fava–Netto semisolid medium consisting of 0.3% protease peptone, 1% peptone, 0.5% beef extract, 0.5% yeast extract, 4% glucose, 0.5% NaCl and 1.6% agar, pH 7.2 (Fava-Netto, 1961). The mycelium form of *P. brasiliensis* was maintained on potato agar medium at 22 °C. For *in vivo* assays, the yeast form of *P. brasiliensis* was grown in Fava–Netto semisolid medium for 7 days before infection of murine macrophages or use as control. Heat-shocked yeast cells were obtained by

changing the temperature of 15 mL aliquots of a YPD (1% yeast extract, 2% peptone, 2% glucose, pH 7.2) culture (10^7 cells mL^{-1}) from 37 to 42 °C. The dimorphic transition from mycelium to yeast was induced as described by Venancio *et al.* (2002).

Identification of *P. brasiliensis ras* genes

A BLAST search at the Genbank (http://www.ncbi.nlm.nih.gov) of the putative protein sequence Ras1 from *Cryptococcus neoformans* (AF294647) identified a 450-bp expressed sequence tag (EST) (BQ493380) deposited by Goldman *et al.* (2003) corresponding to the 3′ *ras1* region of the *P. brasiliensis* isolate Pb18. Based on the fragment found in GenBank, the primers were designed (ras1-GSP1-GCGA GAACAATACATGCGCACAGGCG and ras1-GSP2- GATAC CACACGTTCCTTCTCCAAGTCGC) for RACE experiments. Both 5′ and 3′ RACE fragments (BD Smart TM Race cDNA Amplification Kit, TaKaRa Clontech) were obtained and sequenced using a 'MegaBACE 1000' (GE Healthcare). The sequences were analyzed through the BioEdit alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

The *ras2* of *P. brasiliensis* (Pb01 isolate) was first identified in the Pb01 transcriptome project analysis (Felipe *et al.*, 2003, 2005). The *Cryptococcus neoformans RAS2* gene sequence was used to BLAST search on the *P. brasiliensis* assembled EST (PbAEST) Bank, thus identifying PbA-EST983, which shows 76.5% identity to the probe and had an alignment *E*-value of 1e − 09. The corresponding clone was sequenced and found to be the *ras2* gene of *P. brasiliensis* isolate Pb01. Based on *ras1* and *ras2* sequences, PCR amplification was carried out using genomic DNA as a template to reconstitute the complete gene sequences and to evaluate the presence of introns.

Total RNA extraction of *P. brasiliensis*

Paracoccidioides brasiliensis total RNA was extracted using Trizol reagent (Invitrogen), following the suppliers' recommendations, as described previously (Venancio *et al.*, 2002). To remove any genomic DNA contamination, RNA was treated with RNAse-free-DNAse I (Promega), followed by enzyme inactivation (EDTA 2.5 mM; 65 °C/10 min) and ethanol precipitation.

Infection of the J774 macrophage cell line with *P. brasiliensis* yeast cells and total RNA extraction

The experiments were performed according to Tavares *et al.*, 2007. Briefly, the macrophage-like cell line J774.1 (ATCC TIB-67) was cultured as an adherent monolayer in RPMI-1640, and then infected with 2×10^6 opsonized yeast cells,

with a yeast-to-macrophage ratio of 1 : 5, and incubated for 9 h at 37 °C in an atmosphere containing 5% CO_2. Yeast cells that were not internalized in the period were removed by washing with warmed RPMI-1640 and macrophages were lysed with a guanidine thiocyanate-based solution (Monahan *et al.*, 2002). Intact fungi were harvested by a rapid centrifugation, followed by Trizol RNA extraction, and amplification using the MessageAmp aRNA kit (Ambion) was performed. A single macrophage infection experiment produces about 3 µg of *P. brasiliensis* RNA; because of this limitation, the RNA yields from three to four independent macrophage infection experiments were pooled. Total RNA from *P. brasiliensis* yeast cells cultured in Fava–Netto's medium for 7 days was extracted and amplified exactly as described above, and used as a control.

Southern blot

Total DNA was extracted following Raeder & Broda (1985). One hundred micrograms of DNA were digested with the restriction endonucleases BamHI (QBiogene), EcoRI, HindIII and PstI (all from Promega) in the proportion of 5 U µg^{-1} DNA as recommended by Sambrook & Russel (2001). All digestions were carried out at 37 °C and incubated overnight. Ten micrograms of DNA from each digestion were electrophoresed in a 0.8% agarose gel. The gel was blotted onto a charged nylon membrane (GE Healthcare) by upward capillary transfer. The *ras1* and *ras2* cDNA probes were amplified by PCR using specific primers (for *ras1*: ras1/1-5′-CTTGCTGGTCTACTCCATCACTTC, ras1/3-3′-CAGATGAATACGAGGACATCTC; for *ras2*: ras2/1–5′-CAACGGGACTCAGCTACC, ras2/2–3′-CCACAAGCATTACTG-GAAC). A total of 10 ng of purified product were chemically labelled using the AlkPhos Direct labelling and detection system (GE Healthcare). All procedures were based on the suppliers' recommendations.

Semiquantitative reverse transcription (sqRT)-PCR

The transcriptional analysis of *P. brasiliensis* *ras1* and *ras2* was carried out using the sqRT-PCR methodology. Briefly, the cDNA first strand was synthesized from 2 µg of DNAse-treated total RNA using the SuperScript II Reverse Transcriptase (Invitrogen) following the supplier's recommendations. Five microlitres of reaction were amplified in a final reaction volume of 25 µL containing *Taq* DNA polymerase buffer 1 × , 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM specific primers (*ras1:* ras1/1–5′-CTTGCTGGTCTACTCCATCAC TTC, ras1/3-3′-CAGATGAATACG AGGACATCTC or *ras2:* ras2/1–5′-CAACGGGACTCAGCTACC, ras2/4-3′-GGAGA GCTCCATCATTTCCC), 2 U of *Taq* DNA polymerase (Cenbiot-RS/Brazil) and 0.2 µM *l34* ribosomal protein or clathrin light-chain primers (*clat*) as internal controls (*l34*:

l34-5′-GTCCGCATCATCAAGACTCC, l34-3′- CTTGACA CAGCCAGCGCAG; *clat*: clat-5′-CCTGGGTGAAGATGCG GATC, clat-3′-GGATGTGCCTGTGATGGTTC). The PCR programme consisted of a first step of denaturation (94 °C for 2 min), 24 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The appropriate number of cycles, in which the amplification is on the exponential range, was defined by testing a range from 24 to 35. The optimal number of cycles should be in the same range for the specific gene of interest and for the housekeeping gene used as an internal control (Marone *et al.*, 2001). Because the intensity of specific amplification products reached a plateau at around 30 cycles (data not shown), all semiquantitative analyses used 24 cycles. Amplicons were analysed by 1.5% agarose gel electrophoresis. Each set of reactions always included a negative control containing RNA instead of cDNA, to rule out genomic DNA contamination. The quantification of gene expression levels was performed by densitometry analysis (SCION IMAGE software) available online (http://www.scioncorp.com). Amplified product intensity was expressed as relative absorbance units (AU). The ratio between the relative AU determined for the amplified gene of interest and the internal control was calculated to normalize variations for sample concentration and as a control for reaction efficiency. The mean and SE of all performed experiments were calculated relative to the amplification of the control gene. Statistical analyses were performed using the software 'MYNOVA', version 1.3 (S. Brooks, Copyright 1993). The statistical test applied was Student's *t*-test. A *P* value ≤ 0.05 was considered to be significant.

Drug susceptibility testing

Drug susceptibility tests were adapted from the international standard M27-A2 (National Committee for Clinical Laboratory Standards, 2002). *Paracoccidioides brasiliensis* yeast cells were grown on Fava–Netto semisolid medium for 5–7 days at 37 °C, harvested and diluted in RPMI-1640 medium to a final concentration of 10^5 cells mL^{-1}. The experiment was carried out in U-shaped 96-well plates with 100 µL of final volume per well. Amphotericin B was also used as a control and the concentrations assayed varied from 4 µg mL^{-1} to 0.39 ng mL^{-1}. The FPT inhibitor III ((E,E)-[2-oxo-2-[[(3,7,11-trimethyl-2,6,10-dodecatrienyl)oxy]amino] ethyl]phosphonic acid, (2,2-dimethyl-1-oxopropoxy) methyl ester, Na) (Calbiochem) was resuspended and diluted in the culture media at concentrations varying from 500 to 0.43 µM. The microplate was incubated for 7–10 days at 37 °C. Yeast growth inhibition was quantified via the OD$_{595 nm}$, and each drug concentration was tested in triplicate.

To assess the effect of FPT inhibitor III on the vegetative growth of yeast and mycelium of *P. brasiliensis*, the same

procedure was performed, except that the culture medium used was the defined McVeigh Morton (MVM) medium (Restrepo & Jiménez, 1980) supplemented with 2% glucose as described previously by Hahn & Hamdan (2000). For the mycelium, hyphal fragments were inoculated into brain heart infusion (BHI) and growth was allowed for 10 days at 22 °C under agitation of 150 r.p.m. The supernatant was then removed and hyphae were washed three times with 0.9% saline solution and dispersed by passing through a hypodermic syringe with a 30-gauge needle to produce small mycelium fragments, which were counted in a haematocytometer. The density was adjusted to about 10^5 fragments mL^{-1}. The microplate was incubated for 10 days at 22 °C.

FPT inhibitor III testing during the *P. brasiliensis* differentiation

The effect of the farnesylation inhibitor on the *P. brasiliensis* temperature-driven dimorphic transition was determined on the same concentrations assayed previously for the MIC testing. Inocula for yeast and mycelium were also prepared as described above. After preparation, yeast and mycelium plates were kept for 48 h at 37 and 22 °C, respectively. Each plate was then shifted to the other temperature to allow dimorphic transition and incubated for 10 days. After the incubation period, cell morphology was observed under a Nikon microscope. Pictures were taken using a Sony DSC-W5 digital camera and threefold optical zoom.

Results and discussion

Identification and molecular characterization of *ras1* and *ras2* genes in *P. brasiliensis*

The *ras1* gene is composed of two exons separated by a single intron of 99 bp. The *ras1* 636-bp ORF encodes a putative protein sequence of 212 amino acids with four conserved GTP-binding domains, an effector domain and the farnesylation site (CVIM). The complete *ras1* sequence was deposited at GenBank under accession number DQ157363. The *ras2* gene contains two exons separated by one intron of 89 bp, an ORF of 714 bp that codifies a putative protein of 238 amino acids also with the same five domains and a farnesylation site (CLIL). All these consensus motifs are characteristic of Ras proteins (Bourne *et al.*, 1991). The complete *ras2* sequence was deposited at Gen-Bank under accession number AY910576 (Fig. 1a).

The *in silico* analysis of the *P. brasiliensis* Ras1 and Ras2 deduced protein sequences revealed through a multi-alignment programme that all Ras-GTPases are closely related. A BLAST search showed that both *P. brasiliensis* Ras are highly conserved relative to other fungi, with similarity varying

from 45.7% to 79.3% for Ras1 and 39% to 82% for Ras2; the best matches were to *A. fumigatus* RasA and RasB, respectively. A similarity and identity of 41.3% and 51.4%, respectively, were observed between *P. brasiliensis* Ras1 and Ras2. Molecular masses were estimated at 24.19 kDa (Ras1) and 26.51 kDa (Ras2), well in the range of small GTPases.

The comparison of Ras sequences shows them to be quite conserved on the four GTP-binding sites and the effector domain, but the prenylation site is variable. It is important to note that the prenylation site (CAAX) of Ras2, which is CLIL, has leucine for X, which favours the addition of a geranyl group instead of a farnesyl group as expected for the Ras-GTPase family. The presence of a leucine as X in the carboxy region is also observed in *Cryptococcus neoformans* Ras1 (CVVL) and *A. fumigatus* RasB (CVIL). Special attention must be given to the Ras2 region of about 20–30 amino acids that is absent in Ras1 (Fig. 1a). This region (22 amino acid residues in *P. brasiliensis*) is found in almost all Ras2 sequences analysed, including *A. fumigatus* RasB, *Cryptococcus neoformans* Ras2 and *Neurospora crassa* Ras2. A phylogenetic tree based on sequence homology has been generated in which it is clear that Ras1 and Ras2 groups are clustered apart. Both *P. brasiliensis* Ras are grouped alongside Ras proteins from *A. fumigatus* and *N. crassa*, suggesting that these fungi are in close proximity (Fig. 1b).

To confirm the presence and the copy number of the two *ras* genes in *P. brasiliensis* isolate Pb01, EcoRI, HindIII and PstI were chosen, which do not have cut within either sequence, to digest genomic DNA. BamHI has one site in *ras2* but not in *ras1*. It is important to mention that the probe for *ras2* hybridization was the 75 bp corresponding to the 22-residue region that is not present in the *ras1* sequence, thus enabling specific hybridization of *ras2* and not *ras1*. For the *ras1* probe, the 300 bp of the 3' region was we selected because of its low similarity to *ras2*.

Hybridization detection revealed a single band in all lanes probed for each gene (Fig. 2). These results demonstrate that *P. brasiliensis* has two different *ras* genes, *ras1* and *ras2*, on its genome as observed for most other fungi, except for *Candida albicans* and *Schizosaccharomyces pombe*, in whose genome just one *RAS* gene was identified (Fukui & Kaziro, 1985; Leberer *et al.*, 2001).

Transcriptional profiles of *P. brasiliensis ras1* and *ras2* under different conditions

To investigate the *ras* transcriptional pattern a Northern-blot analysis was first performed using RNA extracted from mycelium and yeast of *P. brasiliensis* but could be detected no transcripts of either *ras* genes under any conditions, even with 15 μg of total RNA per lane (data not shown). Such an impossibility using this same approach was previously reported for the *ras* genes of *Cryptococcus neoformans*
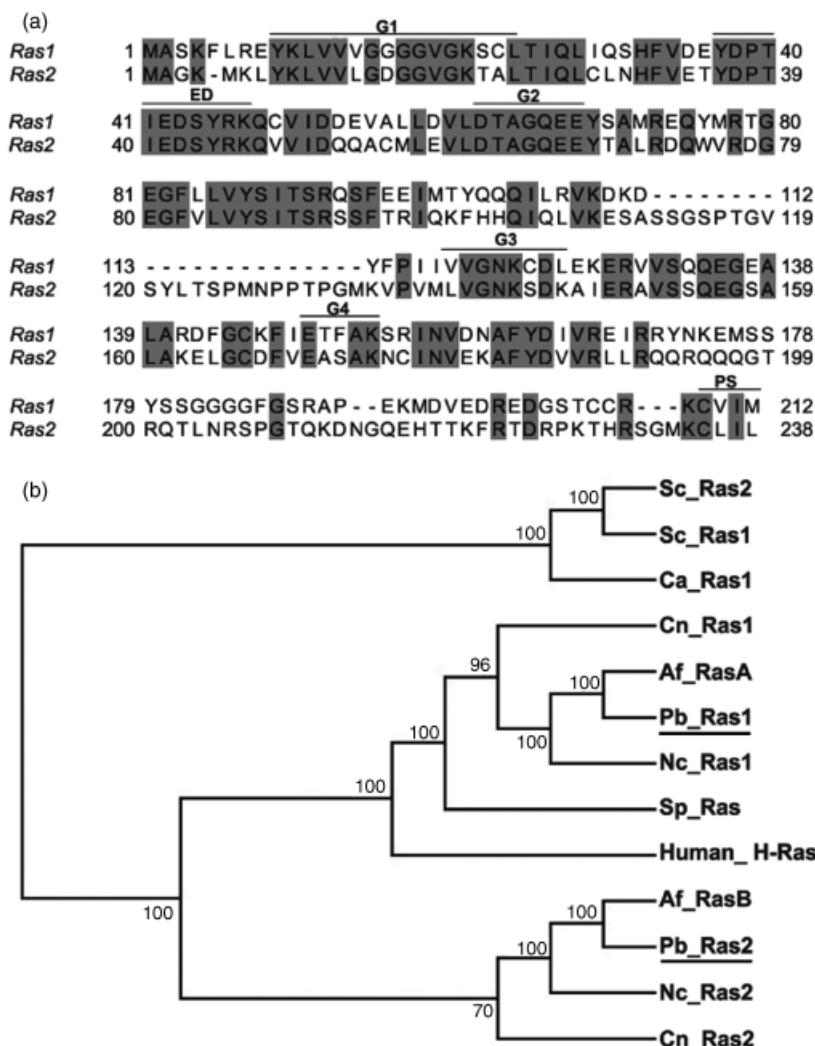
Fig. 1. *Paracoccidioides brasiliensis* Ras1 and Ras2 sequences analysis. (a) CLUSTALW alignment of Ras1 and Ras2 from *P. brasiliensis*. The grey boxes indicate identical residues between the two sequences, and the conserved Ras domains are indicated above the sequences: G1–G4 are the GTP-binding domains, ED is the GTPase effector domain and PS is the prenylation site. (b) Phylogenetic tree analysis based on a clustering algorithm to evaluate the relatedness of *P. brasiliensis* Ras proteins to those of different fungi. Bootstrap values are indicated on each branch of the tree. Af_RasA: *Aspergillus fumigatus* RasA (AAB07703), Af_RasB: *A. fumigatus* RasB (AAP94030), Ca_Ras1: *Candida albicans* Ras1(AF177670), Cn_Ras1: *Cryptococcus neoformans* Ras1 (AF294647), Cn_Ras2: *Cryptococcus neoformans* Ras2 (AF294349), Human H-Ras (AF493916), Nc_Ras1: *Neurospora crassa* Ras1 (P22126), Nc_Ras2: *N. crassa* Ras2 (BAA03708), Pb_Ras1: *P. brasiliensis* Ras1 (DQ157363), Pb_Ras2: *P. brasiliensis* Ras2 (AY910576), Sc_Ras1: *Saccharomyces cerevisiae* Ras1 (CAA99298), Sc_Ras2: *S. cerevisiae* Ras2 (AAA34959), Sp_Ras: *Schizosaccharomyces pombe* Ras (CAA27399).
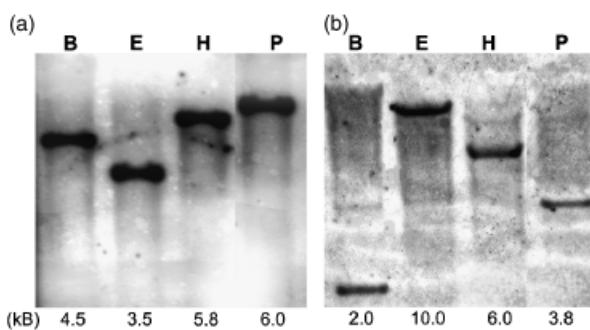
Fig. 2. *Paracoccidioides brasiliensis ras1* and *ras2* copy numbers by Southern blot analysis. Ten-micrograms total DNA samples digested with the restriction endonucleases BamHI (B), EcoRI (E), HindIII (H) and PstI (P) were loaded and separated by 0.8% agarose gel electrophoresis, blotted onto a charged nylon membrane and hybridized against chemically labelled *ras1* (a) and *ras2* (b). The sizes in kb of hybridized bands are estimated at the bottom of each panel.

(Waugh *et al.*, 2002), *A. fumigatus* (Fortwendel *et al.*, 2004) and *P. marneffei* (Boyce *et al.*, 2005). It was decided to evaluate the *ras* transcriptional pattern by semiquantitative RT-PCR. As was no information about the *ras1* and *ras2* in the pathobiology of *P. brasiliensis*, we decided to analyse the transcriptional pattern of *ras* genes on the temperature-dependent dimorphic transition from mycelium to yeast, under heat shock at 42 °C, and during internalization of yeast in murine macrophage cells, which mimics several aspects of host infection.

In vitro expression of *P. brasiliensis ras1* and *ras2* during dimorphism and under heat shock at 42 °C

As Ras proteins are key components of cascades controlling cellular events such as serum-induced pseudo-filament

formation in *Candida albicans* (Feng *et al.*, 1999; Leberer *et al.*, 2001) and haploid fruiting in *Cryptococcus neoformans* (Alspaugh *et al.*, 2000; Waugh *et al.*, 2002), it was speculated that they would suffer some regulation, pointing to the course of the dimorphic transition in *P. brasiliensis*. Expression levels of *ras1* and *ras2* on cells undergoing the first 24 h of the mycelium-to-yeast transition were evaluated. It is important to mention that the gene coding for the chlathrin light chain (*clat*) was chosen as the internal control because its corresponding PbAESTs were equally distributed in the mycelium and yeast partial transcriptomes analysed by the authors' laboratory (Felipe *et al.*, 2003 and Felipe *et al.*, 2005). The sqRT-PCR confirmed its usefulness as a constitutive expression control (data not shown). Total RNA from each point of the differentiation was used in sqRT-PCR experiments with specific primers directed to *ras1* and *ras2* genes and to the internal control *clat*. Amplicons corresponding to *ras1*, *ras2* and *clat* transcripts were quantified by densitometry. Figure 3a shows the quantification levels of the *ras1* or *ras2* product during the temperature-dependent dimorphic transition. After appropriate statistical analysis, no significant difference was observed in the expression levels of *ras1* or *ras2* at any point of the differentiation. The cellular differentiation dependent upon temperature change in dimorphic fungi is an event in which many genes must be tightly regulated. Nunes *et al.* (2005) identified by microarray 2583 genes with a modulated expression at some point of the differentiation of *P. brasiliensis* isolate Pb18. Among them figured as positively regulated in the M to Y direction: the calcineurin regulatory subunit, the Ca^{2+}/calmodulin-dependent protein phosphatase, protein kinase A and G proteins α and β. These proteins are common components of known signalling cascades that are involved in morphogenesis, differentiation, cell integrity, growth at a high temperature and virulence in several pathogens. The results indicate that *P. brasiliensis ras* genes do not have the expression modulated by temperature shifts and appear as constitutive genes during the dimorphic transition.

The involvement of Ras protein-controlled pathways in the heat shock response was reported previously for *S. cerevisiae* (Engelberg *et al.*, 1994). Independent of heat shock factor (HSF), the authors showed induction of heat shock proteins (HSPs) when Ras/cAMP/PKA signalling was blocked in yeast cells exposed to heat shock conditions, resulting in a thermo-tolerant phenotype. To analyse whether *P. brasiliensis ras* genes undergo any transcriptional modulation under heat shock, yeast cells were subjected to temperature shifts from 37 to 42 °C for 0, 15, 30, 60 and 120 min. The result showed that transcription of *P. brasiliensis ras1* is modulated by heat shock at 42 °C. After 30–120 min, *ras1* expression decrease sharply, falling to the lowest level at the 60-min point (Fig. 3b). Conversely, *ras2* is
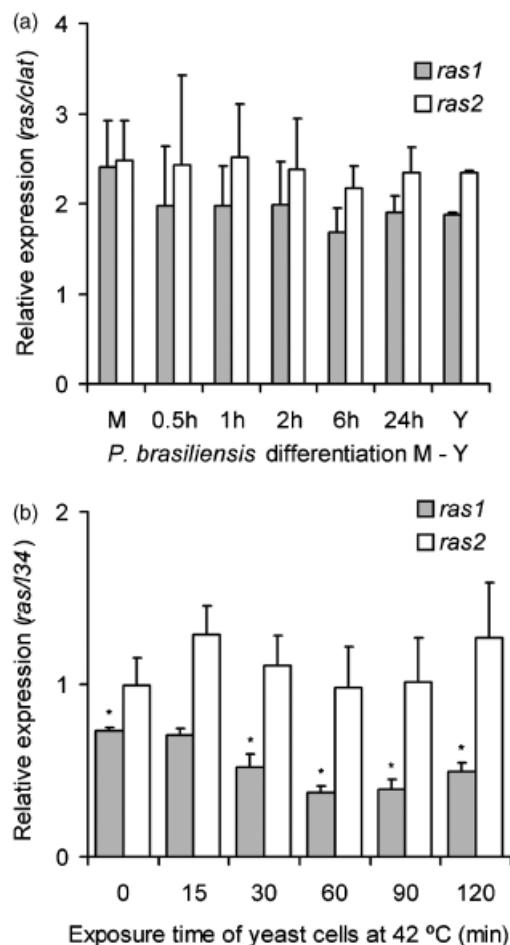
Fig. 3. *ras1* and *ras2* expression analysis during the dimorphic transition from mycelium to yeast (a) and in response to heat shock at 42 °C (b). (a) Represents the relative quantification of *ras1* and *ras2* transcripts measured in triple RT-PCR experiments at several time points (0.5, 1, 2, 6 and 24 h) of the dimorphic transition as well as during the vegetative growth of mycelium (M) at 22 °C and yeast cells (Y) at 37 °C. (b) Depicts similar quantifications of *ras* mRNA transcripts from yeast cells heat-shocked for 0, 15, 30, 60, 90 or 120 min at 42 °C. The semiquantitative analysis of *Paracoccidioides brasiliensis ras* gene expression was performed by densitometry using the SCION IMAGE software (http://www.scioncorp.com). The *ras1* and *ras2* transcript levels were calculated relative to the internal control, *l34* for heat shock and *clat* for dimorphic transition. The grey columns correspond to *ras1* transcripts, and the white columns correspond to *ras2* transcripts. Scale bars represent SEs and asterisks indicate $P < 0.05$.

not induced or repressed during heatshock. As Ras proteins are important signalling molecules needed for diverse responses to different stimuli, it may be hypothesized that *P. brasiliensis* yeast cells keep *ras2* as a constitutive expressed gene to respond immediately to stimuli other than a temperature shift and *ras1* may be modulated specifically by heat shock at 42 °C.

In vivo expression of *ras1* and *ras2* genes following *P. brasiliensis* yeast cells, internalization by murine macrophages

When *P. brasiliensis* infects the host, the first defence line against the pathogen is lung macrophages. Inside nonactivated macrophages fungal propagules have to deal with the harsh phagosome environment before switching to the yeast pathogenic form. These cells may develop a latency state enabling survival until environmental conditions favours fungal replication and dissemination. The ability to survive is crucial for the establishment and progression of the infection in susceptible hosts and, according to Tavares *et al.* (2007), it appears to be a consequence of genetic reprogramming of the pathogen. Those authors provided evidences that the fungus responds to the microenvironment in order to adapt mainly to the nutritional and oxidative stress generated by the phagosome.

In order to study the transcriptional profile of *ras* genes in the setting of the host–pathogen interaction, RNA obtained from *P. brasiliensis* yeast cells after 9 h of internalization by murine J774 macrophages was used. The semiquantitative analysis revealed that *ras1* and *ras2* are highly repressed – sixfold and three times, respectively, when compared with cells grown *in vitro* in Fava–Netto medium as a control (Fig. 4). It is the first time that fungal *ras* expression modulation has been detected in the macrophage microenvironment.

The present results lead to the hypothesis that the modulation of *ras* expression inside macrophage cells is a response to the nutritional starvation that can be noted by the repression of the glycolytic pathway (Tavares *et al.*, 2007), accompanied by the induction of the glyoxylate cycle (L.S. Derengowski, pers. commun.) and the overall gluconeogenetic status of the yeast cell, as reported before. In good accordance with the present data, Breviario *et al.* (1988) demonstrated that *RAS1* and *RAS2* transcripts are downregulated under glucose starvation in *S. cerevisiae*. In addition, Wang *et al.* (2004), by analysing the transcriptional framework when Ras2 is induced, showed a repression of gluconeogenesis in *S. cerevisiae*. In fact, the phagosome is a nutritionally hostile environment with an extremely low pH and a surfeit of oxidative reactive species produced to counter the presence of the pathogen. Yeast cells respond to this aggression by modulating the transcriptional apparatus to survive even in the presence of the macrophage defences. The pathogen latency state seems to be the most reasonable strategy of the yeast cells to evade the host immune system. Based on this fact, downregulation displayed by both *ras* genes in yeast engulfed by host cells completely makes sense, because the situation is unfavourable to proliferation or differentiation.
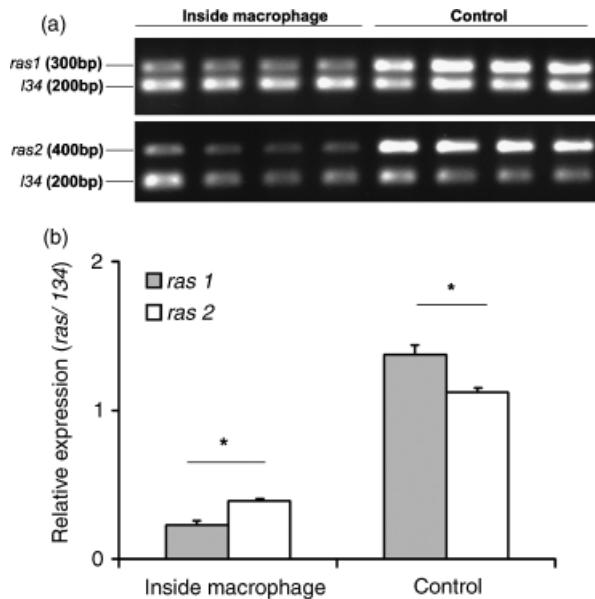
Fig. 4. *ras1 and ras2* transcriptional responses after yeast internalization by murine macrophages. *Paracoccidioides brasiliensis* yeast cells were exposed to murine macrophage J774 cells for 9 h. The total RNA from internalized yeast cells was extracted and used in RT-PCR experiments, as described in 'Materials and methods'. The control was the total RNA from *P. brasiliensis* yeast cells cultivated *in vitro* in Fava–Netto's medium for 7 days. (a) Shows the agarose gel electrophoresis analysis of RT-PCR products obtained for each condition tested, i.e. inside macrophage and control. (b) Represents the semiquantitative analysis of *P. brasiliensis* *ras1* and *ras2* mRNA expression under each condition. The *ras1* and *ras2* transcript levels were calculated relative to the internal control *l34*. The grey columns correspond to *ras1* transcripts, and the white columns correspond to *ras2* transcripts. The semiquantitative analyses were performed exactly as described in Fig. 3. Bars represent SEs of RT-PCR quadruple experiments, and asterisks indicate $P < 0.05$.

Potential role of farnesylation in the dimorphism of *P. brasiliensis*

The high frequency of Ras mutations in human cancer and the elucidation of the role of farnesylation in proper membrane addressing of Ras motivated an increased search for farnesyltransferase inhibitors against malignant cells (Basso *et al.*, 2006). For the present studies, we chose the commercially available FPT inhibitor III (Calbiochem) due to its cell permeability and the previous results with the fungal pathogens *Candida albicans* (McGeady *et al.*, 2002) and *Cryptococcus neoformans* (Vallim *et al.*, 2004). This compound blocks farnesyltransferase by competing with FPP for the active site of the enzyme. It did not produce any effect on the vegetative growth of *Candida albicans*, but led to a decreased rate in serum-induced conversion from yeast to pseudohyphae by this pathogen (McGeady *et al.*, 2002). Subsequently, Vallim *et al.* (2004) also detected no interference by FPT inhibitor III on the vegetative growth of

Cryptococcus neoformans, but it was able to impair two cellular differentiation events in this pathogen: the haploid fruiting and mating. Because Ras proteins must be farnesylated to proper membrane signalling, it was decided to use FPT inhibitor III in *P. brasiliensis* to investigate a role for Ras and other possible farnesylated proteins in this pathogen.

FPT inhibitor III was tested during the vegetative growth of mycelium at 22 °C and yeast at 37 °C and during dimorphic transition from mycelium to yeast and back again. The MIC for Pb01 yeast cells was higher than 500 μM. The MIC data corroborate with Vallim *et al.* (2004) and McGeady *et al.* (2002), who reported that high

(a) Mycelium Vegetative Growth
500 μM FPT Inhibitor III   Control

(b) Mycelium → Yeast
500 μM FPT Inhibitor III   Control

(c) Yeast Vegetative Growth
500 μM FPT Inhibitor III
Control

(d) Yeast → Mycelium
500 μM   250 μM   125 μM   Control

(e)
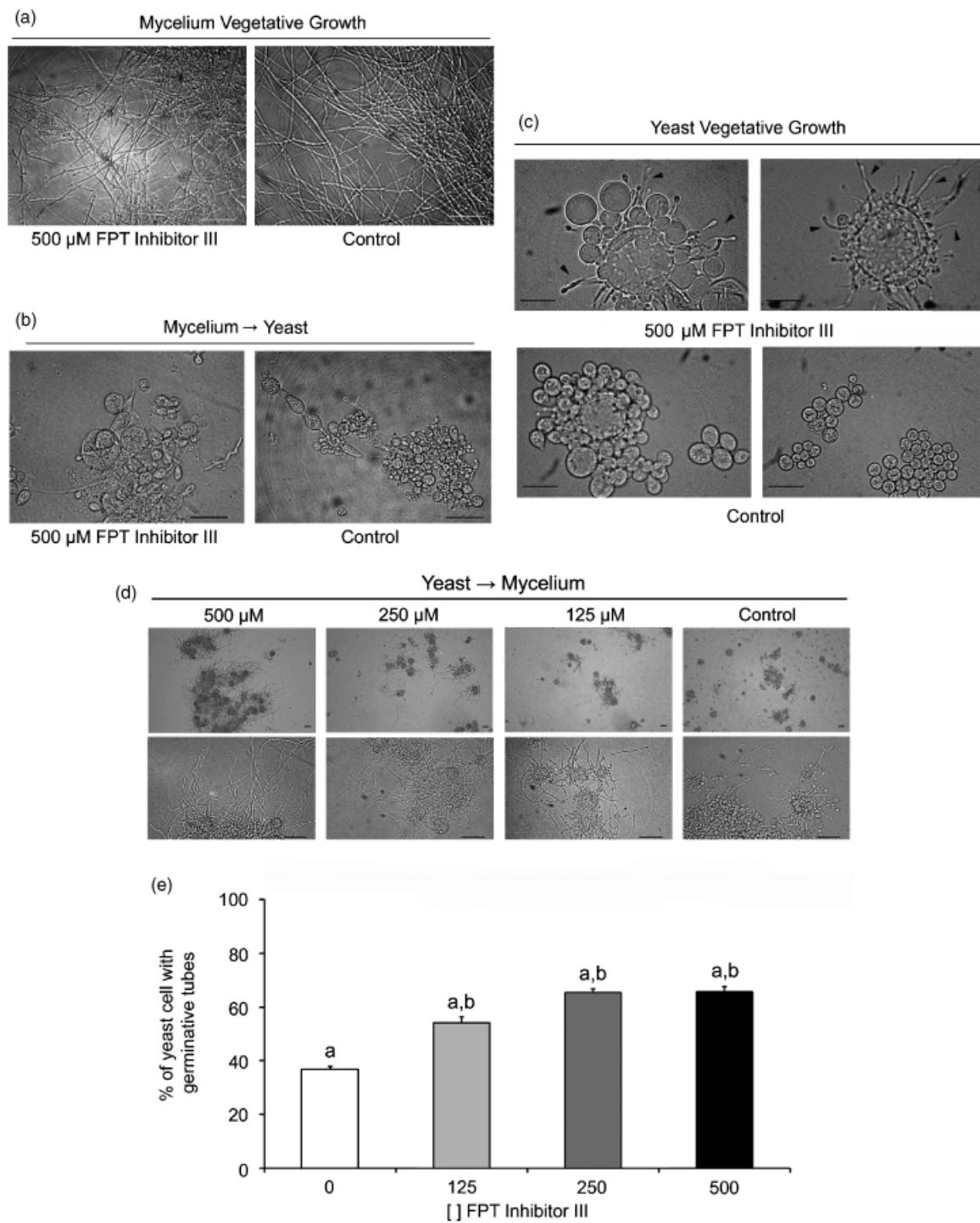
concentrations of the compound act on the vegetative growth of *Cryptococcus neoformans* and *Candida albicans*, respectively. No morphological difference to the control was detected on the vegetative growth of mycelium cells at 22 °C in the presence of the inhibitor even at 10 days of incubation at 500 μM (Fig. 5a). Similarly, no alteration was detected during differentiation from mycelium to yeast at any of the concentrations tested (Fig. 5b).

However, when the vegetative growth of yeast cells was evaluated at different concentrations of the inhibitor at 37 °C, the cells presented germinative tubes typical of mycelium that were not observed in the control (Fig. 5c). It is important to note that this phenomenon in *P. brasiliensis* is strictly dependent on the temperature shift from 37 to 22 °C and that the blockage of farnesylation mimicked the process, thus favouring the filamentous form of the fungus.

In the yeast-to-mycelium transition experiment, yeast cells were incubated at 22 °C in the presence of 0, 125, 250 and 500 μM of the inhibitor. As can be seen, yeast cells also presented germinative tubes developing to produce true hyphal filaments in the presence of the inhibitor (Fig. 5d). However, an increased amount of yeast cells with germinative tubes or true filaments was detected. The data show that the percentage of yeast cells displaying germinative tubes increased up to nearly twice the normal transition values for the highest concentration (Fig. 5e). It is concluded that the inhibitor favours transition to mycelium in a dose-dependent fashion and perturbs the yeast phase by making the yeast-to-mycelium transition a temperature-independent event.

As the farnesylation inhibitor is not specific for Ras protein incorporation into the membrane, the defects observed might result from the inhibition of other Ras-related proteins that are also farnesylated. It is improbable that FPT Inhibitor III has any direct interference on the Rho-GTPase superfamily that includes RhoA, Rac and Cdc42, as they are geranylated instead of farnesylated (Arellano *et al.*, 1999). From the 30 proteins known to be farnesylated in the human cells (Appels *et al.*, 2005), two of

them, Rheb and Ras, are reported to be involved in the growth and differentiation in response to different stimuli in fungi (Aspuria & Tamanoi, 2004). The Rheb protein has been proposed to activate the Tor signalling pathway, which in turn controls cell differentiation in *S. pombe* (Alvarez & Moreno, 2006; Uritani *et al.*, 2006), *Cryptococcus neoformans* and *Candida albicans* (Rohde & Cardenas, 2004). As for Ras, in a wide range of fungi, it is an upstream component of several signalling cascades. Ras proteins activate Cdc42 to promote cell polarity and mating process in *S. pombe* (Marcus *et al.*, 1995), filamentous growth in *S. cerevisiae* (Mosh *et al.*, 1996), vegetative growth and serum-induced dimorphic transition in *Candida albicans* (Leberer *et al.*, 2001), spore germination and polarized growth in *P. marneffei* (Boyce *et al.*, 2005) and high-temperature growth and cellular differentiation in *Cryptococcus neoformans* (Vallim *et al.*, 2005).

In this work, it has been shown that impaired farnesylation promoted alterations in the vegetative growth of *P. brasiliensis* yeast cells and induced dimorphic transition from yeast to mycelium, but it could not be determined as to how and which farnesylated protein, whether Ras, Rheb or both, is controlling these events. It is known that *P. brasiliensis* possesses all signalling components involved in cellular differentiation and morphogenesis described for other fungi (Fernandes *et al.*, 2005), including Tor- and Ras-controlled pathways, but the way in which they work and are connected and which signals activate them are yet to be elucidated.

The present studies show that *P. brasiliensis* has two *ras* genes that codify the Ras1 and Ras2 proteins. These genes are modulated negatively when yeast cells of this pathogen are in contact with host cells and this probably stems from the nutrient limitation and the harsh environment encountered by the fungus. However, only *ras1* displayed heat-shock triggered mRNA suppression, although neither gene responds to dimorphic transition. These data suggest a modulation of *ras* based on the signal that is conveyed to the cell. Still, the cascades that Ras controls and their

Fig. 5. Farnesylation inhibition effects on mycelium and yeast cells and during the differentiation of *Paracoccidioides brasiliensis*. (a) Farnesylation inhibition on the vegetative growth of mycelium, where fragments of mycelium were incubated in the presence of 500 μM FPT inhibitor III in MVM medium at 22 °C for 10 days. The control was mycelium fragments grown in MVM medium; the scale bar is 10 μm. (b) the dimorphic transition from mycelium to yeast, in which fragments of mycelium were incubated for 48 h at 22 °C and shifted to 37 °C for 10 days to induce yeast differentiation in the presence or absence of 500 μM FPT inhibitor III, magnification bar, 10 μm. (c) perturbation of yeast vegetative growth of *P. brasiliensis* in the presence of farnesylation inhibitor. Yeast cells were incubated in the presence of 500 μM FPT inhibitor III in MVM medium at 37 °C for 7–10 days (upper panel). The control was yeast cells grown in MVM medium under the same conditions described above (bottom panel). The black arrowheads indicate germinative tubes emerging from yeast cells when farnesylation is blocked, magnification bar, 10 μm. (d) Dimorphic transition from yeast to mycelium in the presence of farnesylation inhibitor. The yeast cells were inoculated into MVM medium and incubated for 48 h at 37 °C and then shifted to 22 °C for 10 days to induce differentiation to the mycelium form in the presence of 500, 250 and 125 μM of FPT Inhibitor III and in the absence of the compound (control). At all concentrations, the yeast cells produce germinative tubes; the magnification bar in the upper panel stands for 1 μm and that in the bottom panel for 10 μm. To quantify differences in the germination rate for each inhibitor concentration, a random counting of yeast cells with germinative tubes was performed and their proportion was calculated relative to the total cell number in four randomly chosen fields of three different lamina in three independent experiments. The results were plotted in a graphic as percentage of yeast cells with germinative tubes vs. the inhibitor concentration assayed (e). Scale bars represent SEs of triple experiments, with $a = P < 0.001$ and $b = P < 0.01$.

connections must be studied further, but the importance of Ras proteins in the pathobiology of *P. brasiliensis* is already notable. To address the many questions that have arisen from this work, the authors are developing an RNA interference strategy for functional gene analysis in this pathogen. Gene silencing is preferable to classical disruption due to the unfeasibility of knock-out approaches on *P. brasiliensis* multinucleated and multibudding yeast cells. This first analysis of *ras* genes provides an avenue of possibilities for the characterization of *P. brasiliensis* signalling cascades and their components, which will certain lead to new insights into adaptive responses, morphogenesis control, virulence, and pathogenicity of this fungus.

Acknowledgements

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Regulatory networks in the host-fungal pathogen interaction

Fernandes, L.[1], Bocca A.L.[1], Ribeiro A.M.[1], Silva S.S.[1], Paes H.C.[1], Amaral A.C.[1,2], Polez V.L.P.[1], Martins N.F.[3], Soares C.M.A.[4], Felipe M.S.S.[1]

[1] Depto. de Biologia Celular, Universidade de Brasília, 70910-900, Brasília, DF, Brazil.

[2] Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, 70790-160, Brasília, DF, Brazil.

[3] Núcleo Temático Biotecnologia, Cenargen-Embrapa , 70770-900 Brasília, DF, Brasil

[4] Depto. de Bioquímica e Biologia Molecular, Universidade Federal de Goiás, 74001–970, Goiânia, GO, Brazil

Correspondence: Maria Sueli S. Felipe, Laboratório de Biologia Molecular, Departamento de Biologia Celular, Universidade de Brasília – Brasília, DF, Brazil, 70910-900, Tel.: +55 61 3307-2423, Fax: +55 61 3349-8411 E-mail: msueli@unb.br

Running title – Signaling in host-fungal pathogen interaction

Abstract

The host innate immune response is critical for protection against infection, responsible for recognizing and countering microbial invasion. Macrophages and neutrophils mainly participate in this response, producing and releasing cytokines and chemokines. In addition, they present microbial antigens to lymphocytes, leading to the development of a highly specific immune response. Receptors are required for detection of microbial components triggering signaling pathways to activate the host immune response. On the other hand, sensing and responding to the environment is required for fungal survival. Fungi have sophisticated and conserved signaling cascades to sense and respond to different types of stress including osmotic shock, temperature, oxidative or nitrosative damage. This review focuses in the main pathways that host cells uses to recognize, interact and respond to the infecting fungal pathogens and highlights the cascades that regulate environmental response by the most studied human fungal pathogens.

Introduction

Signal transduction is a very important mechanism by which the cell exercises its regulatory response depending on the stimulus. The signal transduction communicates extracellular signals to the cell interior through the alternative phosphorylation of numerous proteins, including transcription factors, cytoskeleton proteins, kinases and other enzymes, influencing gene expression, metabolism, cell division, cell morphology, cell survival and homeostasis (Cobb and Goldsmith, 1995).

Fungi are models on the environmental response as the signal transduction pathways were evolutionarily conserved and are often equivalent to multicellular eukaryotic organisms. Fungi may be divided according to the presence or absence of a differentiation pattern that marks the switch from a mycelium to a yeast form. This division overlaps poorly with phylogenetic relationships; for example, in the group of dimorphic fungi we find both *Candida albicans* and the distantly related, thermo-dimorphic ascomycetes – *Blastomyces dermatitidis, Coccidioides immitis, Histoplasma capsulatum, Paracoccidiodes brasiliensis, Penicillium marneffei and Sporothryx schenkii*; whereas in the non-dimorphic group we find both the filamentous ascomycete *Aspergillus fumigatus* and the basidiomycete *Cryptococcus neoformans*. There is no obvious connection to the life cycle of the fungi, since *C. albicans* and *C. neoformans* are to be found in commensalism with men and birds or *Eucalyptus* trees, respectively, while the others are known or supposed to exist as free-living moulds.

Here we review the current state of the knowledge on environmental adaptation focusing in the host-pathogen interaction cascades in fungi. In this review we discuss the main pathway that host cells recognize and interact to respond to the infecting fungal pathogens. Additionally, we also compare and highlight cascades that regulate environmental response by those human fungal pathogens in relation to morphogenesis, thermal differentiation, oxidative stress adaptation, virulence and pathogenicity.

1. Signaling on pathogen recognition by the host cells

The anti-pathogen innate immune response is critical for protection against infection. It is the first line of host defense, responsible for recognizing and countering microbial invasion. This response is comprised mainly of phagocytic cells, such as macrophages and neutrophils, which can ingest and kill the invading pathogens. These cells produce and release cytokines and chemokines. In addition, they present microbial

antigens to lymphocytes, leading to the development of a highly specific immune response (Janeway, 1992).

The first interaction occurs between Pattern-Recognition Receptors (PRRs) and conserved molecular structures of microbes know as Pathogen-Associated Molecular Patterns (PAMPs), which are generally essential for the survival of the microbes. The ability of the innate immune system to distinguish between pathogens has been of considerable interest, although much is still not understood. The discovery of the Toll-Like Receptor (TLRs) and the non-TLR has given an insight into the mechanisms of intracellular signaling following microbial sensing **(Table 1).**

Insert table 1

PRRs are localized in serum or tissue fluid, membrane or cytoplasm, and are subdivided into related families according to their structure and/or function. Generally, each receptor recognizes a range of microorganisms based on its individual ligand specificity. Leukocytes use these receptors to recognize pathogen either directly (non-opsonic recognition) or indirectly (opsonic recognition). During infection, microbial recognition occurs through multiple interactions at multiple sites and involves many receptors (Janeway and Medzhitov, 2002; Romani, 2004). Following recognition, some PRRs promote microbial uptake. Shortly thereafter, microbial killing occurs in the phagosome through various mechanisms such as drop in pH and attack by hydrolytic enzymes and toxic metabolites. The fungal cell wall consists mainly of carbohydrates, including mannose-based structures, β-glucan (more than 50%) and chitin. The recognition of β-glucan could contribute to an effective immune response.

1.1. Recognition of fungal structures by Toll-Like Receptor (TLR)

TLRs are a family of ten innate immune recognition receptors that are required for detection of a broad range of microbial products including LPS, flagellin, and bacterial lipoproteins. They are distinct from each other in ligand specificities, expression patterns, and signaling pathways, but all act in the initiation and activation of immunity.

TLR2 and TLR4 have been implicated in anti-fungal immunity and recent studies have demonstrated a crucial involvement of these in the recognition of fungal pathogens such as *C. albicans*, *A. fumigatus* and *C. neoformans.* (Netea *et al.*, 2004a;

Netea *et al.*, 2006). Fungal infection in knock-out mice deficient in either TLRs or TLR-associated adaptor molecules showed that specific TLRs such as TLR2 and TLR4 play differential roles in the activation of the various arms of the innate immune response. Recent data also suggest that TLRs offer escape mechanisms to certain pathogenic microorganisms, especially through TLR2-driven induction of antiinflamatory cytokines (Netea *et al.*, 2004b). TLR2 is the most powerful receptor and recognizes a wide variety of PAMPs from bacteria, yeast, fungi, parasites and viruses. The natural ligands of the TLRs are evolutionary conserved microbial components. Those recognizing bacterial and fungal PAMPs are generally displayed on the surface of cells, and those recognizing viral components are found inside the cells **(Figure 1).**

Insert Figure 1

TLRs are believed to function as dimers. Though most TLRs appear to function as homodimers, TLR2 forms heterodimers with TLR1 or TLR6, each dimer having different ligand specificity. TLRs may also depend on other co-receptors for full ligand sensitivity, such as in the case of TLR4's recognition of LPS. When activated, TLRs recruit adapter molecules within the cytoplasm of cells in order to propagate a signal. Four adapter molecules are known to be involved in signaling, known as MyD88, TIRAP, TRIF, and TRAM. The adapters activate other molecules within the cell, including certain protein kinases (TAK1, IKK complex, IRAK1, IRAK4, TBK1) that amplify the signal, and ultimately lead to the induction or suppression of genes that orchestrate the inflammatory response. In all, various genes are activated by TLR signaling, and it constitutes one of the most powerful and important gateways for gene modulation. Following activation by ligands of microbial origin, several reactions are viable. Immune cells produce signaling factors as cytokines which trigger inflammation.

Study of TLRs has significantly enhanced the understanding of innate and adaptive immune responses and provides novel therapeutic approaches against infectious and inflammatory diseases. Recent developments provide crucial information for clarifying the mechanisms of fungal recognition by cells of the immune system, and provide hope for designing new therapeutical approaches to fungal infections**.**

1.2. Recognition of fungal structures by non-Toll Like Receptor (non-TLR)

1.2.1. Receptors that recognize mannose

C-type lectins are classical molecules characterized by their ability to bind carbohydrates in a Ca^{2+}-dependent manner. They have a common domain, named carbohydrate recognition domain (CRD), which contains 18 highly conserved amino acid residues including two disulfide bonds. Phagocytes express many kinds of C-type lectin receptors on their surface for antigen capture. Some of these domains are known by the acronym CLEC (C-type lectin domain family) (Kanazawa, 2007).

The mannose receptor (MR) is one of such receptors, consisting of an N-terminal cystein-rich domain, a fibronectin type II domain and 8 or 10 CRDs. MR preferentially recognizes α-linked oligomannose with branched rather than linear structures (Kanazawa, 2007) and is capable of recognizing a variety of antigens, ranging from self-molecules to pathogens (Geijtenbeek *et al.*, 2004). Recognition of *C. albicans* by human/murine macrophages is mediated by a mannose receptor that can bind to N-linked mannosyl residues and by TLR-4 bound to O-linked mannosyl residues (Netea *et al.*, 2006). It was demonstrated that the protective T-cell response to the pathogenic yeast *C. neoformans* is dependent on the recognition of mannoproteins by multiple MR present on the surface of dendritic cells. Mansour *et al.* (2006) suggest that these cells provide the crucial link between innate and adaptive immune responses to *C. neoformans* via a process that is dependent upon the efficient uptake of mannoprotein by MR. Following the interaction between *Pneumocystis carinii* and human alveolar macrophages, IL-8 is released. This event is partially dependent on the transcription factors NF-kappaB, ERK1/2 and JNK MAPKs. It also requires the interaction between the fungus and both receptors MR and TLR2 (Tachado *et al.*, 2007) **(Figure 1)**. As for *P. brasiliensis,* the higher mannose receptor expression is important for macrophage phagocytic capacity (Jiménez *et al.*, 2006). Another receptor able to recognize mannose residues present on the surface of a wide spectrum of microorganisms is the CLEC-1b (C-type lectin like receptor 1). The gene encoding this receptor is induced during macrophage infection with *P. brasiliensis* (S.S. Silva, unpublished).

1.2.2. Receptors that recognizes β-glucan

The recognition of β-glucan occurs by a family of non-classic C-type lectin receptors called Dectin-1. Dectin-1 receptors lack a cysteine residue in its stalk region and contain an immunoreceptor tyrosine-based activation motif (ITAM) in the

cytoplasmic tail, which is involved in cellular activation. After phosphorylation of the second tyrosine occur various effects such as induction of phagocytosis, production of reactive oxygen species (ROS) and cytokine production mediated by nuclear factor (NF)-kB activation (Brown, 2006).

The synergism of Dectin-1 and TLR2 signaling enhances NF-kappaB activation and regulates cytokine production **(Figure 1).** Dectin-1 signaling via CARD9, that is an adaptor molecule containing an N-terminal caspase-recruitment domain (Dostert & Tschopp, 2007), and also directly modulates gene expression via activation of NFAT (Goodridge *et al*., 2007). Dectin-1 was originally thought to be a dendritic cell-specific receptor, from which its name "<u>de</u>ndritic-<u>c</u>ell-associated C-type lec<u>tin</u>-1" was derived (Ariizumi *et al*., 2000). This receptor recognizes fungal β-glucan without opsonic molecules. It therefore recognizes several fungal species, including *C. albicans* (Taylor *et al.,* 2007), *P. carinii* (Saijo *et al*., 2007), *Coccicioides posodaii* (Viriyakosoi *et al*., 2005) and *A. fumigatus* (Gersuk *et al*., 2006). Their expression on macrophage is up-regulated by IL-4 and IL-13 and down-regulated by LPS, IL-10 and dexamethasone. The loss of this receptor might explain why some fungi escape the host immune surveillance (Brown, 2006).

The protection role of dectin-1 against all fungal infections is not a consensus. Taylor *et al.* (2007) described that dectin-1-knockout mice were more susceptible than wild-type mice to *C. albicans* infection. In contrast, Saijo *et al*. (2007) showed that wild-type and dectin-1-knockout mice were equally susceptible to *Candida* infection. The recognition of *C. albicans* by macrophages is a complex process involving multiple receptor systems that recognize sequentially the various layers of the outer portion of the fungal cell wall. The components of the cell wall of a pathogenic fungus (*N*-linked mannans, *O*-linked mannans, and β-glucans) are involved in the recognition by monocytes/macrophages by MR, TLR4 and dectin-1/TLR2 respectively and in the subsequent induction of pro- and anti-inflammatory cytokine release (Netea *et al*., 2006).

The absence of interaction between dectin-1 and β-glucan contributes to fungal evasion of immune system. The yeast form of *C. albicans* induces a host-protective response while hyphae do not, since the last lacks the surface exposed β-glucan (Heinsbroek *et al*., 2005). *C. neoformans* encapsulation also mask the cell-wall β-glucan (Cross and Bancroft, 1995) and the changes in proportion of cell wall α/β glucan of *P. brasiliensis* (San-Blas *et al*., 1984; Silva *et al*., 1994) contributes to the evasion of

specific response. The subversion of host immune response is effective by dectin-1 receptor blockage by cell wall α-glucan, as demonstrated for *H. capsulatum* (Rappleye *et al.*, 2007). The macrophage response to resting spores and invasive form of the *A. fumigatus* is dependent on the dectin-1 interaction with β-glucan present in the cell wall. β-glucan is absent on the surface of dormant conidia, but is present after cellular swelling and loss of the hydrophobic proteinaceous cell wall. Dectin-1 binding to germ tubes increases, but is not required, for TLR2-mediated inflammatory cytokine secretion. In addition, it stimulates TNF-α production in the absence of both TLR2 and MyD88 signaling (Gersuk *et al.*, 2006).

Cells that do not express dectin-1 receptor and still have an important role in anti-fungal immunity express other receptors, such as the complement receptor 3 (CR3), lactosylceramide and scavenger receptors. All these receptor also recognize β-glucan. CR3 (also called CD11b-CD18 or MAC1), the complement fragment inactivated C3b (C3bi), β-glucan and endogenous ligands are involved in cell adhesion and migration. CR3 consists of several domains, including a lectin domain that mediates carbohydrates recognition and regulates adhesion (Taylor *et al.*, 2002; Xia *et al.*, 1999). The importance of CR3 in fungus uptake is demonstrated for *P. brasiliensis* when the opsonic conidia are extensively phagocyted by macrophages. The heat-inactivation of serum used for opsonization or the treatment of macrophages with anti-CR3 decreased phagocytosis by macrophages (Jiménez *et al.*, 2006).

Lactosylceramide, also called CDw17 and Galβ4Glcβ1Cer, is a glucosphingolipid PRR found in plasma membrane micro-domains of many cells types. Lactosylceramide-mediated recognition of β-glucan induces various cellular responses *in vitro,* including cytokine production, the respiratory burst and NFkB activation (Wakshull *et al.*, 1999.). The scavenger receptors are a family of cell-surface glycoprotein PRRs that differ greatly in their structure but are all capable of recognizing modified low-density lipoproteins. These receptors have also been implicated in β-glucan recognition (Rice *et al.*, 2002).

Phagocytic cells are crucial for host defence against invading pathogens such as bacterial and fungus. The macrophage phagosome is believed to be a poor source of glucose and amino acids (Fan *et al.*, 2005; Lorenz *et al.*, 2004). Such nutrient deprivation inside the macrophage induces a similar adaptive response of intracellular bacterial and fungal pathogens (Chatterjee *et al.*, 2006; Fan *et al.*, 2005; Lorenz *et al.*,

2004). Further, we will focus in the signaling pathways underwent by fungal pathogens in order to activate the genetic re-programming necessary to the survival in the host cells.

2. Signaling response in fungal pathogens

2.1. Morphogenesis and differentiation

In this topic we focused on signaling components that have been demonstrated by knock-out experiments to be involved in fungal pathogenesis using animal models. The differentiation process observed in fungal pathogens is that of thermo-dimorphic fungi. The species that cause human disease probably follow a similar genetic programme that causes them to change from mycelium to yeast upon exposure to the higher temperature of the host. Recently, Nemecek *et al.*, 2006 identified a histidine kinase (HK) receptor as the dimorphism switch - denominated Drk1, for <u>D</u>imorphism-<u>r</u>elated <u>k</u>inase - in *B. dermatitidis*. They also provided evidence that the mechanism is conserved in the other species when they reproduced the effects of silencing the corresponding drk1 gene in *H. capsulatum*. These effects were to lock the fungi in the mycelial phase, which was correlated with a severe impairment of their ability to cause infection. HKs have only been proposed as membrane receptors in *B. dermatitidis* and *H. capsulatum*. Exactly how these receptors sense environmental changes and which factors function downstream in morphogenetic process remains to be elucidated.

MAPKinase cascades are responsible for fungal differentiation and virulence. The key regulator molecules in this case are the small GTPase Ras and its immediate downstream effectors best known is the Rho-GTPase Cdc42. This pathway was first characterized in *C. albicans* (Feng *et al.*, 1999) and *C. neoformans* (Alspaugh *et al.*, 2000), and more recently in *A. fumigatus* (Fortwendel *et al.*, 2004; Fortwendel *et al.*, 2005) and *P. marneffei* (Boyce *et al.*, 2005). The Ras-controlled processes seem to be both more global and more conserved among species. Cdc42 regulates the actin component of the cytoskeleton and thus the vegetative growth of all pathogens studied so far. The pathway is also indispensable for growth at 37°C and therefore knock-out strains are avirulent in animal models. Still, some Ras-triggered events are known to be species-specific: in *C. albicans*, the pathway is necessary for serum-induced filamentation (Feng *et al.*, 1999; Leberer *et al.*, 2001); in *P. marneffei*, no influence in dimorphism has been identified for Ras and its effectors to date, but the maintenance of cell polarity by this cascade has been well characterized for that fungus (Boyce *et al.*,

2005); and in *A. fumigatus*, spore germination and virulence are determined by Ras-dependent mechanisms (Fortwendel *et al.*, 2005).

There is ample evidence that Ras acts not only as a relay protein in signaling processes, but also that it functions in the cell as a bifurcation, exerting modulation over several pathways. Recent data indicate that more than one MAPKinase cascade is modulated by Ras through downstream Rho-GTPases other than Cdc42 (Nichols *et al.*, 2007). Furthermore, a completely different branch cascade is also modulated by Ras, namely the cAMP/PKA pathway **(Figure 2)**. In that case the immediate effector of Ras is adenylate cyclase, and there seems to be functional redundancy with the MAPKinase pathway, for both seem to influence the same morphogenetic events and pathogenicity. Apart from *P. marneffei*, the only thermo-dimorphic pathogen where members of the Ras-mediated pathways have been studied *in silico* is *P.brasiliensis* (Fernandes *et al.*, 2005; Felipe *et al.*, 2005).

Insert Figure 2

2.2. Signaling in response to oxidative stress

Phagocytosing macrophages is responsible to release oxygen and nitrogen compounds as part of the antimicrobial burst (Densen and Mandell, 1995). In this way, the study of the oxidative stress in fungi, which suffers phagocytosis, is expected to elucidate how this pathogen survives the oxidative killing mechanism by the host macrophage system.

Reactive oxygen species (ROS) such as hydrogen peroxide, produced externally or during normal metabolism can damage different cell components and usually trigger a counteracting antioxidant response (Aguirre *et al.*, 2005). Fungi have several mechanisms to handle ROS, such as the presence of a large number of antioxidant enzymes and the ability to produce secondary metabolites with antioxidant function (Kawasaki and Aguirre, 2001; Lee *et al.*, 2005).

A number of studies indicate that reactive nitrogen species (RNS) are important antimicrobial effectors produced by macrophages (Missall *et al.*, 2004). In contrast to the redundancy in the ROS elimination, there are few mechanisms for the disposal of NO (de Jesus-Berrios *et al.*, 2003). To survive the oxidative and nitrosative attack initiated by phagocytic cells of the host, pathogens must respond appropriately. This antimicrobial attack is established by two main systems including the nitric oxide

synthase, which generates reactive nitrogen species and the NADPH oxidase pathway, generating reactive oxygen species (Fang, 2004). To cause infection, pathogens must evade the immune system by initiating a response to the stress encountered.

2.2.1. Signal transduction mechanisms in fungal responses to ROS and RNS

An important example of an environmental challenge to which fungi must rise is the high levels of ROS produced by neutrophil cells during the oxidative burst. The oxidative killing of fungal cells by this host defense mechanism represent an important line of elimination of pathogens, as demonstrated by the correlations between the function of oxidative stress response in pathogenic fungi and their ability to proliferate in host cells (Wysong *et al.*, 1998). Studies have demonstrated that eukaryotes evolved novel mechanism to perceive and eliminate ROS. Those studies had identified three major modules that govern the response to oxidative stress, the stress responsive MAPKinase cascade (also denominated SAPK for Stress Activated Protein Kinases), the most well-characterized signal transduction cascades regulating fungal adaptation to the environmental stress, a histidine kinase (HK) system (a prokaryotic-type multistep phosphorelay system) and the AP-1-like transcription factors (Posas *et al.*, 1996; Grefen and Harter, 2004).

2.2.2. Fungal MAPK and HK components systems

In eukaryotic cells, phosphorelay systems (HKs) have been found in slime molds, fungi and plants where they connect environmental stress signals to the MAPKinase cascades (reviewed in Kruppa and Calderone, 2006). In a prototypical two-component system, a sensor HK phosphorylates a histidine residue within its kinase domain, referred as an H-box; subsequently the HK phosphorylates a phosphotransfer protein (HPt), which in turn transfers the phosphate group to an aspartate residue in a receiver domain of a response regulator protein (RR). Most characterized HKs, both bacterial and eukaryotic, require the additional phosphorelay step through the HPt domain protein and a second RR protein. This additional phosphorelay step may allow the organism to integrate multiple input signals into a single output. In fungi, a classical target of the two-component system is the Hog1 MAPKinase pathway and the phosphotransfer from the HK is mediated by Ssk proteins **(Figure 3)**, which possess aspartate residues that are phosphorylated by the kinase (Kruppa and Calderone, 2006).

Insert Figure 3

The ascomycete *C. albicans* is the most common fungal pathogen in immunocompromised patients (Fridkin and Jarvis, 1996). In order to defend against colonization of this pathogen, the host immune system utilizes the ROS production. Studies have suggested that macrophages ingest and kill the fungus through an oxygen-dependent mechanism (Vazquez-Torres and Balish, 1997). One of the major signal transduction pathways in *C. albicans* is the HOG pathway, which senses oxidative stress and is also involved in sensing osmotic stress (Bahn *et al.*, 2007). Three HK genes, Sln1, Chk1 and Nik1, which are distinct and redundant, as well as the homologue of the HPt protein, Ypd1, have been reported in *C. albicans* (Nagahashi *et al.*, 1998; Calera *et al.*, 1998; Calera *et al.*, 2000). Additionally, *C. albicans* presents two RR, Ssk1 and Skn7; the last is supposed to regulate its action as a transcriptional factor (Lee *et al.*, 1999). Ssk1 mediates gene activity by interacting with the MAPKKK Ssk2 of the MAPK pathway (Chauhan *et al.*, 2003). The MAPKinases module in *C. albicans* is composed of Ssk2 (MAPKKK), Pbs2 (MAPKK) and Hog1 (MAPK). When phosphorylated, Ssk1 (RR) is not able of inducing activation of the MAPKKK, Ssk2. Under oxidative/osmotic stress, phosphotransfer through Sln1-Ypd1-Ssk1 activates Ssk2, which activates Pbs2 and finally Hog1 (reviewed in Chauhan *et al.*, 2006). Observations are consistent with the idea that *C. albicans* MAPKinase (Hog1) pathway is required for the fungal resistance to oxidative stress. Hog1 mutant strains are sensitive to the oxidants menadione and H_2O_2 (Alonso-Monge *et al.*, 2003). Also, the Pbs2 MAPKK component is crucial for the fungal anti-oxidant adaptation (Arana *et al.*, 2005). Transcriptional analysis of ssk1 mutants has shown that a considerable portion of the altered genes encode for proteins related to the stress adaptation (Chauhan *et al.*, 2003). The role of Hog1 in the regulation of global transcriptional responses to stress has been studied in *C. albicans*. DNA microarrays were used to characterize the global transcriptional responses of HOG1 and hog1 cells to osmotic, oxidative and heavy metal stress. Inactivation of HOG1 attenuated transcriptional responses to osmotic and heavy metal stresses, but not to oxidative stress. It has been suggested that Hog1 plays a central role in the regulation of osmotic and heavy metal stress-induced gene expression, but a less central role in the regulation of oxidative stress genes in *C. albicans* (Enjalbert *et al.*, 2006).

The basidiomycete *C. neoformans* is encapsulated yeast that predominantly infects the central nervous system in immunocompromised patients causing life threatening fungal meningitis (Mitchell and Perfect, 1995). In *C. neoformans* a two-component phosphorelay system has been investigated (Bahn *et al.*, 2006). Unlike the ascomycete fungi, the HOG pathway of *C. neoformans* is constitutively phosphorylated under normal conditions, via the Pbs2 MAPKK and an unknown MAPKKK. In response to diverse stress conditions a variety of sensor kinases, including the characterized Tco1 and Tco2, activates Ssk1, which in turn activates a Hog1-specific phosphatase resulting in rapid dephosphorylation of Hog1 and the consequent induction of appropriated cellular response to the environmental stimuli **(Figure 3)**. Tco2 is partially responsible for response to oxidative damage and osmotic shock, as described (Bahn *et al.,* 2006).

By comparison to known sequences of the MAPK module in other fungi, all the components of the MAPK module had been identified in *A. fumigatus* (Aguirre *et al.*, 2006) and homologues of the HOG-MAPK pathway proteins had been studied in this fungal pathogen. The sakA gene (also called hogA), has a role in protection against oxidative stress, since SakA mutants of *A. fumigatus* are oxidant sensitive (Xue *et al.*, 2004). Additionally, *A. fumigatus* contains a second SAPK, called MpkC; its role in the oxidative stress response and pathogenicity has not yet been evaluated (May *et al.*, 2005).

P. brasiliensis is an important dimorphic fungal pathogen located predominantly in Central and South America. *In silico* search of *P.brasiliensis* database has revealed a number of two-component signal transduction proteins, as well as MAPK pathways including HOG1 (Felipe *et al.*, 2005; Fernandes *et al.*, 2005) Accordingly the fungal database predicts a sln1 sensor kinase orthologue, one ypd1 orthologue, as well as ssk1 (RR), ssk2 (MAPKKK), pbs2 (MAPKK), hog1 (MAPK), suggesting that this fungal pathogen can utilize the modules that govern the response to oxidative stress, the stress responsive MAPKinase cascade and the multistep phosphorelay system for dealing with oxidative stress, which constitutes another hint at the phylogenetic conservation of this mechanism.

2.2.3. Fungal AP-1-like transcription factors

Fungal cells have targeted nuclear localization of several components of their oxidative-stress response machinery for modulation. AP-1 (Activating Protein 1) describes a group of members of the Jun, Fos and ATF family proteins that form homo/heterodimers to regulate cell proliferation and differentiation, apoptosis and stress response (Karin *et al.*, 1997). Studies had demonstrated the role of the AP-1 homologues in the redox homeostasis in fungi, putatively as a positive regulator of gene expression (Harshman *et al.*, 1988).

Fungal AP-1-like factors had been first identified in *Saccharomyces cerevisiae* (Yap1, for yeast AP-1) and had been characterized as a transcription factor, which regulates antioxidant genes, such as thioredoxin and gluthatione peroxidase (Kuge and Jones, 1994; Tsuzi *et al.*, 2004). *C. albicans* presents a homologue of the *S. cerevisiae* Yap1, denominated Cap1. The role of Cap1 in the fungal adaptation to oxidant stress has been investigated. The protein is induced in the presence of oxidative stress and localizes to the nucleus, in an oxidant–responsive fashion, inducing a set of genes necessary to the response to this stress (Zhang *et al.*, 2000). Fungal cells lacking *CAP1* are hypersensitive to diamide and H_2O_2 (Zhang *et al.*, 2000). Although the detailed mechanisms of *CAP1* regulation needs to await further studies, the oxidative stress sensing by Cap1 seems to involve a cysteine residue in the C-terminal domain, as described for its homologues (Zhang *et al.*, 2000). In *C. albicans*, Cap1 has been shown to activate transcription via the Yap response element (YRE: TKACTAA) (Fernandes *et al.*, 1997) and *cap1* mutant cells display increased sensitivity to reactive oxygen species (Alonso-Monge *et al.*, 2003). Cap1 seems to act independently from the HOG pathway in *C. albicans*, since deletion of *HOG1* has no effect on the Cap1 translocation to the nucleus and *CAP1* deletion does not inhibit Hog1 phosphorylation (reviewed in Chauhan *et al.*, 2006). Other pathways, such as the Cap1 pathway, could play key roles in the regulation of oxidative stress genes, as suggested (Enjalbert *et al.*, 2006). Homologues of Yap1 have been described in other fungi. *A. fumigatus* contains Yap1 homologues, but its function is still unknown (Aguirre *et al.*, 2006).

In contrast to the redundancy in the systems for H_2O_2 elimination in response to ROS, there are few mechanisms for the disposal of NO. Resistance to nitric oxide has been shown to be important to the stress defense and virulence of *C. neoformans*. It has been shown that macrophages produce nitric oxide in response to cryptococcal cells and that the macrophage antifungal activity of macrophages is mostly dependent on RNS (Gross *et al.*, 1999; Tohyama *et al.*, 1996). Proteomic and transcriptional response of *C.*

neoformans to nitric oxide stress have been investigated. One stress-related protein up-regulated in the presence of nitric oxide stress was glutathione reductase (GLr1); the corresponding mutant was sensitive to nitrosative stress and macrophage killing. Additionally the mutant was avirulent in mice. Interestingly, mutants deficient in Glr1 are not sensitive to peroxide stress, suggesting the specificity of this antioxidant enzyme to the nitrosative stress response in this fungal pathogen (Missall *et al*., 2006). The fungus depicts a single gene for NO oxigenase and also a single gene for 5-nitroglutathione reductase. It has been described that NO generated in mammal hosts exerts a fungistatic effect against *C. neoformans* and that NO oxigenase is able of protecting fungal cells during infection and promotes fungal virulence (de Jesus-Berrios *et al*., 2003).

Two candidate factors, an alternative oxidase, presumably providing a means of fungal cells to continue respiratory growth in the face of RNS, and a nitric oxide reductase has been suggested in *H. capsulatum* mechanism for combating nitrosative stress. Also a P450 nitric oxide reductase, Nor1, is induced in *H. capsulatum* exposed to nitrosative stress. In support of its role in combating host defenses, overexpression of NOR1 in *H. capsulatum* shows slightly increased growth rates in the presence of a nitrosative agent compared with wild-type cells (Nittler *et al*., 2005).

2.2.4. Antioxidant enzymes as effectors of oxidative stress of the pathogens

Fungi have enlisted several classes of antioxidant enzymes defenses to cope with the variety of ROS that is available in the phagocytes. O_2^- is eliminated by superoxide dismutases (SOD), present at cytosol, mitochondria and also secreted forms. A battery of enzymes decomposes H_2O_2, such as catalases, thioredoxin, glutathione reductase and gluthatione peroxidase. Those enzymes are considered for the majority fungal pathogens virulence factors due to their ability to handle with the oxidative stress implied by the host environment. Some of the genes that encode those antioxidant enzymes when impaired produce mutants with virulence attenuated or completely avirulent (Chauhan *et al*., 2006). The virulence factors from the fungal pathogens are going to be discussed below.

3. Signaling in response to temperature stress

Thermotolerance in fungi is the ability to grow at 37°C, which is a *sine qua non* condition for human infection and dissemination. The ability to exist in different forms

and to reversibly switch from one to the other during infection provides the metabolic flexibility reflecting as an evolutionary adaptation to different environments (Tekaia and Latge, 2005). The evolutionary mechanisms may involve the expression of temperature-induced genes or even the stress resistance ones that might confer pathogenicity and/or virulence. Since the thermotolerance is a universal virulence trait across pathogenic fungi, the understanding of how pathogenic fungi grow at high temperatures may provide important clues to pathogenesis in humans.

3.1. MAPKinases

MAPKinases are key elements in the environmental stress in fungi. Their essential role in sensing different signals enables the pathogenic fungi to integrate, amplify and modulate the adaptive response and virulence (Roman *et al*, 2007). The genetic manipulation in several pathogenic fungi revealed the important relationship between signal-transduction pathways, virulence and stress response.

In *C. albicans*, the HOG pathway is able to respond to a myriad of environmental stimuli, including sexual development, oxidative stress response, morphological differentiation and growth at low temperature (for revision, see Bahn *et al*, 2007). *MKC1* gene encodes a mitogen-activated protein (MAP) kinase, which is phosphorylated in response to low-temperature shock **(Figure 4)**. Recently it has been found that the oxidative-stress-mediated phosphorylation of Mkc1 is partially dependent on an intact HOG pathway (Arana *et al.*, 2005), which might indicate a crosstalk between the temperature and oxidative stress responses.

Insert Figure 4

In *C. neoformans*, MAPKinases together with calcineurin pathway are reported to be involved in cell wall integrity and remodeling in response to elevated growth temperature (Kraus *et al.*, 2003). The *C. neoformans* Mpk1 is required for growth at 37ºC *in vitro*. The *mpk1* mutants were unable to grow at 37ºC and also the virulence was attenuated in the mouse model of cryptococcosis. Mpk1 is activated by phosphorylation in response to drugs that are able to alter the cell wall integrity, such as calcofluor white, nikkomycin Z and caspofungin (Kraus *et al*, 2003). The cross-talk between both pathways was recently reinforced by the isolation of a hypervirulent *eca1* mutant that is unable to grow at mammalian body temperature. Eca1 is likely involved

in the calcium maintenance at the ER. The mutant behavior for virulence was tested in different host models where the ability of infection was attenuated at 37ºC (Fan *et al.*, 2007).

In *P. brasiliensis*, Bastos *et al.* (2007) described the differentially expressed sequences during transition from mycelium to yeast cells, which is essentially temperature-dependent event. MAPKinase ESTs were identified in the transition transcriptome. A MAPKinase and PKC proteins were induced in the transition library suggesting their involvement in the fungal cell wall biosynthesis as a response of adaptation to the environment and also consequently to survive and proliferate within the host.

3.2. Ca $^{2+}$-Calmodulin-calcineurin

Calcineurin is a major player in calcium-dependent signal transduction pathways in eukaryotes. Calcineurin is a conserved serine-threonine-specific Ca^{2+}-calmodulin activated protein phosphatase that mediates cell-stress responses. Calcineurin B binds to a α-helical extension of calcineurin A distinct from the active site. In response to calcium influx, calmodulin binds to calcineurin A and blocks the action of the auto-inhibitory C-terminal domain of calcineurin A, resulting in the formation of the active calcineurin complex.

Calcineurin acts on transcription factors and governs the expression of genes in a species-dependent fashion. It is critical for virulence in pathogenic fungi that infect humans as *C. neoformans* and *C. albicans* (Kraus *et al.*, 2005) and *A. fumigatus* (Steinbach *et al.*, 2007). It was recently reviewed by Steinbach and coworkers (2007) the role of calcineurin as a novel drug target against invasive fungal infections caused by *C. neoformans, C. albicans* and *A. fumigatus* which most causes infection in immunocompromised patients. De Carvalho *et al.* (2003) have shown the inhibitory effect of several drugs in the calcium-calmodulin signalling pathway in *P. brasiliensis* during the mycelium-to-yeast transition. Additionally, an *in silico* search of the *P. brasiliensis* database has also revealed a calcium-calmodulin signalling pathway **(Figure 4)** that might be controlling the temperature shift (Felipe *et al.*, 2005; Fernandes *et al.*, 2005). The potential role of this pathway in the dimorphism control and possible in pathogenesis of *P. brasiliensis* was re-inforced by the up regulation of calcineurin B during the transition from mycelium to yeast (Bastos *et al.*, 2007).

Calcineurin is critical for virulence and pathogenicity of *C. neoformans*, since calcineurin A mutant strains are viable but fail to grow in high temperature and are avirulent in animal model of cryptococcal meningitis (Odom *et al.*, 1997). Down-regulation of calmodulin was associated with impaired growth at 37°C **(Figure 4)** and, in the absence of calcineurin function the *C. neoformans cam1* mutant displayed impaired bud formation, which implicates the involvement of Ca^{+2}-calmodulin-calcineurin in morphogenesis and high-temperature growth (Kraus *et al.*, 2005).

Calcineurin is not required for the serum induced transition of *C. albicans*, however the involvement of calcineurin in the virulence and consequently in the growth on the high temperature is dependent on the host niche. In mouse models of systemic disseminated candidiasis the calcineurin is essential to the infection while in vaginal or pulmonary infection the absence of calcineurin function does not affect the virulence of the pathogen (Bader *et al.*, 2006). In addition, in *C. albicans* the deletion of either the catalytic subunit or regulatory subunit abrogates calcineurin activity resulting in cells that are sensitive to multiple stress conditions (Bader *et al.*, 2006).

Recent studies in *A. fumigatus* suggest that calcineurin controls key steps in polarized hyphal growth, and is therefore critically important for tissue invasion. The *Aspergillus* calcineurin pathway was not directly involved with heat stress, but the over-expression of calcineurin was observed under several stresses. The link between calcineurin and *A. fumigatus* pathogenicity was supported by animal models in which the blockage of calcineurin leads to a decrease in fungal growth, halt tissue invasion and decrease host mortality (Steinbach *et al.*, 2006).

3.3. RAS

Ras proteins, small guanine-nucleotide binding proteins, are highly conserved and they control the activation of diverse signaling pathways. Among microorganisms, Ras proteins regulate fundamental and cellular processes such as morphological transitions, mating and microbial pathogenesis.

Ras proteins control several distinct cellular processes in *C. neoformans* including the maintenance of the cytoskeletal integrity, allowing this organism to respond to changing environments and to external stress. *C. neoformans* Ras1 has a role in the high temperature growth since *ras1* mutants arrest as large, unbudded cells with depolarized actin, also are unable to grow at 37ºC and are avirulent in animal models of meningitis. The *RAS2* gene when over-expressed in the *ras1* mutant rescues the ability

to grow at high temperature, indicating a redundant effect in the signaling pathways that responds to temperature (Alspaugh *et al.*, 2000; Waugh *et al.*, 2002). Over-expression of either *RAC1* or *CDC42* also suppresses the high-temperature growth defect of the *ras1* mutant strain **(Figure 4)**. Cdc24 was recently identified as the downstream effector of Ras1 that activates Cdc42 to regulate thermo-tolerance and ability to cause infection in *C. neoformans* (Nichols *et al.*, 2007). Over-expression of *CDC42* but not *RAC1* suppresses the *cdc24* temperature-sensitive growth defect. Therefore, Rac1 is similar to the Cdc42 protein and may play some overlapping functions downstream of Ras1. However, the primary role of Rac1 appears to be as a downstream effector of Ras1 to control hyphal formation during sexual differentiation working in coordination to function with other similar protein as an alternative pathway (Vallim *et al.*, 2005). Differently, Cdc24 and Cdc42 proteins likely mediate Ras function in establishing and maintaining polarized growth in the budding yeast cell in the face of cell stresses, such as elevated temperature.

Ras signaling during morphogenesis of *C. albicans* has been studied extensively and it regulates both mitogen-activated protein kinase-dependent and cAMP–PKA-dependent responses (Phillips *et al.*, 2006). The morphogenetic switching relies on the activation of Ras-GTPase by extracellular signals as environmental sensing and induces two downstream cascades: a MAPkinase and the cAMP pathway, resulting in hypha-specific gene expression (Martin *et al.*, 2005). The deletion of RAS gene in *C. albicans* is not essential; however the mutants presented defects in morphology, impaired ability to switch from yeast to filaments and reduced pathogenicity (Leberer *et al.*, 2001). The defects observed in the *ras* mutant suggest the involvement of Ras signaling in control the high temperature growth and morphogenesis in the fungal pathogen *C. albicans*. Also, two *ras* genes were identified in *A. fumigatus* and despite the morphogenetic defects and the virulence attenuated observed in the *ras* mutant, there is just a suggestion of the indirect involvement of Ras on the high temperature growth control of this pathogen (Fortwendel *et al.*, 2005).

In the dimorphic human pathogens as *P. marneffei* and *P. brasiliensis*, two *ras* genes were identified. In the first case, *ras* genes have been implicated in a signaling cascade to regulate morphogenesis (Boyce *et al.*, 2005); however there is no evidence of the direct role of those genes in the dimorphic process of *P. marneffei*. In the case of *P. brasiliensis ras* genes were not regulated during the transition temperature dependent from mycelium to yeast, however *ras1* and not *ras2* showed a negative regulation after

heat shock at 42ºC. Also both genes, *ras1* and *ras2*, were repressed inside the macrophage cells indicating the potential role of Ras in the response to temperature and host environment stimuli in *P. brasiliensis* (L. Fernandes, unpublished).

3.4. cAMP - PKA

Known as a nutrient sensing pathway the cAMP-PKA cascades involves a G protein couples receptor, Gpr1, the G proteins Gpa2 and Ras2, adenylyl cyclase, cyclic AMP (cAMP) and cAMP-dependent protein kinases. The role of cAMP is to activate protein kinase A (PKA), a heterotetramer consisting of two subunits, cAMP binds to two sites on each regulatory subunit and causes the release of free and active catalytic subunits, which may phosphorylate serine and threonine residues on target proteins which, in turn, stimulates enzymes involved in a myriad of cellular events. For example in *C. albicans* this pathway controls the utilization of storage carbohydrates, represses stress-activated genes, regulates entry into stationary phase and promotes pseudohyphal morphogenesis (Jung and Stateva, 2003).

The cAMP-dependent pathway, which regulates yeast-to-hypha morphogenesis in *C. albicans*, is controlled by changes in cAMP levels. Both low-and high-affinity cAMP phosphodiesterases are encoded in the *C. albicans* genome. Deletion of *PDE2* causes elevated cAMP levels and responsiveness to exogenous cAMP, higher sensitivity to heat shock, severe growth defects at 42^0C and highly reduced levels of *EFG1* transcription (Jung and Stateva, 2003).

In order to understand the cyclic adenosine 5′-monophosphate (cAMP)/protein kinase A (PKA) signaling pathway, Hu *et al*., 2007 studied the transcriptional regulation of *PKA* in *C. neoformans* by the evaluation of the transcriptional changes in the *pka* mutants. They reported alterations of gene response involved with virulence, ribosome biogenesis, the response to stress, vesicle (protein) trafficking, membrane transport, and cell wall biogenesis. Among the genes related to high temperature growth that suffered any modulation by PKA were the *TPS1* that codes for trehalose-6-phosphate synthase, required in trehalose synthesis, and is involved in the ability of the fungus to grow at 37°C; *ILV2* encoding acetolactate synthase and *SPE3/LYS9* chimeric gene (spermidine synthase/saccharopine dehydrogenase) which are involved in amino acid metabolism and also necessary for growth at elevated temperature and virulence, and finally the cyclophilin A which is related to growth at host temperature.

3.5. Other genes

Up to now two genes have been directly related to thermophyly in *Aspergillus*: the *thtA* gene that allows the fungus to grow at 48°C (Chang *et al.*, 2004) and the *cgrA* gene (Bhabhra and Askew, 2005). The *thtA* encodes a putative protein of 141 kDa with unknown function and the HA-tagged ThtA protein accumulated to similar levels in cultures grown at either 37°C or 48°C. ThtA was shown to be essential for growth of *A. fumigatus* at high temperatures but does not contribute to the pathogenicity of the species. In contrast, the *cgrA* gene seems to be implicated in the virulence of *A. fumigatus*. CgrA is a highly conserved nucleolar protein involved in ribosome biogenesis. The disruption of *cgrA* decreases the growth rate at 37°C *in vitro* and *in vivo* (Bhabhra *et al.*, 2004).

The use of reverse-genetic allowed the access to the contribution of stress response genes implicated in high-temperature growth of *C. neoformans*. Giles and coworkers (2005) showed that Sod2, as a critical component for cell viability, is also implicated in the adaptation to growth at elevated temperatures. The authors showed that Sod2 acts as a regulator of the steady-state concentration of reactive oxygen species at higher temperatures.

4. Signal transduction pathways as regulators of fungal virulence.

Virulence factors and events as dimorphism, growth at elevated temperatures, adherence to host cells, cell wall components, enzyme production are important for the ability of human pathogenic fungi to create a microenvironment that is favorable for its penetration and dissemination into the host, by digesting cell membranes, absorbing the nutrients derived from this digestive process, suppressing the host immune system, and finally growing through the host tissues. By the definition, a gene to be considered involved in virulence of one pathogen, its null mutant has to cause an attenuated infection compared to the wild type and reconstituted strains, based on "Molecular Koch's postulates" (Falkow, 1988; Falkow, 2004). Fungal pathogens have adapted the conserved signaling components involved in diverse cellular functions to activate their virulence factors in order to survive and establish the pathogenesis. Despite many virulence genes have been reported previously for diverse fungi, it is still unknown all signaling cascades events and components that elicits the activation of the pathogen virulence. Then we decide to explore the signaling pathways involved with virulence and also the virulence factors that still do not have an activation mechanism elucidated.

4.1. Signaling cascades

There are several genes in pathogens the contribution of which to oxidant adaptation in vitro can be correlated with their survival and virulence *in vivo*. In *C. albicans* the HOG pathway is activated in response to oxidative stress and *hog1* mutants are more sensitive to oxidants *in vitro* (Alonso-Monge *et al.*, 2003). A strain of C. *albicans* lacking *SLN1* has reduced virulence in an animal model (Yamada-Okabe *et al.*, 1999) and mutants in the two component response regulator Ssk1 are more easily killed by human neutrophils (Du *et al.*, 2005), further confirming the role of both genes in the transmission of the phosphorylation signal to Hog1 MAPKinase.

The Skn7 response regulator has been described in *C. neoformans* (Wormley *et al.*, 2005), in which it works independently of the Hog1 MAPK pathway (Bahn *et al.*, 2006). Animal model experiment revealed that *SKN7* disruption strongly attenuates cryptococcal virulence *in vivo*, promoted increased susceptibility to oxidant stress and decreased intracellular survival in endothelium (Coenjaerts *et al.*, 2006).

Only recently have histidine kinases (HK) – with receptor function or not – been identified as virulence determinants in fungi (**Figure 2**). They trigger "two-component" systems that relay the received stimulus to other signaling pathways that target both nucleus and cytoplasm. Two such kinases, Tco1 and Tco2, have been implicated in genetic reprogramming and adaptation to the host by *C. neoformans* (Bahn *et al.,* 2006). Similarly, the kinase Chk1 from *C. albicans* is known to control cell wall biosynthesis, biofilm formation and adaptation to heat and oxidative stress, all of which are determinants of virulence (Chauhan *et al.*, 2003; Kumamoto and Vinces, 2005a). In *A. fumigatus*, work with the HK (Fos1) has begun to unravel its function in the ability of the fungus to grow invasively (Clemons *et al.*, 2002). In all these fungi, a classical target of the two-component system is the Hog1 MAPkinase pathway and the phosphotransfer from the HK is mediated by Ssk proteins, which possess aspartate residues that are phosphorilated by the kinase (Kruppa and Calderone, 2006). Members of both the two-component system and of the Hog1 pathway have been identified *in silico* for *P. brasiliensis* (Fernandes *et al.*, 2005), which constitutes another hint at the phylogenetic conservation of this mechanism.

The cyclic AMP-dependent protein kinase A (PKA) is also a regulator of the morphogenesis, differentiation, stress response and virulence in eukaryotic microorganisms. In *A. fumigatus* deletion of one or more catalytic subunits of PKA has been shown to decrease growth, to reduce tolerance to oxidative stress and also

virulence of this pathogen (Liebmann *et al.*, 2003). A *pkaR* mutant, deleted for the regulatory subunits of PKA, presents reduced growth and germination rates, increased susceptibility to oxidative stress and attenuated virulence (Zhao *et al.*, 2006). The *C. neoformans* Gα subunit, Gpa1, plays a key role in cAMP signaling and virulence (Alspaugh *et al.*, 1997). A mutant in the Gpa1 protein showed reduced virulence and inability to synthesize melanin, which could be partially restored by the addition of extracellular cAMP, suggesting that Gpa1 regulates cAMP production (Alspaugh *et al.*, 1998).

The calmodulin/calcineurin pathway is another determinant of virulence. Its action in *C. neoformans* seems to be preserving cell integrity at host temperature and thus protect the fungus against the new environment (Kraus and Heitman, 2003). As expected, mutants are avirulent. Both calmodulin and calcineurin seem to be essential for fungal pathogenicity and a similar role has been defined for the pathway in *C. albicans* and *A. fumigatus* (Bader *et al.*, 2006; Steinbach *et al.*, 2006).

4.2. Melanin as virulence factor

Many pathogenic fungi produce melanin, which are hydrophobic compounds though to confer protection from harsh environmental conditions. Melanin formation might protect fungal cells by quenching released free radicals, acting as a buffer against external ROS. Different types of melanin have been described as important virulence factors for numerous fungi. Mutants of *C. neoformans* lacking melanin pigment showed a reduced virulence in a murine animal model (Wang *et al.*, 1995). DOPA-melanin protects the cells from damage by hypochlorite or hydrogen peroxide, which is part of the immune response (Wang *et al.*, 1995). *C. neoformans* melanin has been postulated to inhibit TNF-α production and proliferation of lymphocytes, thus reducing the immune response to fungal infection (Huffnagle *et al.*, 1995). *C. neoformans* encodes two laccases, with are both regulated by oxidative and nitrosative stress and deletion of both encoding genes reduces survival of *C. neoformans* in primary macrophages (Missall *et al.*, 2005).

Both the MAPkinase and the cAMP signaling cascades regulate two important and very well described virulence factors - the melanin and capsule - in *C. neoformans*. Melanin and capsule genes are co-regulated at the level of transcription by cAMP pathway. In *C. neoformans*, Pka1 is required for both melanin and capsule production (D´Souza *et al.*, 2001). Unlike *gpa1* mutant strains, exogenous cAMP does not restore

melanin or capsule production in *pka1* mutant strains, suggesting that Pka1 is the target of cAMP activation. Strains with defects in the cAMP signaling cascade are unable to induce melanin and capsule formation and these strains are highly attenuated for virulence (Pukkila-Worley *et al.*, 2005). A striking feature of the *C. neoformans* Hog1 MAPK pathway is the cross talk with the cAMP signaling pathway in controlling biosynthesis of capsule and melanin (Bahn *et al.,* 2005). In addition, Tco1 was found to be a key sensor histidine kinase negatively regulating melanin synthesis; disruption of the *TCO1* gene dramatically enhanced melanin production via the Pbs2-Hog1-pathway and the *tco1* mutant was less virulent in a mice model of infection, as cited above (Bahn *et al.*, 2006).

In *A. fumigatus* DHN-melanin is though to protect the conidia from the host immune system by quenching reactive oxygen species. A mutant producing pigment-less conidia showed a reduced virulence (Tsai *et al.*, 1998). The *pksP* mutant is more sensitive to hydrogen peroxide and sodium hypochlorite and more susceptible to damage by murine macrophages *in vitro* than the wild strain (Jahn *et al.*, 1997; Jahn *et al.*, 2000). Despite those descriptions, melanin seems not to be a major factor for *A. fumigatus* virulence, since deletion of additional genes in the DHN-melanin pathway do not result on fungal virulence decrease (Tsai *et al.*, 1999).

P. brasiliensis melanin-like pigments were detected in cells growing *in vitro* and during infection (Goméz *et al.*, 2001) and also melanization of *P. brasiliensis* yeast cells reduces their paghocytosis by macrophages in culture (Silva *et al.*, 2005). Transcripts encoding tyrosinase and aromatic-L-amino acid decarboxylase are up regulated in *P. brasiliensis* yeast cells recovered from murine infected tissues suggesting an active synthesis of melanin during experimental infection (Bailão *et al.*, 2006). The other dimorphic fungus, *P. marneffei*, produces melanin *in vitro* as well as *in vivo*. Skin tissue of infected patients with this fungus was demonstrated to contain yeast cells that were labeled by melanin-binding antibodies (Youngchim *et al.*, 2005). Melanization can also be detected in conidia and yeast cells of *H. capsulatum in vitro* and during infection (Nosanchuk *et al.*, 2002). Despite of the presence of melanin like pigments in those dimorphic human pathogens the pathways that activate the melanin synthesis is still not elucidated. However, as the components of the cellular cascades are much conserved among the species probably that involved in melanization in those fungi may be the same as described for others as for *C. neoformans*.

4.3. Cell wall components – chitin, glucans and lipids

Cell wall, for almost fungi, is a complex structure, made by the ordered arrangement of different components. This composite provides protection to the cell against physical, chemical and biological aggression, and is responsible for the morphology and as an important factor for the virulence.

The crystalline arrangement of the polysaccharide chitin and its linkage to β-1,3-glucan constitute the basic cell-wall scaffold to which mannoprotein are covalently associated. The chitin amount in cell wall contributes to the virulence of *P. brasiliensis*, since the yeast contains three-fold higher chitin than mycelium (San-Blas and San-Blas, 1977). Proteins, related with fungal virulence, binds to chitin such as Yps3 from virulent strains of *H. capsulatum* (Bohse and Woods, 2005) and Bad1 from *B. dermatitidis* (Brandhorst and Klein, 2000). These yeast-phase-specific proteins are homolog and are involved in adhesion of fungi and can suppress cytokine production (Rappley and Goldman, 2006). Chitin synthases have been proposed to be a target for the control of mycosis considering the importance of chitin in the structure of the fungal cell wall and its absence in the host.

Glucans are the most abundant cell wall component. The β-(1,3)-glucan and α-(1,3)-glucan are essential components of the cell wall. The α-(1,3)-glucan, a virulence determinant, is a cell wall polysaccharide common to most medically important fungi, including all the dimorphic pathogenic species. Spontaneous loss of α -(1,3)-glucan correlated with decreased virulence, indicating this may be a conserved mechanism of fungal pathogenicity as described to *B. dermatitidis*, *C. neoformas, A. fumigatus, C. immitis* and *P. brasiliensis* (San-Blas and San-Blas, 1977; Klimpel and Goldman, 1988; Hogan and Klein, 1994, Bernard and Latge, 2001; Cole and Hung, 2001). The role of α-(1,3)-glucan in virulence was demonstrated by RNA interference in *H. capsulatum* (Rappleye *et al.*, 2004) and also in *C.neoformans* (Reese and Doering, 2003). The silencing of *AGS1* expression in *H. capsulatum* cells impaired the ability of the fungus to proliferate, to kill macrophage in culture and showed decreased TNF-α production (Rappleye *et al.*, 2004; Rappleye *et al.*, 2007). The activity of the β-1, 3-glucan synthase is regulated by Rho-GTPase, and protein kinase C (PKC) signaling molecules. The Rho –GTPase was already identified in pathogenic fungi such as *Candida, Aspergillus, Cryptococcus, Pneumocystis* and *Paracoccidioides*, constituting a promising drug target against fungal diseases (Liu and Balasubramanian, 2001).

Lipids, in general, constitute the minor components of the fungal cell wall. In *C. albicans* the phospholipomannan are involved in organization of the glucan chains and have relevance in adhesion, protection and signaling (Ruiz-Herrera *et al*., 2006). The phospholipomannan mediated the *C. albicans* escape from macrophages and induction of cell apoptosis (Mille *et al*., 2004).

4.4. Antioxidant enzymes as virulence factors in response to oxidative stress

Copper-and zinc-containing superoxide dismutase (Cu/ZnSOD) is suspected to be one of the anti-oxidant enzymes and virulence determinants active in some pathogenic microorganisms. The *C. albicans SOD1* encoding a Cu/ZnSOD was disrupted; the resulting *sod1/sod1* mutant showed increased sensitivity to menadione compared to the isogenic wild-type strain. Furthermore the *C. albicans* lacking the Cu/ZnSOD showed increased susceptibility to macrophage attack and had attenuated virulence in mice, which suggests that the Cu/ZnSOD is required for the protection of *C. albicans* against oxidative stresses and for the full virulence of the organism (Hwang *et al*., 2002). Furthermore, *C. albicans* virulence is attenuated by the inactivation of catalase (*CAT1*) and *SOD5* (Wysong *et al*., 1998; Martchenko *et al*., 2004). Nevertheless, the significance of oxidative stress responses in the virulence of *C. albicans* has been emphasized by the observations that mutations that inactivated such responses attenuate the virulence of this fungal pathogen. In *C. neoformans* the expression of the *SOD1* gene increases at the host temperature and the mutant lacking this Cu/ZnSOD can be killed by ROS in a cell free system and is less virulent than the wild-type strain in a murine model of infection (Cox *et al*., 2003). Strains of *C. neoformans* variety *gattii* lacking *SOD1* and *SOD2* are unable to produce experimental disease (Narasipura *et al*., 2005). Although little is known about the role of Sod in *A. fumigatus*, a Cu/Zn Sod is known to be immunoreactive with sera of patients with aspergillosis (Holdom *et al*., 2000). Four genes encoding for two Sod isoenzymes, a Mn containing and a Cu/Zn containing protein, had been described in the *P. brasiliensis* transcriptome (Campos *et al*., 2005). The *sod3* gene was induced upon internalization of *P. brasiliensis* yeast cells by *in vitro* cultured macrophages (Tavares *et al*., 2007). *H. capsulatum* has three catalase genes encoding *CATA*, *CATB* and *CATP*, presenting differential expression. Among them, *CATB* and *CATP* are constitutively expressed, whereas *CATA* expression is restricted to the mycelial phase on when yeast cells are exposed to H_2O_2 (Johnson *et al*., 2002). *P. brasiliensis* presents three catalases, one of

which *catP* is up-regulated in the parasitic phase and when yeast cells are exposed to H_2O_2 (Moreira *et al.*, 2004; Campos *et al.*, 2005). The transcript encoding a homologue of *catA* is induced upon incubation of yeast cells in human plasma (Bailão *et al.*, 2007).

The gene encoding for a thiol peroxidase (*TSA1*) of *C. neoformans* was shown to be necessary to hydrogen peroxide fungal resistance. In cryptococcosis inhalation mouse model the gene *TSA1* was proven to be necessary for virulence (Missall *et al.*, 2004). Also the ability of the fungal pathogen *C. neoformans* to evade the mammalian innate immune response and cause disease is partially due to its capacity to respond and survive under nitrosative stress. A fungal deletion mutant for the glutathione reductase gene (*GLR1*) is sensitive to nitric oxide stress, but not peroxide stress, revealing specificity between oxidative and nitrosative stress response in *C. neoformans*. Additionally the *glr1* mutant is sensitive to macrophage killing being avirulent in mice (Missall *et al.*, 2006). Peroxiredoxin homologues had been identified in the *P. brasiliensis* transcriptome (Campos *et al.*, 2005), although the role in the antioxidant mechanisms remains to be elucidated. Also, a member of the thioredoxin family was up-regulated in the *P. brasiliensis* yeast cells recovered from liver of infected mice (Soares, CMA, unpublished).

4.5. Secreted virulence factors: phospholipases, proteases and other metabolites

Pathogenic fungi use extracellular enzymes to degrade the cell membrane, which is the main structural barrier of the host. The main enzymes are phospholipases and proteases. Since phospholipases target membrane phospholipids and digest these components, direct host cell damage and lyses have been proposed as a major mechanism contributing to virulence. Such host cell injury would be expected to facilitate the penetration of the infecting agent (Ghannoum, 2000; Ganendren *et al.*, 2006).

Phospholipases constitute a heterogeneous group of enzymes which can hydrolyze one or more specific ester bonds in phospholipids. These enzymes are classified as: phospholipaseA (PLA_1, PLA_2), B, C and D. Besides the lysophospholipase activity, PLB also bears a transacylase activity (LPTA). The phospholipases observed in C. *neoformans* are similar to those found in *C. albicans* (Chen *et al.*, 1997). *A. fumigatus* secretes PLA, PLB and PLC (Birch *et al*, 1996). In *P. brasiliensis* transcriptome a *plB* orthologue to *C. albicans* was found (Tavares *et al.*, 2005). In this case, it is well known that *P brasiliensis* invades endothelial cells (Mendes-Giannini *et*

al., 2004) and the action of phospholipases would therefore contribute to tissue invasion (Tavares *et al*., 2005). In *C. albicans* the amount of phospholipase varies according to the isolate and also is related with the infection location. For instance, blood isolates produce higher levels of phospholipases when compared with wound or urine isolates (Price *et al*, 1982). In *C. albicans*, PLB-deficient mutants when compared to parental strains revealed that the disruption of *PLB* does not affect growth and germination, but reduces the ability to secrete this enzyme (Leidich *et al*, 1998). In *C. neofarmans* a disruption gene (*PLB1*) also does not affect significantly fungi growth, but the inhibition of enzyme secretion reduces invasion levels at the lungs (Noverr *et al*., 2003; Santangelo *et al*., 2004). Besides contributing to virulence by causing cell damage in the host, phospholipases are also involved in survival inside macrophage, destruction of lung and in the production of eicosanoids with modulate phagocyty activity (Cox *et al*., 2001; Noverr *et al*., 2003).

In animal tissues the structural barriers are composed by phospholipids and proteins. Thus the fungus requires proteases to invade them, secreting large amounts of these enzymes during infection. Fungal extracellular proteases constitute a large and heterogeneous group of proteins. SAP protease (aspartyl peptidase) was broadly studied in *C. albicans* and was demonstrated to be related with the degradation of several human proteins such as albumin, hemoglobin, keratin and secreted immunoglobulin A (Hube *et al*., 1998). *C. albicans* presents nine different *SAP* (*SAP* 1 to 9) genes (Yang , 2003). *In vitro* studies demonstrated that *SAP* 1, 2 and 3 are expressed by yeast cells only, but *SAP* 4, 5 and 6 are expressed in the transition from yeast to hyphae at neutral pH (Hube *et al*, 1998, Schaller *et al*., 1999). The *SAP* gene in *C. albicans* is regulated at the transcriptional level and processed by a signal peptidase and Kex2-like protease (Newport and Agabian, 1997). Thus, production of Sap is a highly regulated and tightly controlled process, which appears to be an important virulence factor and is indicative of the multiple functions of this gene family, including digesting molecules for nutrient acquisition, destruction of host cell membrane aiding host tissue invasion, degradation of host surface molecules to enhance adhesion and degradation of cells and molecules of the host immune system (Naglik *et al*., 2003, dos Santos *et al*., 2006). *C. neoformans* protease activities degrade host proteins such a collagen, elastin, fibrinogen, immunoglobulins and complement factors (Chen *et al*., 1996). Degradation of host cells may protect *C. neoformans* from the host immune response and possibly aids in the escape of the fungal cells from phagosomal compartments (Chen *et al*., 1996,

Steenbergen and Casadevall, 2003). Fungal proteases may induce local airway inflammation by recruiting inflammatory cells via activation of epithelial cells (Tomee *et al.*, 1997). Serine proteases have been described in human pathogenic fungi such as *C. albicans* (Santos *et al.*, 2006), *P. brasiliensis* (Carmona *et al.*, 1995), *C. neofarmans* (Rodrigues *et al.*, 2003) and *A. fumigatus* (Larcher *et al.*, 1992). The serine protease activity in *A. fumigatus* causes cell desquamation and induces production of IL-8 , IL-6 and monocyte chemotactic protein 1 (MCP-1) in human pulmonary epithelial cell lines (Tomee *et al.*, 1997). The production of these pro-inflammatory cytokines may trigger an inflammatory cascade involving macrophages, monocytes, lymphocytes and neutrophils causing local inflammation which may create a microenvironment for fungal attachment, penetration and mobilization of nutrients for fungal growth. The fungal attachment and penetration may further enhance inflammation and create a self-perpetuated cycle of inflammation (Tomee and Kauffman, 2000).

Besides phospholipases and proteases, another secreted enzyme which has to be highlighted is the urease. This enzyme catalyses the hydrolysis of urea yielding carbon dioxide (CO_2) and ammonia (NH_3) and further ammonium hydroxide, therefore creating an alkaline microenvironment. This enzyme is found in several fungi species such as *C. immitis, C. neoformans* and *P. brasiliensis* (Yu *et al*, 1997; Cox *et al*, 2000, Tavares *et al.*, 2005). In *C. neoformans,* when the urease gene (*URE1*) was disrupted, the mutants did not show differences regarding capsule size or colony formation unit counts in the central nervous system of rabbit model (Cox *et al.*, 2000). In contrast, in murine intravenous and inhalation models, mutants were attenuated when compared to the parental strain, which imply the urease in the pathogenesis of *Cryptococcus*, probably in a species and /or infection site manner (Tavares *et al.*, 2005).

Other important virulence factors are the metabolites such as gliotoxin, fumagillin and restictocin which facilitate fungal growth by causing generalized immunosuppression. One of the best studied fungal toxins is gliotoxin, first described as an antibiotic. Gliotoxin may be lethal at relative low concentrations and is produced by many fungi including *A. fumigatus*. Gliotoxin immunossupressive activity includes inhibition of phagocytosis, T cell proliferation, cytolytic T lymphocytes activation and adherence of macrophage, blood monocytes, fibroblasts ((Tomee and Kauffman, 2000). This inhibitory activity of gliotoxin may be exerted by inducing DNA damage through production of reactive oxygen species ((Tomee and Kauffman, 2000). Other special classes of toxins are the ribotoxins produced by *Aspergillus*. These toxins are small

basic ribosome-binding proteins that inactivate the large ribosomal subunit impairing protein synthesis.

4.6. Biofilm as a virulence factor in fungal pathogens

The ability to modify the surrounding environment by secretion of an extracellular matrix is the landmark of biofilm formation. *C. albicans* is capable to structure biofilms on several kinds of surface, both organic and inorganic, and this accounts for the resilience of systemic candidiasis and its resistance to treatment in several cases (Kumamoto and Vinces, 2005b). The secretion of matrix components seems to be regulated by the Histidine kinase Chk1, but in this case more can be said about the triggering mechanism. Morphogenetic and virulence processes in this pathogen are influenced by the recently described mechanism of eukaryotic quorum-sensing. Tyrosol (Chen *et al*., 2004) and farnesol (Hornby *et al*., 2001) are the quorum-sensing molecules (QSM) of *C. albicans*. The former induces biofilm synthesis and the latter represses it (Alem *et al*, 2006). Recent data indicate that farnesol exerts its inhibitory action through the Chk1 two-component system (Kruppa *et al*., 2004). The mechanism of action of tyrosol remains unknown, but its actions go beyond matrix production. In an apparent positive feedback system, tyrosol is known to induce tube germination and biomass expansion in *Candida* biofilms (Alem *et al*, 2006). As for farnesol, there seems to be at least one kind of cell response to this molecule that could contribute to fungal virulence: farnesol-conditioned media have conferred *in vitro* resistance of *C. albicans* to oxidative stress (Westwater *et al*., 2005).

Concluding remarks

Understanding the host-pathogen interaction may provide insights to host defenses and the tactics used by pathogens to circumvent these defenses. The discovery of the TLR and the non-TLR implicated in anti-fungal immunity has given an insight into the mechanisms of intracellular host signaling following microbial sensing. In counterpart, signal transduction pathways provide mechanisms for fungal adaptation to stress conditions. Transduction pathways, including the MAPkinases, calcineurin, Ras and cAMP/PKA, are responsible for activating transcription factors to control gene expression in cell survival, osmotic shock, morphogenesis, thermal and oxidative stresses and virulence in fungi. Molecular understanding of those signaling networks increases the knowledge in cell communication and provides new landscapes for drug

targets. Besides, understanding the mechanisms of signal transduction pathways may facilitate the development of new drugs and therapies for fungal infections disease.

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Figure Legends

Figure 1 – Membrane receptors recognizing yeast components – The fungi molecules bind to the toll-like (TLR), Dectin and/or mannose receptors inducing intracellular signaling cascades that result in cell-specific responses such as phagocytosis, burst respiratory and cytokines production. **TAK1 -** TGF-β-Activated Kinase 1; **NIK** - NF-kβ-Inducing Kinase; **NF-κB -** Nuclear factor κB; **IKK -** I-kB kinase; **SYK -** Spleen Tyrosine Kinase; **CARD9 -** adaptor molecule containing an N-terminal caspase-recruitment domain.

Figure 2 – Proposed mechanism of cAMP/PKA and MAPkinases pathways cross-talking by Ras-GTPases in pathogenic fungi that regulates morphogenesis, high temperature growth and virulence. In *P. brasiliensis* the dotted arrowhead indicates that the mechanism was not still demonstrated, however all the components listed on the pathways were found by *in silico* annotation of the pathogen transcriptome.

Figure 3 – Mechanism of stress-response in pathogenic fungi. The diagram depicts the multi-step phosphorelay pathways in *C. albicans*, *C. neoformans* and *P. brasiliensis*. Arrowheads depict known routes; dotted arrowheads indicate unconfirmed signaling pathways.

Figure 4- Comparative cascades signaling that control temperature stress response pathways in *C. neoformans*, *C. albicans* and *P. brasiliensis*. The Cam1/Cna1 corresponds to the calcium-calmodulin/calcineurin and PKC1/Mkk1/Mkc1 to the MAPkinase pathways, respectively. Arrowheads depict known routes; dotted arrowheads indicate unconfirmed signaling pathways.

Table 1 – <u>P</u>atter-<u>R</u>ecognition <u>R</u>eceptors (PRR) and <u>P</u>athogen-<u>A</u>ssociated <u>M</u>olecular <u>P</u>atterns (PAMPs) involved in the host-fungal pathogen interaction

PRR	Fungal PAMPs	Microorganisms
Non-TLR		
CD14	glucuronoxylomannan	*Cryptococcus neoformans*
CR3	mannose, β-glucan, N-acetylglucosamine, methylmannoside, methylglucoside, complement opsonized pathogen	*C. albicans, C. neoformans, P. brasiliensis*
Classical C-type lectin receptor	mannose	*C. albicans, P. carinii,*
Non-classical C-type lectin receptor	β -glucan	*C. albicans, P. carinii, H. capsulatum, Coccidioides posadasii, Aspergilus fumigatus.*
Lactosylceramide	β -glucan	*P. carinii, C. neoformans, C. albicans*
Mannose-binding lectin	mannose, fucose, glucose	*C. neoformans, A. fumigatus, C. albicans*
TLR		
TLR2	phospholipomannan, zymosan, lipoproteins, lipopeptides, glycolipids	*C. neoformans, C. albicans, A. fumigatus, H. capsulatum.*
TLR4	mannan, glucoronoxylomannan	*C. neoformans, C. albicans, A. fumigatus*

Adapted and modified from Brown, 2006. CD14 – cluster of differentiation 14; CR3 - complement receptor 3; PAMP, pathogen-associated molecular pattern; PRR, pattern-recognition receptor; TLR, Toll-like receptor.

A

Estresse nutricional
Glicólise

Citosol de
P. brasiliensis

Glicose-6-fosfato
pgi
Frutose-6-fosfato
↓*pfkA*↓ ↑*fbp1*

Biossíntese de
aminoácido
metG↑

Frutose-1,6-bifosfato
fba1
Gliceraldeído-3-fosfato
↓*gapdh*↓↑

Biossíntese de
parede celular
fks↓

3-fosfo-D-glicerolfosfato
↓*pgk*↓↑
3-fosfoglicerato
↓*gpmA*↓↑
2-fosfoglicerato
↓*eno*↓↑
Fosfoenolpiruvato

Estresse
oxidativo
sod3 *hsp60*↓

Macrófago

B

Estresse oxidativo
hsp30
hsp70
hsp90
fator de transcrição Ap1-like

Biossíntese de aminoácidos
metE1

Parede celular/
Sensor osmótico
gln1
pas-like
septina1
sho1
gapdh

Citosol de
P. brasiliensis

Sangue

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