**UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO
DEPARTAMENTO DE BIOLOGIA CELULAR E MOLECULAR**

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**Paracoccina: uma quitinase importante para a patobiologia e virulência de
*Paracoccidioides brasiliensis***

**Ribeirão Preto
2018**

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Tese apresentada à Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo para obtenção do título de Doutor em Ciências.

Área de Concentração: Biologia Celular e Molecular.

Orientadora: Profa Dra Maria Cristina Roque Antunes Barreira.

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AUTORIZO A REPRODUÇÃO E DIVULGAÇÃO TOTAL OU PARCIAL DESTE TRABALHO, POR QUALQUER MEIO CONVENCIONAL OU ELETRÔNICO, PARA FINS DE ESTUDO E PESQUISA, DESDE QUE CITADA A FONTE.

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*Um cientista que também é um ser humano não deve descansar enquanto o conhecimento que
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GONÇALES, R. A. **Paracoccina: uma quitinase importante para a patobiologia e virulência de *Paracoccidioides brasiliensis***, 2018. 154 f. Tese (Doutorado) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2018.

Espécies do gênero *Paracoccidioides spp* são fungos patogênicos, termodimórficos, agentes etiológicos de doença endêmica em diversas regiões da América Latina. O indivíduo infectado desenvolve uma resposta específica que, quando associada à alta produção de TNF- α e IFN- γ , favorece a resistência ao fungo. Componentes de alguns fungos patogênicos foram caracterizados, por técnicas de *knockdown* gênico, como importantes para a virulência fúngica. Nosso grupo identificou paracoccina (PCN) como um componente de leveduras de *P. brasiliensis*; trata-se de uma proteína com um domínio enzimático, dotado de atividade quitinase e um domínio lectínico, ligante de GlcNAc. PCN é dotada das seguintes propriedades: (a) contribui para o crescimento do fungo; (b) promove a adesão da levedura à matriz extracelular, por ligar-se à laminina; (c) interage com N-glicanas de TLR2 e TLR4 e promove ativação celular; (d) estimula macrófagos a produzirem mediadores pró-inflamatórios como IL-12, TNF- α e NO; (e) promove a polarização M1 de macrófagos; (f) induz atividade fungicida em neutrófilos, bem como formação de NETs e supressão da apoptose, eventos que se mostraram dependentes da síntese de novo de proteínas pelos neutrófilos estimulados. Dada a relevância das atividades biológicas de PCN, promovemos recentemente o silenciamento do gene que codifica essa proteína, através de metodologia que usa RNA anti-sense e transformação mediada por *Agrobacterium tumefaciens* (ATMT). Uma vez PCN silenciada, a levedura perdeu a capacidade de fazer a transição para micélio e diminuiu a resistência à atividade fungicida de macrófagos. A infecção de camundongos com as cepas silenciadas, em comparação com as WT, causou doença de menor gravidade, com carga fúngica reduzida e baixa taxa de mortalidade. Essas observações sugerem de que PCN funcione como um fator de virulência em *P. brasiliensis*, que afeta a patogênese da infecção. Neste trabalho, ampliamos as

ferramentas moleculares de manipulação do fungo e viabilizamos a superexpressão de PCN em leveduras de *P. brasiliensis*, tendo como objetivos estudar seu papel na virulência e na patogênese da infecção, bem como determinar os mecanismos responsáveis por tais atividades. A inoculação de leveduras que superexpressam PCN (ov-PCN) em camundongos causou doença pulmonar muito grave, em comparação à doença leve e moderada causada por leveduras silenciadas em PCN e leveduras WT, respectivamente. Nesse sentido, nossos esforços se dedicaram à busca dos mecanismos dos mecanismos através dos quais PCN influencia o curso da infecção experimental. Na tentativa de identificar o papel exercido pelo domínio quitinase da PCN, coletamos o sobrenadante de culturas de leveduras ov-PCN e WT. Partículas de quitina presentes nesses sobrenadantes foram purificadas por afinidade à lectina WGA (*wheat germ agglutinin*). Através de medida da área das partículas capturadas, através de microscopia eletrônica e aplicação do programa ImageJ, verificamos que a superexpressão de PCN resultou em clivagem mais eficiente da quitina da parede de leveduras, uma vez que apenas partículas muito pequenas (mediana das medidas = 2 nm²) foram detectadas, enquanto as áreas das partículas de quitina obtidas de leveduras selvagens (WT) forneceram mediana 3 vezes maior (6 nm²). As partículas de quitina foram então utilizadas para estimular macrófagos a produzirem citocinas. As obtidas de ov-PCN estimularam preponderantemente a secreção da citocina anti-inflamatória IL-10, enquanto os macrófagos estimulados com partículas de leveduras WT produziram mais TNF- α e IL-1 β , ambas de efeito pró-inflamatório. Esses resultados permitiram a identificação de um mecanismo importante para que a superexpressão de PCN se associe à ocorrência de doença pulmonar muito grave: o microambiente anti-inflamatório criado pelo estímulo de macrófagos por PCN leva ao desenvolvimento de resposta imune não protetora do tipo Th2 e lesões mais graves. Um segundo mecanismo foi identificado ao compararmos a resistência de leveduras ov-PCN e WT às respostas efectoras de macrófagos. A superexpressão de PCN associou-se à maior internalização das leveduras e maior resistência à atividade

fungicida exercida por macrófagos. O estudo demonstra que diferentes níveis da expressão de uma quitinase (como PCN) levam à resistência a atividades antifúngicas de macrófagos e a diferentes graus de clivagem de quitina. A clivagem, por sua vez, pode alterar a estrutura da parede celular fúngica e a geração de fragmentos de quitina, cujos tamanhos e concentrações influenciam a produção de citocinas pelos macrófagos. Sob a ação de citocinas pró- ou anti-inflamatórias liberadas pelos macrófagos e, conseqüentemente, a montagem de respostas adaptativas pode ser decisiva para haver suscetibilidade ou resistência à infecção por *P. brasiliensis*. Este trabalho proporciona um importante avanço no conhecimento do papel de quitinases na resposta anti-fúngica do hospedeiro.

Palavras-chave: fator de virulência; heveína; lectinas; macrófagos; neutrófilos humanos; *Paracoccidioides brasiliensis*; quitinase; superexpressão e silenciamento de paracoccina.

Abstract

GONÇALES, R. A. **Paracoccin: a major chitinase for the pathobiology and virulence of *Paracoccidioides brasiliensis***. 2018. 154 f. Thesis (*Doctorade Scientiae degree*) – School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, 2018.

Species of the genus *Paracoccidioides spp* are thermodynamic fungi that cause a systemic disease, which is endemic in several regions of the Latin America. The infected individual develops a specific response that, when associated with the high production of TNF- α and IFN- γ , favors resistance to the fungus. Components of some pathogenic fungi were characterized by gene knockdown techniques as important for fungal virulence. Our group has identified a component of *P. brasiliensis*, named Paracoccin (PCN); it is a bifunctional protein with an enzymatic domain, endowed with chitinase activity and a lectin domain, which binds GlcNAc and chitin, a GlcNAc polymers. PCN has the following properties: (a) contributes to the fungus growth; (b) promotes the yeast adhesion to the extracellular matrix, by binding to laminaria glycans; (c) interacts with TLR2 and TLR4 N-glycans, which triggers cell activation; (d) stimulates macrophages to produce proinflammatory mediators, such as IL-12, TNF- α and NO; (e) promotes the M1 polarization of macrophages; (f) induces the neutrophils fungicidal activity, NETs formation, and suppression of neutrophils apoptosis, which are depending events on the *de novo* protein synthesis by neutrophils. Given the relevant biological activities exerted by PCN, we have performed recently the silencing of the gene that codes for this protein through a system that uses RNA anti-sense and *Agrobacterium tumefaciens* mediated transformation (ATMT). Once having the PCN gene silenced, yeast lost the ability of doing the transition to mycelium and decreased its resistance to macrophages fungicidal activities. Mice infection with PCN-silenced yeasts, compared to the infected with WT yeasts, exhibited a milder pulmonary disease with reduced fungal burden and low mortality rate. These observations suggest that PCN acts as a *P. brasiliensis* virulence factor that affects the pathogenesis of the fungal infection. In the present study, we expanded the molecular tools for the fungus manipulation

and enabled the overexpression of PCN in *P. brasiliensis* yeasts, aiming to elucidate the PCN role in the fungus virulence and the infection pathogenesis, as well as determining the responsible mechanisms for the PCN activities. Inoculation of the PCN overexpressing yeasts (ov-PCN) into mice caused a very severe lung disease, compared to the mild and moderate diseases caused by PCN-silenced and WT yeasts, respectively. Then our efforts became dedicated to the search of mechanisms through which PCN influences the course of the experimental fungal disease. In an attempt to identify the role of the PCN chitinase domain, we harvested the supernatant of the ov-PCN and WT yeasts cultures. Chitin particles contained in the supernatants have been captured by affinity to the immobilized WGA (wheat germ agglutinin) lectin. By measuring through electron microscopy and application of the ImageJ program the area of the isolated chitin particles, we verified that the overexpression of PCN resulted in a more efficient cleavage of whole chitin molecules contained in the yeast cell wall, since only very small particles (median of the measurements = 2 nm²) were detected, while the the chitin particles areas obtained from WT-yeasts provided a median 3 fold higher (6 nm²). Then, the preparations of chitin particles were taken to stimulate macrophages to produce cytokines. The particles obtained from ov-PCN have stimulated preponderantly the secretion of the anti-inflammatory cytokine IL-10, whereas the macrophages stimulated with WT yeast particles have produced higher concentrations of TNF- α and IL-1 β , which are known pro-inflammatory cytokines. These results allowed the identification of an important mechanism for the association of PCN overexpression to the occurrence of very severe pulmonary disease: the anti-inflammatory microenvironment created by the macrophages stimulation with PCN leads to the development of a non-protective Th2-type immune response and the more severe pulmonary injury. A second mechanism was identified as implicated in the severity of the lung disease associated to PCN overexpression. We compared the sensitivity of ov-PCN and WT yeasts to macrophages effector functions. PCN overexpressing yeasts were better internalized

by macrophages and more resistant to the fungicidal activity of these cells, events that contributes for the high pulmonary fungal load verified in mice infected with ov-PCN yeasts. The study demonstrates that different levels of a chitinase (PCN) expression and enzymatic activity lead yeasts to change their sensitivity to macrophages antifungal activities as well as to different grades of chitin cleavage. The cleavage, in its turn, leads to changes in the structure of the fungal cell wall and generation of chitin fragments, whose sizes and concentrations influence the cytokines production by macrophages. Under the influence of pro-inflammatory or anti-inflammatory cytokines released by macrophages, the mounted adaptative responses can be decisive in conferring susceptibility or resistance to the *P. brasiliensis* infection. This study provides an important advance in the knowledge on the role of a chitinase in the host antifungal response.

Keywords: virulence factor; hevein; lectins; macrophages; human neutrophils; *Paracoccidioides brasiliensis*; chitinase; overexpression and silencing of paracoccin.

Lista de Abreviaturas

A/J: camundongos resistentes a infecção por *Paracoccidioides brasiliensis*.

ATMT: *Agrobacterium tumefaciens mediated transformation*.

B10.A: camundongos suscetíveis a infecção por *Paracoccidioides brasiliensis*.

BAD-1: *Blastomyces Adhesin 1*.

BHI: *brain heart infusion*.

CFU: *Colony-forming unit*.

DO: densidade óptica.

ELISA: *Enzyme linked immuno sorbent assay*.

FBS: *fetal bovine serum*.

Fc-γ: *Fc-gamma receptor*.

GlcNAc: N-acetilglicosamina.

GM-CSF: granulocyte-macrophage colony-stimulating factor.

GMS: *Grocott mehenanime silver*.

gp43: Glicoproteína de peso molecular de 43 kDa de *P. brasiliensis*.

h: hora.

HE: hematoxilina-eosina.

HPH: *higromycin*.

HSP: *heat shock protein*.

IFN-γ: interferon-γ.

IL: interleucina.

kDa: quilodaltons.

LPS: lipopolissacarídeo bacteriano.

min: minuto.

MR: *manose receptor*.

NAGase: N-acetilglicosaminidase.

NETs: *neutrophils extracellular traps*.

NK: *natural killer*.

NO: *nitric oxide*.

NOD2: *nucleotide-binding oligomerization domain containing 2*.

ORF: *open reading frame*.

ov-PCN: *overexpressing of paracoccin*.

Pb: *Paracoccidioides brasiliensis*.

Pb27: proteína de 27 kDa de *P. brasiliensis*.

PBS: *phosphate buffered saline*.

PCM: *paracoccidioidomycose*.

PCN: *paracoccina*.

Pl: *Paracoccidioides lutzii*.

PMNs: *polimorfonucleares*.

p-rPCN: *paracoccina recombinante produzida em Pichia pastoris*.

qRT-PCR: *reação em cadeia da polimerase quantitativo em tempo real*.

ROS: *reactive oxygen species*.

r-PCN: *paracoccina recombinante produzida em Escherichia coli*.

s: *segundos*.

Th: *T-helper*.

TLR: *toll like receptor*.

TNF- α : *necrosis factor alpha*.

WGA: *wheat germ agglutinin*.

WT: *wild type*.

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1. INTRODUÇÃO

1.1. Paracoccidioidomicose (PCM)

Fungos do gênero *Paracoccidioides spp* são agentes etiológicos da paracoccidioidomicose (PCM), uma micose grave que é endêmica na América Latina (San-Blas, Niño-Vega et al. 2002). Esses fungos são termodimórficos e se desenvolvem na forma filamentosa, de 25 a 30°C, e na forma leveduriforme, de 35 a 37°C (Rappleye and Goldman 2006) (Figura 1). As espécies de *Paracoccidioides* foram classificadas taxonomicamente a partir de isolados bem conhecidos e estudados (Matute, McEwen et al. 2006; Bagagli, Theodoro et al. 2008; Teixeira, Theodoro et al. 2009).

Figura 1. A. Microscopia de campo claro de hifas de *P. brasiliensis*. B. Microscopia de campo claro de levedura de *P. brasiliensis*.

Recentemente, a análise molecular de 17 isolados *P. brasiliensis* Pb01-like da Região Centro-Oeste do Brasil corroborou a ideia de um evento de especiação dos isolados Pb01-like a partir dos isolados S1, PS2 e PS3 por volta de 32 milhões de anos atrás (Teixeira, Theodoro et al. 2009). Fungos com características próprias da linhagem Pb01 são endêmicos na região Centro-Oeste e Sul do Brasil e atualmente denominam-se *Paracoccidioides lutzii*, uma homenagem ao pesquisador brasileiro Adofl Lutz (Teixeira Mde, Theodoro et al. 2013; Mendes, Klafke et al. 2017). Por outro lado, fungos com características próximas às da

linhagem Pb18 são encontrados nas regiões Sul, Sudeste e Norte do país; eles são tradicionalmente conhecidos como *Paracoccidioides brasiliensis* (Teixeira, Theodoro et al. 2009; Theodoro, Teixeira Mde et al. 2012).

O nicho de *Paracoccidioides spp* na natureza ainda não foi estabelecido, porém o fungo já foi isolado de mamíferos e acredita-se que estes animais, como o tatu (*Dasypus novemcinctus*) são importantes reservatórios primários naturais (Nishikaku, Peracoli et al. 2008). Estudo recente, realizado no Rio Grande do Sul com soro de animais domesticados (cavalos, cachorros) e animais selvagens (tatu), mostrou soropositividade para infecção por *P. lutzii* de 13, 26 e 31.8 %, respectivamente (Mendes, Klafke et al. 2017).

Os isolados de *Paracoccidioides spp* mantêm-se restritos a áreas geográficas específicas. Há predominância em regiões de clima tropical e subtropical, com temperaturas moderadas entre 17 e 24°C, e índices pluviométricos acima de 1.400 mm ao ano (Sifuentes-Osornio, Corzo-Leon et al. 2012).

A PCM é a oitava causa de mortalidade entre as doenças infecciosas e parasitárias e apresenta a maior taxa de mortalidade entre as micoses sistêmicas (Colombo, Tobon et al. 2011). O Brasil é o país com o maior número de áreas endêmicas (regiões Sul, Sudeste e Centro-Oeste) e responsável por 85% do total de casos (Restrepo 1985; Verli, Marinho et al. 2005; Martinez 2017) (Figura 2). A PCM é a quinta causa de morte em pacientes com HIV no Brasil (Prado, Silva et al. 2009). A infecção pulmonar em humanos ocorre devido a inalação de conídios ou fragmentos de hifas encontrados no solo (McEwen, Bedoya et al. 1987; San-Blas, Niño-Vega et al. 2002). O desenvolvimento da PCM está relacionado a fatores do hospedeiro, níveis de virulência dos isolados e condições ambientais de crescimento (San-Blas, Niño-Vega et al. 2002; Benard 2008).

Figura 2. Áreas geograficamente endêmicas para paracoccidioidomicose na América Latina. Fonte: Martinez, R., 2017. (■) Primeiras áreas reconhecidas como endêmicas; (■) Alta endemividade observada desde as últimas décadas do século 20; (■) Áreas com alguma evidência recente de endemividade crescente; (■) Áreas com endemividade moderada; (■) Baixa endemividade; (□) Nenhum caso, ou casos raros de PCM nesses Países ou regiões (Martinez 2017).

A maioria dos indivíduos infectados com *Paracoccidioides spp* desenvolve a forma assintomática, podendo ou não progredir para a doença, sendo que esta gera manifestações clínicas muito variáveis (Benard, Neves et al. 1995). Há duas formas clínicas de PCM: a aguda e a subaguda (crônica). A forma aguda afeta pacientes jovens, de ambos os sexos, envolvendo principalmente órgãos do sistema retículoendotelial. Essa forma aguda da doença, que é grave e de mau prognóstico, causa desordem linfoproliferativa e depressão da resposta imunológica mediada por células (Brummer, Castaneda et al. 1993). A forma crônica, muitas vezes fatal, é prevalente em adultos do sexo masculino, havendo envolvimento predominantemente pulmonar e/ou mucocutâneo (Alves, Azevedo et al. 2009).

1.2. Imunologia da Paracoccidioidomicose

As considerações aqui feitas referem-se à infecção por *P. brasiliensis*, a espécie melhor conhecida.

A resposta imunitária celular está diretamente envolvida na resistência do hospedeiro contra a PCM e difere em doenças de diferentes gravidades e formas clínicas. Em 1998, com os estudos de Calich & Kashino sobre citocinas produzidas frente à infecção experimental de camundongos resistentes ou suscetíveis a *P. brasiliensis*, adquiriu-se a noção de que resistência estava ligada à secreção de altos níveis de TNF- α e IFN- γ [próprias de respostas inflamatórias Th1 (*T-helper 1*)], enquanto suscetibilidade e progressão da doença associam-se à produção de níveis baixos e efêmeros de TNF- α e IFN- γ e secreção de IL-5, IL-10 and TGF- β [citocinas próprias de respostas anti-inflamatórias Th2 (*T-helper 2*)] (Kashino, Fazioli et al. 2000). Tal diversidade de padrões de expressão de citocinas também foi verificada na PCM humana, em associação com as diferentes formas clínicas da doença ou apenas com a infecção fúngica, não seguida de doença (Mamoni and Blotta 2005; Mamoni and Blotta 2006). Castro e colaboradores (2013) caracterizaram mais detalhadamente a resposta imunológica na PCM humana e identificaram ocorrer, na forma crônica/adulta, respostas de padrão misto. A predominância de resposta Th17 (*T-helper 17*), além da resposta Th1, leva à resistência parcial ao fungo, como esquematizado na Figura 3 (de Castro, Ferreira et al. 2013).

Figura 3. Modelo proposto para a geração de diferentes respostas imunológicas observadas nas diferentes formas clínicas da paracoccidiodomicose humana (de Castro, Ferreira et al. 2013).

Voltando a abordar estudos com modelos experimentais de PCM murina, os resultados obtidos reforçaram, no decorrer das duas últimas décadas, a ideia de que camundongos resistentes apresentem baixa carga fúngica na maioria dos órgãos e que a infecção tenda a ser resolvida por desenvolvimento de imunidade predominantemente Th1, manifesta por alta produção de IFN-γ. Esta citocina atua sobre macrófagos, potencializando sua atividade antifúngica (Alves, Azevedo et al. 2009). Reações granulomatosas são predominantes na PCM têm importante papel na contenção dos fungos. Células dendríticas se dispõem em torno do granuloma e colaboram para a produção de TNF-α, justificando o fato de que essa citocina esteja precocemente envolvida na infecção (Pagliari and Sotto 2003). Estaleceu-se o conceito

de que TNF- α e IFN- γ sejam cruciais para o controle da infecção, por estimularem a formação de granulomas e a produção de óxido nítrico. Camundongos deficientes para tais citocinas tiveram comprometida a formação de granulomas e, conseqüentemente, a contenção das células fúngicas (Souto, Figueiredo et al. 2000).

A ocorrência de resposta Th2 está associada à produção de IL-4, IL-5 e IL-10, citocinas que estimulam linfócitos B a produzirem imunoglobulinas, moléculas que, além de não protegerem o hospedeiro, inibem as funções de macrófagos e a diferenciação de células Th1 (Calich and Kashino 1998). Indivíduos acometidos por formas graves de PCM desencadeiam predominantemente a resposta humoral, caracterizada por altos títulos de anticorpos circulantes não específicos, decorrentes de ativação policlonal de linfócitos B, associada à supressão da imunidade celular, fenômeno característico da infecção crônica (Benard 2008). A ineficiência da resposta humoral em proporcionar resistência a PCM foi demonstrada tanto em pacientes (Benard, Mendes-Giannini et al. 1997) como em camundongos suscetíveis infectados (Calich and Kashino 1998). A PCM associa-se frequentemente à hipergamaglobulinemia, que acarreta em aumento dos níveis de complexos imunes circulantes e ativação do sistema complemento pela via clássica (Arango and Yarzabal 1982; Munk, Kajdacsy-Balla et al. 1992).

Consolidou-se ainda o relevante papel exercido por respostas Th17 para que haja resistência à PCM (de Castro, Ferreira et al. 2013). Uma vez que um evento relevante de respostas Th17 é a infiltração tecidual por neutrófilos, os estudos do papel dessas células na PCM ampliaram-se. Kurita e colaboradores, em 2005, demonstraram que neutrófilos humanos matam leveduras de *P. brasiliensis*; os autores verificaram que células polimorfonucleares (PMNs) obtidas do sangue periférico de indivíduos saudáveis exercem papel anti-fúngico, atividade esta que é aumentada pela adição de IFN- γ , GM-CSF, IL-1 β ou IL-4 ao sistema de ensaio utilizado e antagonizada por IL-10 (Kurita, Oarada et al. 2005; Rodrigues, Dias-Melicio et al. 2007). Essa atividade foi inibida quando o desafio por *P. brasiliensis* ocorreu na presença

de catalase e superóxido dismutase, observação que reforça a ideia de que neutrófilos humanos exerçam papel efetor no processo de morte das leveduras (Rodrigues, Dias-Melicio et al. 2007). Em 2008, o estudo de Dias e colaboradores demonstrou a importância de neutrófilos para a montagem da resposta do hospedeiro à infecção por *P. brasiliensis*, resposta essa que envolve a produção e liberação de fatores anti-microbianos, citocinas, quimiocinas, bem como influencia os processos de ativação e regulação da imunidade inata e adaptativa (Dias, Mesquita et al. 2008). Soma-se a tais observações, o estudo mais recente (2015) de Fernandes e colaboradores, que mostrou que neutrófilos humanos, frente ao desafio com leveduras de *P. brasiliensis*, produzem IL-12, IL-10, PGE2 e LTB4, de uma maneira que envolve TLR2, receptor de manose e dectina-1. Esse trabalho constitui-se em demonstração de que neutrófilos, além de atuarem como células efetoras da imunidade inata, têm a capacidade de modular a imunidade inata e adaptativa contra o fungo, exercida através da produção de citocinas e mediadores lipídicos. Tal modulação se dirige ao padrão pró- ou anti-inflamatório, na dependência do isolado de *P. brasiliensis* utilizado e do receptor envolvido no reconhecimento do fungo pelos neutrófilos (Fernandes, Bachiega et al. 2015). Os estudos, ainda que pouco numerosos, deixam claro que neutrófilos exercem um papel importante na infecção por *P. brasiliensis*, tanto pela ação fungicida direta como pela interação com outros tipos celulares, levando à modulação da resposta imunitária adquirida, que é montada a seguir.

Estudos em modelo murino de PCM contribuíram muito para que fosse estabelecida a importância de neutrófilos para o combate à infecção por *P. brasiliensis* (Meloni-Bruneri, Campa et al. 1996). Foi demonstrado que camundongos resistentes à PCM, após quinze dias de infecção, têm maior número de neutrófilos nos pulmões, maior *burst* oxidativo e maior produção de ROS, do que camundongos suscetíveis (Meloni-Bruneri, Campa et al. 1996). Mais recentemente, o estudo de Sperandio e colaboradores (2015), utilizando modelo de infecção do camundongo em bolha subcutânea de ar preparada no dorso do animal, mostrou que

camundongos resistentes apresentam respostas inflamatórias distintas das verificadas nos suscetíveis. Neutrófilos dos resistentes têm padrões adequados de maturidade e migração, de maneira que respondem prontamente a estímulos inflamatórios, contrariamente ao observado em camundongos suscetíveis. Assim, os neutrófilos de resistentes ligam-se mais rapidamente às leveduras de *P. brasiliensis* e dirigem a resposta adquirida para o padrão protetor Th1. Mais uma vez, os neutrófilos de camundongos suscetíveis foram menos competentes, tanto para internalizar as leveduras, como para modular a imunidade adquirida, que se dirigiu predominantemente para o padrão Th2, não protetor (Sperandio, Fernandes et al. 2015).

A imunidade contra *P. brasiliensis* também é influenciada por interações de componentes da parede celular fúngica com receptores presentes em células da imunidade inata. Tal fato é ilustrado pelos resultados obtidos por Feriotti e colaboradores (2015), ao investigarem a interação de Dectina-1 (expressa em macrófagos) e β -glucana (encontrada na superfície de leveduras, na camada mais externa da parede celular) e seu efeito sobre a imunidade inata de camundongos resistentes e suscetíveis à infecção por *P. brasiliensis*. Para tanto, a ativação de macrófagos provenientes das duas raças de camundongos pela infecção com leveduras foi observada após incubação dos macrófagos com um açúcar agonista do receptor (curdlan) ou com um açúcar antagonista (laminarina). Os autores constataram que curdlan não exerce efeito significativo sobre a ativação dos macrófagos de animais suscetíveis, mas aumenta as capacidades fagocíticas e fungicidas de macrófagos de resistentes, além de aumentar sua expressão de PRRs, incluindo dectina-1, eventos esses que não ocorrem em macrófagos de suscetíveis. Adicionalmente, curdlan inibe a expressão de arginase-1 e aumenta a de NO-sintase em macrófagos de camundongos resistentes, mas não tem efeito sobre células do animal suscetível. Ao contrário, laminarina reforçou os respectivos perfis M2/M1 dos macrófagos de camundongos resistentes e suscetíveis. Em seguida ao tratamento com curdlan, macrófagos de animais resistentes aumentaram a fosforilação de Syk kinase e a expressão intracelular de pro-

IL-1 β , em comparação a células dos suscetíveis. Foi avaliada então eventual diferença na ativação do inflamassoma NRLP3 nos dois tipos de macrófagos. Realmente, em comparação ao observado em macrófagos de camundongo suscetível, as células do resistente exibiu expressão aumentada de NALP3, ASC e IL-1 β , além de atividade caspase1 aumentada e secreção de altos níveis de IL-1 β madura e de IL-18. O trabalho demonstrou que β -glucanas solúveis ou particuladas exercem atividades moduladoras opostas em macrófagos de diferentes padrões genéticos. Conclui-se que a ação sinérgica de dectina-1 e do inflamassoma NALP3 estão associadas com a resposta imune inata de hospedeiros resistentes à infecção por *P. brasiliensis*.

Interações entre PRRs e componentes fúngicos. Reconhecimento como o aqui descrito induz a produção de citocinas, as quais ativam mecanismos de defesa que culminam na eliminação do fungo e na regulação da intensidade da reação granulomatosa (Feriotti, Bazan et al. 2015). No entanto, citocinas produzidas no contato inicial entre o fungo e as células fagocíticas podem favorecer o crescimento do patógeno, levar à sua disseminação e facilitar a progressão da doença. Já foi observado, por exemplo, que a infecção fúngica de monócitos que foram pré-incubados com IL-6 resulta em inibição da produção de TNF- α e IL-10 e maior recuperação de células fúngicas na cocultura. Esses resultados sugerem que IL-6, ao exercer um efeito modulador da produção de TNF- α , torna os monócitos mais permissivos ao crescimento fúngico (Siqueira, Campos Soares et al. 2009).

1.3. A parede celular de fungos

Os fungos são organismos eucarióticos que se distinguem de células de mamíferos por diversas características, sendo que uma das mais marcantes corresponde à existência, além da membrana plasmática, de um envoltório externo que é a parede celular (Xie and Lipke 2010). A parede celular localiza-se na interface entre o micro-organismo e o meio ambiente; é altamente dinâmica e finamente regulada. Ela desempenha um papel importante para o crescimento, morfogênese e interação do fungo com o ambiente e com células do hospedeiro.

Em *Saccharomyces cerevisiae* a parede celular representa 15-30% do peso seco e 25-50% do volume da célula (Orlean 1997). De maneira semelhante, a parede celular de *Candida spp* e *Aspergillus spp* está disposta em uma bicamada cuja face interna é composta de polissacarídeos e a externa de proteínas. Essa disposição em bicamada pode ser observada na maioria dos ascomicetos, incluindo fungos dimórficos e filamentosos (De Groot, Ram et al. 2005).

Em fungos patogênicos a parede celular tem função importante na relação entre o fungo e o hospedeiro, constituindo-se em alvo para o desenvolvimento de antígenos e vacinas. Uma característica importante de vários fungos patogênicos é a capacidade de alternar sua morfologia de filamentosa para levedura, mudança que se acompanha de alterações na composição da parede celular (San-Blas, Niño-Vega et al. 2002).

Polissacarídeos são predominantes na parede celular. Dentre eles, destacam-se β -1,3- e β -1,6- glucanas (polímeros de glicose), quitina (polímero de *N*-acetilglicosamina) e mananas (De Groot, Ram et al. 2005).

As ASL-CWP (*alkali sensitive linkage-cell wall protein*) constituem um grupo de proteínas que se ligam covalentemente às moléculas de β -1,3-glucanas da parede celular de fungos, através de uma ligação sensível a alcali (de Groot, de Boer et al. 2004). As ASL-CWP incluem dois grupos de proteínas. O primeiro grupo é composto de proteínas sensíveis a pH alcalino e que não apresentam repetições internas de sequências. O segundo grupo é composto pelas proteínas Pir (*protein with internal repeats*), que compartilham a característica de serem constituídas de um peptídeo sinal N-terminal, um sítio de reconhecimento para a protease Kex2p e uma ou mais repetições internas (Toh-e, Yasunaga et al. 1993; Mrsa, Seidl et al. 1997). Proteínas Pir são altamente *O*-glicosiladas e mantidas na parede em associação direta com β -1,3-glucanas, através de ligação de natureza desconhecida, também sensível a pH alcalino (Toh-e, Yasunaga et al. 1993; Mrsa, Seidl et al. 1997; Kapteyn, Van Egmond et al. 1999; Kandasamy, VEDIYAPPAN et al. 2000; Kapteyn, Hoyer et al. 2000).

A parede celular de *Paracoccidioides spp* contém moléculas envolvidas na virulência e patogenicidade fúngicas e na modulação da resposta imune do hospedeiro (Ruiz-Herrera, Elorza et al. 2006). Mudanças na composição e rearranjo da parede celular são disparadas por sinais ambientais (De Groot, Ram et al. 2005; Levitz 2010) e levam à transição morfológica, que desempenha papel essencial na patobiologia fúngica (Castro Nda, de Castro et al. 2009).

A parede celular de *P. brasiliensis*, assim como mencionado para outros fungos, é composta por polissacarídeos, quitinas e glucanas, sendo que a proporção entre os componentes varia com a fase morfológica e a virulência do isolado (Carbonell, Kanetsuna et al. 1970; Kanetsuna and Carbonell 1970). Acredita-se que alguns polissacarídeos da parede celular de *P. brasiliensis* sejam essenciais para a patogênese (Puccia, Vallejo et al. 2011; Longo, Nakayasu et al. 2013) (Figura 4). Nas leveduras, α -glucana é o principal constituinte da parede celular, enquanto a β -glucana é escassa e caracterizada como apresentando ligações glicosídicas do tipo β -1,6 (Kanetsuna, Carbonell et al. 1969; San-Blas, San-Blas et al. 1984). Por outro lado, a parede do micélio é composta principalmente de β -1,3-glucana e possui somente 7 a 18% de quitina, associada a essas β -glucanas e proteínas (San-Blas and San-Blas 1977). Durante a fase de crescimento micelial, as glucanas permanecem com ligações glicosídicas do tipo β -1,3, havendo baixa quantidade de β -1,6 (Kanetsuna, Carbonell et al. 1969; San-Blas and San-Blas 1977). A mudança para β -1,6-glucana vai ocorrer em decorrência da transição para leveduras, formas fúngicas que, como já mencionado, expressam caracteristicamente β -1,6-glucanas.

A singularidade estrutural e essencial dos componentes da parede celular de fungos indica que proteínas envolvidas em síntese e/ou degradação são alvos potenciais de drogas, como α -1,3-glucana sintase, glucanases [exo- β -1,3, endo- β -1,3(4)], α -1,2 e α -1,6-manosil transferases, quitinases e dolicol fosfato-D-manose (Desjardins, Champion et al. 2011; Puccia, Vallejo et al. 2011). Análises de hibridização por *microarray* identificaram algumas sintases de quitinas reguladas positivamente durante a transição de micélio para levedura, enquanto há

regulação negativa de quitinases e endoquitinases (Nunes, Costa de Oliveira et al. 2005; Puccia, Vallejo et al. 2011).

Figura 4. Representação esquemática de componentes da parede celular de *P. brasiliensis*. (Puccia, Vallejo et al. 2011).

Análises genômicas revelaram a existência de sete quitina-sintases em *Paracoccidioides spp*, uma de cada classe, incluindo a classe III funcional (Barreto, Sorais et al. 2010; Puccia, Vallejo et al. 2011), não descrita previamente (Nino-Vega, Munro et al. 2000) e Pbrchs6, originalmente identificada em estudos de transcriptoma em micélio (Nunes, Costa de Oliveira et al. 2005; Puccia, Vallejo et al. 2011).

Além da variação na composição de polissacarídeos, a parede celular de *P. brasiliensis* também apresenta variação na quantidade de proteínas (24-41% em leveduras e 7-14% em hifas) (Kanetsuna, Carbonell et al. 1969) e composição de lipídeos (duas vezes maior em leveduras do que em hifas) (Manocha 1980). A quitina corresponde de 37 a 48% da parede celular e encontra-se na camada interna dessa estrutura (San-Blas and San-Blas 1977). O conteúdo de quitina na parede celular das leveduras é cerca de três vezes maior do que o verificado em hifas (San-Blas and San-Blas 1977).

Alguns componentes de *Paracoccidioides spp* têm sido bem caracterizados, com destaque para a glicoproteína gp43 (Puccia, Schenkman et al. 1986; De Camargo, Unterkircher et al. 1988; Giannini, Bueno et al. 1990; Travassos, Puccia et al. 1995; Cisalpino, Puccia et al. 1996). Outros componentes são Pb27 (Fernandes, Martins et al. 2011), Pb40 (Fernandes, Martins et al. 2012), Hsp60 (Izacc, Gomez et al. 2001; Cunha, Zancoppe-Oliveira et al. 2002; Fernandes, Oliveira et al. 2016) e Paracoccina (estudada por nosso grupo, veja suas características no item que se segue).

1.4. Paracoccina de *P. brasiliensis*: uma quitinase com domínio de ligação a carboidrato

Lectinas são proteínas que possuem um domínio não catalítico que se liga especifica e reversivelmente a mono ou oligossacarídeo (Peumans and Van Damme 1995). As primeiras descrições de lectinas datam do final do século XIX, sendo recente a ideia de que elas medeiam fenômenos biológicos a partir do reconhecimento de glicanas, especialmente as expressas em superfícies celulares. Essa ligação modifica a fisiologia da membrana e/ou desencadeia sinalização celular, de maneira a ativar fenômenos como proliferação, migração ou outras respostas bioquímicas celulares (Sharon and Lis 2004). Interações lectina-carboidrato desempenham importante papel em eventos de adesão celular, que são cruciais na inflamação, nas infecções por vírus, bactérias, fungos e protozoários, na simbiose de plantas com bactérias, na diferenciação celular, na migração de linfócitos, na transformação neoplásica e na ocorrência de metástases (Sharon and Lis 1989), dentre outros processos biológicos e patogênicos.

No que concerne a fungos patogênicos, a lectina melhor estudada é BAD-1 (*Blastomyces Adhesin I*), uma adesina de 120-kDa secretada por *Blastomyces dermatitidis*, que se liga à superfície das leveduras via quitina, correspondente a um importante fator de virulência (Brandhorst, Gauthier et al. 2005). BAD-1 apresenta dois domínios principais: um maior, com um conjunto de repetições de ligação à quitina e outro domínio EGF-like (semelhante ao fator de crescimento epidérmico) C-terminal, que fixa a levedura na superfície celular. Foi

demonstrado que na lectina BAD-1 os domínios de conjuntos de repetições são importantes para ligação à heparina e desempenham importante papel na virulência fúngica. Essa atividade pode favorecer a adesão da levedura *B. dermatitidis* a células do hospedeiro e matriz extracelular; a deleção do gene *bad-1* torna as leveduras avirulentas, por regular negativamente a ativação de células T (Brandhorst, Roy et al. 2013).

Quitinases estão presentes em todos os reinos e dependendo do organismo, apresentam diferentes funções. Quitinases bacterianas estão envolvidas em processos nutricionais e hidrolizam quitina para transferência de carbono e azoto (Fukamizo 2000). Quitinases fúngicas, estão envolvidas principalmente em processos de remodelamento da parede e divisão celular (Kuranda and Robbins 1991).

As quitinases são classificadas em duas famílias de glicosil hidrolases, GH18 e GH19, caracterizadas por diferenças na estrutura primária e presença de motivos conservados (Henrissat 1991). A distribuição das quitinases pertencentes a família GH18 é ubíqua, pois estão presentes em vírus, bactérias, plantas, fungos, mamíferos, incluindo o homem (Perrakis, Tews et al. 1994; Hawtin, Arnold et al. 1995; Terwisscha van Scheltinga, Hennig et al. 1996; Saito, Ozaki et al. 1999; Hollis, Monzingo et al. 2000). Grande parte das quitinases da família GH19 é encontrada em plantas (Truong, Park et al. 2003), ainda que também descritas em bactérias, vírus e nematódeos (Ohno, Armand et al. 1996; Geng, Plenefisch et al. 2002; Ueda, Kojima et al. 2003; Honda, Taniguchi et al. 2008).

Quitinases apresentam diferentes módulos de ligações a carboidratos (CBM – *Carbohydrate Binding Modules*), ou seja, domínios lectínicos. Em fungos, os CBMs usualmente descritos correspondem a (i) CBM18, ligante de quitina, (ii) CBM1, ligante de celulose e quitina, e (iii) CBM50, ligante de peptideoglicano e quitina (também conhecido como LysM) (Seidl 2008). Como mencionado, quitinases fúngicas pertencem à família GH18 e são produzidas em todos os estágios de crescimento ativo de fungos, quais sejam na germinação

dos esporos, na fase de crescimento exponencial e no desenvolvimento do micélio (Gooday, Wei-Yun et al. 1992). Há grande homologia entre quitinases fúngicas e quitinases de classe III de plantas (Hayes, Klemsdal et al. 1994) que, sabidamente, têm importância no desempenho de funções morfogênicas, autolíticas, nutricionais e parasíticas. O número de quitinases varia entre as espécies fúngicas.

Enzimas glicolíticas da família 18 desempenham atividade quitinase e podem estar envolvidas no remodelamento da parede celular (Adams 2004). Ademais, a caracterização de quitinases específicas mostrou que elas podem ser essenciais para a geração de certos fenótipos, como os referentes à separação celular. Por exemplo, a deleção do gene que codifica a proteína Cht3 em *C. albicans* resultou na geração de células dispostas em cadeias inseparáveis na fase de crescimento das leveduras, reproduzindo o fenótipo de *S. cerevisiae* quando teve o gene *cts1* deletado (Dunkler, Walther et al. 2005). Outro exemplo refere-se ao gene *chi42* de *Trichoderma harzianum*, que codifica uma endoquitinase necessária para eliminação de fungos fitopatogênicos (Lorito, Woo et al. 1998). Um exemplo adicional vem da observação de que a deleção do gene *chiB* de *Aspergillus nidulans* resulta em prejuízo do processo de autólise de hifas, verificada no fungo selvagem (Yamazaki, Yamazaki et al. 2007). A clonagem do gene *chiA* de *A. nidulans* permitiu que se identificasse o seu produto, a proteína ChiA, como glicosilada e ancorada via GPI, bem como localizada nas regiões de ramificações e no ápice das hifas, atuando no remodelamento e maturação da parede celular (Yamazaki, Tanaka et al. 2008). Genes de quitinases foram descritos como diferencialmente regulados durante o crescimento e desenvolvimento fúngico (Taib, Pinney et al. 2005), de maneira a permitir que seus produtos atuem em diferentes fases do organismo.

Nosso grupo tem trabalhado na identificação de lectinas de patógenos e, nesse contexto, descreveu a Paracoccina (PCN) de *P. brasiliensis*, que se destacou por exercer efeito imunomodulador que conferiu proteção à infecção por *P. brasiliensis* em camundongos. Esse

efeito foi atribuído à sua atividade lectínica, ligante de N-acetilglicosamina. Com base nas descrições das lectinas da família da heveína, investigamos a atividade enzimática de PCN, com resultados positivos. Estudos genômicos e proteômicos mostraram que a proteína PCN é constituída de 218 resíduos de aminoácidos, dispostos em multidomínios, codificada por gene que apresenta três éxons e dois íntrons e é anotado como PADG_03347 (Gene ID: 22582669). PCN compartilha uma sequência de aminoácidos que é similar às de duas outras proteínas da família 18 de quitinases. A sequência de um segundo domínio de PCN também é compartilhado com algumas proteínas da família 18 de quitinases; trata-se do domínio lectínico, ligante de N-acetilglicosamina (Terwisscha van Scheltinga, Hennig et al. 1996). A PCN relaciona-se ainda com quitinases de *H. capsulatum* (83% de similaridade), *B. dermatitidis* (81% de similaridade) e a quitinase de classe III ChiA1 de *A. nidulans* (83 % de similaridade) (*Blastp*, NCBI – *National Center for Biothecnology Information*). Tal tipo de organização molecular permitiu que se confirmasse a identificação da PCN como um membro da família heveína de lectinas (Itakura, Nakamura-Tsuruta et al. 2017), que recebe tal denominação porque o primeiro membro descrito da família foi encontrado no látex da *Hevea brasiliensis* (seringueira).

Os primeiros estudos sobre o papel de PCN na biologia do fungo foram realizados com preparações enriquecidas da lectina, obtidas por cromatografia de afinidade em coluna de N-acetilglicosamina (Coltri, Casabona-Fortunato et al. 2006) ou em coluna de quitina (dos Reis Almeida, de Oliveira et al. 2010). Essas preparações permitiram verificar que a lectina contribui para a adesão do fungo à matriz extracelular (ECM) e induz macrófagos a produzirem TNF- α e NO (Coltri, Casabona-Fortunato et al. 2006). A ligação à ECM se faz por interação com laminina, de maneira dependente do reconhecimento de carboidrato e da concentração utilizada (Coltri, Casabona-Fortunato et al. 2006).

A habilidade de um micro-organismo em aderir e invadir é reconhecida como um fator importante de patogenicidade. Paracoccina é dotada de atividade N-acetilglicominidase,

revelada por sua capacidade de clivar quitina (Ganiko, Puccia et al. 2007; dos Reis Almeida, de Oliveira et al. 2010). Constatamos ainda que N-glicanos desempenham relevante papel na atividade N-acetilglicosaminidase, que contribui para o crescimento da célula fúngica e biogênese da parede celular de *P. brasiliensis* (Dos Reis Almeida, Carvalho et al. 2011). Desta forma, ela pode se ligar a compartimentos específicos da parede celular, levando à remodelagem de estruturas em resposta as mudanças ambientais (dos Reis Almeida, de Oliveira et al. 2010). Além disso, foi demonstrado que paracoccina localiza-se na superfície de leveduras, com predominância nas regiões de brotamento (Coltri, Casabona-Fortunato et al. 2006). Observamos ainda que após 120 horas da troca de temperatura (25°C para 37°C) de culturas de *P. brasiliensis*, há predominância de leveduras cujo conteúdo de paracoccina predomina no interior dessas formas fúngicas. No micélio, PCN predomina na extremidade das hifas (Oliveira, Fernandes et al. 2017), local de crescimento das formas filamentosas, que ocorre por extensão apical (Gadd 1995; Harris and Momany 2004). Outros estudos também indicam a relevância da lectina no crescimento fúngico, observação proporcionada pela adição de anticorpos anti-paracoccina ao meio de cultivo de *P. brasiliensis*, resultando em inibição do crescimento (Ganiko, Puccia et al. 2007). Estudo realizado com o gene da quitinase *cts1* de *P. brasiliensis* mostrou que ele é mais expresso em leveduras do que em hifas e não é detectado na parede celular (Bonfim, Cruz et al. 2006). O que sugere que PCN tenha papel importante na biossíntese da parede celular de leveduras.

Recentemente, realizamos o silenciamento gênico da PCN e constatamos que essa proteína é um fator de virulência em *P. brasiliensis* (Fernandes, Oliveira et al. 2017). Verificamos ainda que PCN é muito expressa em hifas e nas formas de transição levedura-hifa (Oliveira, Fernandes et al. 2017), o que sugeriu seu envolvimento em processos de transição morfológica em *P. brasiliensis*.

Paracoccina recombinante (rPCN), expressa em *Escherichia coli*, teve sua qualidade demonstrada pela reprodução das atividades biológicas conhecidas da proteína nativa. A disponibilidade da proteína recombinante permitiu avaliar o efeito da administração profilática ou terapêutica de paracoccina em camundongos (Alegre-Maller, Mendonca et al. 2014; Alegre, Oliveira et al. 2014). Dessa maneira foi demonstrada sua capacidade de conferir proteção contra a PCM experimental, manifesta por redução do número e da extensão das lesões granulomatosas pulmonares e por aumento do *clearance* de leveduras no tecido pulmonar. A tais fatos, associam-se elevados níveis pulmonares de IFN- γ , TNF- α e IL-12 (citocinas de perfil Th1). Constatamos que esses efeitos eram atribuídos à interação de rPCN com glicanas N-ligadas a TLR2 e TLR4 (Alegre-Maller, Mendonca et al. 2014).

Subsequentemente, utilizamos a levedura *Pichia pastoris*, linhagem GS115, como modelo de expressão eucariótico para a produção de PCN, já que esse sistema permite que ocorram modificações pós-traducionais, como a glicosilação, similares às da proteína nativa (construção realizada durante o doutorado de Ana Claudia Paiva Alegre em colaboração com o estagiário Bruno Rodrigues de Souza). Motivados por evidências prévias de que a administração de PCN confere proteção a camundongos contra a infecção por *P. brasiliensis*, Freitas e colaboradores (2016) realizaram estudo *in vitro* que mostrou a propriedade de PCN de induzir a polarização de macrófagos para M1, seguida de produção de mediadores inflamatórios (IL-6, TNF- α , IL-12p40 e NO), bem como a capacidade de PCN de reverter a polarização de macrófagos de perfil M2 para M1. Esses resultados contribuíram importante para elucidar os possíveis mecanismos responsáveis pelo efeito imunomodulador da PCN. A alta produção de IL-12 pelos macrófagos M1 e a decorrente diferenciação de células Th1, produtoras de IFN- γ , são responsáveis pela geração de resposta protetora contra *P. brasiliensis*, quando PCN é administrada na forma profilática ou terapêutica (Freitas, Oliveira et al. 2016).

Para que ocorra o estabelecimento da PCM, *P. brasiliensis* precisa vencer uma primeira barreira que é a resposta imune inata do hospedeiro, propiciada por diversas células, que incluem neutrófilos, macrófagos e células dendríticas.

No que concerne a neutrófilos, estudos recentes trouxeram elementos sugestivos de que funções não fagocíticas dessas células sejam importantes para a eliminação de fungos. Leveduras de *C. albicans* induzem e interagem com as redes extracelulares de neutrófilos (NETs) induzindo o remodelamento da arquitetura fúngica, culminando na expressão de antígenos reconhecidos por dectina-1 presentes em outros leucócitos (Hopke, Nicke et al. 2016).

Recentemente, nosso grupo verificou o papel da p-rPCN em neutrófilos humanos. Observamos que a p-rPCN induz a secreção de IL-8, suprimindo a apoptose de maneira dependente da síntese *de novo* de poli-peptídeos (Ricci-Azevedo, Goncales et al. 2017). Além disso, a p-rPCN induz a formação de NETs e a produção de ROS em neutrófilos humanos (Ricci-Azevedo, Goncales et al. 2017).

1.5. Estudos moleculares de caracterização estrutural e funcional de proteínas fúngicas

Numerosos estudos utilizando sistemas de transformações gênicas foram desenvolvidos para caracterizar fatores de virulência em fungos patogênicos. O primeiro relato utilizando a abordagem de eletroporação de fungos foi de Woods e colaboradores (1998), que demonstraram haver eletrotransformação eficiente ao utilizarem plasmídeo contendo o gene *URA5* (*HcURA5*) de *H. capsulatum* (Woods, Heinecke et al. 1998). A técnica por eles empregada forneceu as bases para estudos e análises de expressão gênica em *H. capsulatum* (Retallack 1997; Patel, Batanghari et al. 1998). Em 2004, Rappleye e colaboradores desenvolveram um sistema de silenciamento para genes-alvos por meio de RNA de interferência em *H. capsulatum*; o procedimento proporcionou evidências funcionais de

silenciamento e demonstrou que o gene *agsI* [codificador de α -(1,3)-glucana sintase] corresponde a um importante fator de patogenicidade em *H. capsulatum*.

Dentre as novas abordagens desenvolvidas, a utilização de transformação fúngica mediada por *Agrobacterium tumefaciens* (ATMT) é muito eficiente, pois, nesse caso, é possível realizar inserção randômica do gene de interesse no DNA cromossomal por meio do T-DNA [plasmídeo bacteriano indutor de tumor (Ti)] (Zupan and Zambryski 1995). Recentemente, micologistas moleculares mostraram que o sistema que utiliza *A. tumefaciens* transforma eficientemente alguns fungos filamentosos (Lacroix, Tzfira et al. 2006), o que resulta num potencial método de inserção mutagênica nesses micro-organismos (Walton, Idnurm et al. 2005; Nemecek, Wuthrich et al. 2006). A partir de tais evidências, as técnicas de estudos de expressão gênica foram aprimoradas, conforme demonstrado por Marion e colaboradores (2006), permitindo desenvolver mutantes por meio de inserções no DNA em linhagens virulentas de *H. capsulatum* via *A. tumefaciens*. Nesse trabalho, o grupo estudou o gene α -(1,4)-amilase (*AmyIp*), o qual é requerido para a biossíntese de α -(1,3)-glucana, um fator de virulência em *H. capsulatum* (Marion, Rappleye et al. 2006).

Essa técnica de transformação tem sido aplicada em diversos organismos fúngicos, constituindo-se em valiosa ferramenta para o estudo de fungos dimórficos, patogênicos para o homem, como *Coccidioides immitis*, *Cryptococcus neoformans*, *H. capsulatum* e *B. dermatitidis* (Abuodeh, Orbach et al. 2000; Sullivan, Rooney et al. 2002; McClelland, Chang et al. 2005). O fungo *P. brasiliensis* é suscetível a transformação via ATMT (Leal, Montes et al. 2004; Soares Rde, Velho et al. 2005). Leal e colaboradores (2004) descreveram a transformação de leveduras de *P. brasiliensis* (ATCC-60855) com *A. tumefaciens* (GV3101) carreando o vetor *pAD1625*. A descrição original utilizando o sistema ATMT mediando a manipulação genética de *P. brasiliensis* com o vetor *pUR5750* (Almeida, Carmona et al. 2007), feita sob liderança do Prof. Fernando Rodrigues (Universidade do Minho, Braga, Portugal), foi

associada ao relato da aplicação da ATMT para expressar GFP (*green fluorescent protein*), uma proteína exógena fluorescente, em leveduras de *P. brasiliensis* (Almeida, Carmona et al. 2007). Recentemente, Torres e colaboradores (2013) desenvolveram linhagens *knockdown* do gene *gp43*, utilizando RNA antisense via *A. tumefaciens*, e observaram diminuição da expressão de *gp43*; o estudo resultou em evidências adicionais de que essa proteína seja um fator de virulência em *P. brasiliensis*. Além das proteínas mencionadas, outros silenciamentos gênicos foram obtidos pelo mesmo método (Menino, Saraiva et al. 2013; Torres, Hernandez et al. 2013; Holbrook, Kemski et al. 2014).

A tecnologia ATMT, foi aplicada para promover o *knockdown* da expressão de PbCDC42 em *P. brasiliensis*, resultando em diminuição do tamanho e alteração da morfologia das leveduras, bem como promoveu aumento da suscetibilidade do fungo à fagocitose e diminuição de sua virulência (Almeida, Cunha et al. 2009). Em outro estudo realizado pelo grupo do Prof. Fernando Rodrigues, obtiveram-se transformantes *down-regulados* para *SCONC*, um regulador negativo da via de assimilação de enxofre inorgânico pelo fungo; a *down* regulação da expressão de *SCONC* revelou ser tal componente um fator de virulência não descrito de *P. brasiliensis* (Menino, Saraiva et al. 2013).

A estratégia ATMT passou a ser também empregada por pesquisadores colombianos e brasileiros, interessados na obtenção de transformantes de *P. brasiliensis* (Almeida, Carmona et al. 2007; Almeida, Cunha et al. 2009; Hernandez, Almeida et al. 2010; Menino, Almeida et al. 2012; Menino, Saraiva et al. 2013; Torres, Hernandez et al. 2013; Torres, Hernandez et al. 2014; Marcos, Silva Jde et al. 2016; Tamayo, Munoz et al. 2016). A ampliação do número de aplicações do método, bem como sua otimização, decorrerá, em grande parte, da publicação do genoma completo de três isolados (Pb18, Pb03 and Pb01-like) do gênero *Paracoccidioides* spp.

1.6. Silenciamento gênico da Paracoccina: efeito sobre a virulência de *P. brasiliensis*

As evidências da relevância biológica da PCN, tanto para a biologia do fungo como para a imunidade do hospedeiro, tornaram importante a obtenção de transformantes de *P. brasiliensis*, que tivessem o gene da PCN silenciado ou superexpresso.

No ano de 2015, estabelecemos colaboração com o Prof. Fernando Rodrigues, da Universidade do Minho, em Braga, Portugal, pesquisador de destaque na área de manipulação genética de fungos, incluindo *P. brasiliensis*, para que pudéssemos proceder o silenciamento da paracoccina e, em seguida, promover também a superexpressão do mesmo gene. Três linhagens de leveduras foram obtidas, com níveis de expressão de PCN reduzidos para 60, 60 e 40%, respectivamente, em relação a fungos WT. O silenciamento de paracoccina levou à agregação das leveduras e bloqueio da transição de levedura para hifa (Fernandes, Oliveira et al. 2017). Além disso, ao infectarem camundongos, as leveduras silenciadas provocaram doença mais leve, conforme demonstrado por queda da carga fúngica pulmonar (de 96%), lesões granulomatosas compactas e taxa de mortalidade reduzida, em comparação ao verificado nos camundongos infectados com leveduras selvagens. Concluímos que a paracoccina atua como um fator de virulência de leveduras de *P. brasiliensis*, afetando diretamente a patogênese da infecção (Fernandes, Oliveira et al. 2017).

O silenciamento gênico de PCN em *P. brasiliensis* permitiu que identificássemos PCN como um fator de virulência do fungo; entretanto, não chegamos a elucidar o mecanismo através do qual PCN atua para interferir na virulência de linhagens de *P. brasiliensis*. O êxito no silenciamento nos fez supor que a superexpressão também pudesse ser alcançada, ainda que a superexpressão de moléculas-alvo em *P. brasiliensis* não tivesse sido anteriormente explorada por nenhuma das técnicas mencionadas. A motivação para investirmos na superexpressão veio do fato de acreditarmos que utilizando os novos transformantes pudéssemos determinar o mecanismo através do qual paracoccina atua sobre a patogênese da infecção.

1.7. Superexpressão da Paracoccina: efeito sobre o grau de clivagem e hidrólise de quitina em *P. brasiliensis*

O conteúdo apresentado aqui encontra-se descrito mais detalhadamente no capítulo IV desta Tese.

A colaboração firmada com o Prof Fernando Rodrigues, viabilizou a manipulação genética de *P. brasiliensis* para promover a superexpressão de PCN. Realizamos a clonagem da sequência genômica da PCN, correspondente a ORF hipotética PADG_03347 e partimos para a transformação genética de *P. brasiliensis*. A partir dessa metodologia inédita de transformação, caracterizamos os transformantes através de ensaios de estabilidade mitótica, resistência à higromicina (HPH) e curva de crescimento. Através de qRT-PCR, mostramos níveis de expressão significativamente superiores aos verificados na linhagem selvagem (Gonçales et al., em fase final de preparação).

A superexpressão da PCN foi constatada através de microscopia multifoton, utilizando anticorpo fluorescente anti-paracoccina conjugado com *Alexa-fluor* 294. A espessura da parede celular foi determinada através de microscopia eletrônica de transmissão e a redução da quitina na parede celular de *P. brasiliensis* foi verificada e quantificada por citometria de fluxo de leveduras que reagiram com a lectina WGA (*wheat germ agglutinin*) conjugada ao *TexasRed*. O resultado foi corroborado por microscopia multifoton, utilizando-se calcofluor para revelar a presença de quitina. O aumento da virulência das linhagens superexpressoras foi constatado através de infecções em camundongos por tais linhagens, que foram comparadas a infecções por leveduras selvagens. Nos camundongos infectados com linhagem superexpressora foram verificados (1) aumento da carga fúngica pulmonar (CFU), confirmado através da observação microscópica de cortes pulmonares impregnados por prata; (2) maior extensão das lesões granulomatosas pulmonares, determinada por análise morfométrica em microscópio óptico; (3)

maior gravidade da infecção, verificada através de curva de sobrevivência de camundongos infectados (Gonçales et al., em fase final de preparação).

A partir da superexpressão de PCN em *P. brasiliensis* tornou-se possível estudar o mecanismo de inflamação desencadeado pela hidrólise aumentada de quitina, decorrente da superexpressão de uma quitinase, no caso a PCN. A maior hidrólise gera fragmentos menores de quitina, indutores de polarização M2 de macrófagos. A relação entre o tamanho das partículas de quitina e a geração de um ambiente anti-inflamatório já foi demonstrada *in vitro*, utilizando quitina comercial e quitinase humana comercial, bem como em outro fungo patogênico, que é *C. albicans*. Em todos os casos, fragmentos menores de quitina induziram alta produção de IL-10, enquanto fragmentos maiores induziram macrófagos a produzirem TNF- α (Da Silva, Chalouni et al. 2009). Dado o impacto que a polarização de macrófagos tem sobre a resposta imune, assumimos que esse seja um importante mecanismo através do qual PCN atue como um fator de virulência fúngica. A virulência aumentada das leveduras superexpressoras de PCN também se relaciona ao fato de que elas são altamente suscetíveis à internalização por macrófagos e altamente resistentes à ação fungicida dessas células (Gonçales et al., em fase final de preparação).

Nosso estudo proporcionou a geração de leveduras de *P. brasiliensis* que superexpressam PCN e que ao infectarem camundongos causam grave acometimento pulmonar. O aumento de virulência foi atribuído a dois mecanismos, quais sejam a maior resistência à função efetora de macrófagos e a geração de um microambiente pulmonar anti-inflamatório, atribuído à atividade quitinase aumentada e à decorrente quebra de quitina em partículas de tamanho muito reduzido, indutoras de ativação alternativa de macrófagos (perfil M2).

Objetivos

2. OBJETIVOS

A) Investigar o efeito da paracoccina sobre células da imunidade inata, buscando identificar mecanismos que contribuam para a imunomodulação exercida pela proteína fúngica:

i) Determinar o efeito da paracoccina sobre a polarização de macrófagos murinos;

ii) Determinar o efeito da paracoccina sobre funções não fagocíticas de neutrófilos humanos;

B) Estudar a relevância da paracoccina para a biologia de leveduras de *P. brasiliensis* e patogênese da doença por elas causada, através da utilização de transformantes do fungo cujos genes codificadores da paracoccina foram silenciados ou superexpressos:

i) Promover o silenciamento e a superexpressão do gene que codifica a paracoccina em leveduras *de P. brasiliensis*;

ii) Avaliar os efeitos do silenciamento e da superexpressão da paracoccina sobre a patobiologia de leveduras de *P. brasiliensis*;

iii) Investigar os mecanismos responsáveis pelas alterações no curso da doença causada pela inoculação de leveduras transformantes, em comparação à causada por levedura selvagem de *P. brasiliensis*.

Material e Métodos / Resultados

1. Capítulo I

Diz respeito a artigo publicado com as especificações que se seguem.

Título: Paracoccin induces M1 polarization of macrophages via interaction with TLR4.

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Paracoccin Induces M1 Polarization of Macrophages via Interaction with TLR4

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The fungal human pathogen *Paracoccidioides brasiliensis* contains paracoccin (PCN), a multi-domain protein that has lectin and *N*-acetyl-glucosaminidase activities, which account for its effects on the growth and morphogenesis of the fungus and on the activation of host macrophages through its interaction with TLR *N*-glycans. With the purpose of detailing the knowledge on the effects of PCN on macrophages, we used recombinant PCN expressed in *Pichia pastoris* (p-rPCN) to stimulate isolated murine peritoneal macrophages. The activation of these cells manifested through the release of high levels of inflammatory mediators, such as nitric oxide, TNF- α , IL-12p40, and IL-6. Furthermore, peritoneal macrophages stimulated with p-rPCN increased the relative expression of STAT1, SOCS3, and iNOS2 mRNA (M1 polarization markers). However, the expression of Arginase-1, Ym-1, and FIZZ1 (M2 polarization markers) remained at basal levels. Interestingly, the observed M1 macrophages' polarization triggered by p-rPCN was abolished in cells obtained from knockout Toll-like receptor-4 mice. In this case, the p-rPCN-induced production of pro-inflammatory mediators was blocked too. These results demonstrate that the classical activation of macrophages induced by paracoccin depends on TLR4. Taken together, the results of our study indicate that paracoccin acts as a TLR agonist able to modulate immunity and exerts biological activities that favor its applicability as an immunotherapeutic agent to combat systemic fungal infections.

Keywords: lectin, paracoccin, TLR, macrophage polarization, proinflammatory mediators

INTRODUCTION

Paracoccidioides brasiliensis and *Paracoccidioides lutzii* are thermally dimorphic fungi and the causal agents of paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in Latin America. The infection occurs through the inhalation of conidia, which convert into yeasts in the lungs, causing benign and transient lesions. It may progress into an acute form or, more frequently, reactivate later as a chronic and insidious disease (Restrepo, 2000; de Almeida, 2005; Laniado-Laborin, 2007), which disseminates to many different organs and tissues, particularly the skin, oral cavity, pharynx, larynx, upper gastrointestinal tract, lymph nodes, adrenal glands, and central nervous system (Tuder et al., 1985; Do Valle et al., 1993; Almeida et al., 2003; de Almeida, 2005; Restrepo et al., 2008).

The PCM course depends on factors inherent to the fungus, such as its virulence and antigenic composition, as well as on environmental conditions and the host's immune state (Kurokawa et al., 2005). In this scenario, macrophages are essential in establishing the first barrier to the invading pathogens and in guiding the ensuing development of adaptive immunity (Hussell and Bell, 2014). Macrophages exhibit a high expression of pattern recognition receptors, especially Toll-like receptors (TLRs), whose interaction with agonists triggers cell activation. Macrophages can assume different types of activation depending on certain stimuli. "Classic" M1 macrophages are inflammatory cells that are involved in phagocytosis and killing of microbes, while "alternative" M2 cells favor angiogenesis, tissue remodeling, and repair (Murray and Wynn, 2011). The M1 and M2 subsets are discriminated by the production of nitric oxide (NO) and arginase activity, respectively, as well as by the expression of particular genes, such as iNOS2, STAT1, and SOCS3 for M1, and Arginase1, FIZZ1, YM1, STAT3, and SOCS1 for M2 (Lawrence and Natoli, 2011).

Our group has reported that *P. brasiliensis* yeast extracts contain an *N*-acetylglucosamine- and chitin-binding lectin called paracoccin (PCN) (Coltri et al., 2006). It exerts *N*-acetyl- β -D-glucosaminidase activity and is involved in fungal growth and morphogenesis (Ganiko et al., 2007; dos Reis Almeida et al., 2010; Dos Reis Almeida et al., 2011). More important for the biology of *P. brasiliensis*, PCN stimulates macrophages to produce high levels of NO and TNF- α (Coltri et al., 2006). Moreover, administration of a recombinant form of PCN expressed in *Escherichia coli* (herein named b-rPCN) confers protection against experimental PCM in a manner that depends on TLR2 and TLR4. This protection was primarily associated with the effects of b-rPCN on macrophages, triggered by its interaction with TLR *N*-glycans and culminating with the establishment of Th1 immunity (Alegre et al., 2014; Alegre-Maller et al., 2014).

Pichia pastoris (strain GS115) cells have been extensively used for the expression and large scale production of heterologous proteins (Mattanovich et al., 2012). In this study, we validated a recombinant form of PCN produced in *P. pastoris* (p-rPCN) to mimic the known features of the native protein and identified that the p-rPCN stimulus promotes M1 polarization of macrophages. We verified that this response depends heavily on the interaction between p-rPCN and TLR4.

MATERIALS AND METHODS

Mice and Ethics Statement

Male C57BL/6 (wild-type, WT), TLR2 knockout (TLR2^{-/-}), and TLR4 knockout (TLR4^{-/-}) mice of 6–8 weeks of age were used. They were acquired from the vivarium on the campus of the University of São Paulo at Ribeirão Preto, São Paulo, Brazil, and housed in the animal facility of the Molecular and Cellular Biology Department, Faculty of Medicine of Ribeirão Preto, University of São Paulo, under optimized hygienic conditions. Animal procedures were approved by the Ethical Committee for Ethics in Animal Research (CETEA) of the School of Medicine at

Ribeirão Preto, University of São Paulo, under protocol number 20/2013-1.

Cloning, Expression in *Pichia pastoris*, and Purification of Recombinant Paracoccin

The full-length prediction encoding ORF for paracoccin, cloned in vector pUC57 by Alegre et al. (2014), was used as a template for PCR with forward primer 5'-CTCGAGATGGCGTTTGAAAACCAGATTG-3' and reverse primer 5'-GCGGCCGCCAGCTGCTGGTGCTAAAGC-3', containing the restriction sites of the *XhoI* and *NotI* enzymes, respectively. The reaction was carried out in 30 cycles (30 s at 94°C, 30 s at 57°C, and 60 s at 72°C). The purified PCR product was cloned into the pGEM-T vector (Promega, Fitchburg, WI, USA), and the insert was removed from the vector with the aforementioned restriction enzymes and ligated into the pGAP α A vector (Invitrogen, Carlsbad, CA, USA). The pGAP α A-PCN vector was obtained and sequenced to determine the ligation success and the correct sequence of the insert. This vector was then linearized with the restriction enzyme *AvrII* so as to be used for the transformation of the *Pichia pastoris* GS115 strain, as described by Maleki et al. (2010). In short, 10 μ g of the purified (using Illustra kit plasmidPrep Mini Spin – GE Healthcare, Little Chalfont, UK) and linearized plasmid were electroporated into the yeast in 0.2 cm cuvettes at 1.5 kV (25 μ F and 200 Ω), using Gene Pulser (Bio-Rad, Hercules, CA, USA).

The transformants obtained on the selective YPD medium containing Zeocin (100 μ g/mL) were confirmed by PCR. A clone was grown in 300 mL YPD liquid medium at 30°C, 220 rpm, for 72 h, for paracoccin recombinant expression and secretion. The culture supernatant was collected, dialyzed against phosphate buffered saline (PBS; 10 mM, pH 7.2), and concentrated 10 times using centrifugal filtration devices with a 10,000-molecular weight cut-off (Millipore, Darmstadt, Germany).

For paracoccin purification, the prepared culture supernatant was centrifuged at 10000 $\times$ g, at 5°C, and applied to a chitin column, manufactured as described by dos Reis Almeida et al. (2010), and equilibrated with PBS. After rinsing with 20 column volumes of PBS, the material adsorbed to chitin was eluted with 0.1% acetic acid. The eluate was concentrated and dialyzed against PBS. The product obtained from chromatography was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and submitted to quality control by assessing its lectin and enzymatic activities. The final product was named p-rPCN.

Lectin Activity: Microplate Assay for p-rPCN Binding to Laminin

A high affinity polystyrene microplate (Corning Costar, Badhoevedorp, The Netherlands) was coated with laminin (250 ng/well) in carbonate-bicarbonate buffer (0.1 M; pH 9.6) overnight, at 4°C. After rinsing with PBS-Tween 20 (PBS-T) and blocking with PBS-T with 3% gelatin (blocking buffer) for 1 h at 37°C, rPCN (200 ng) or blocking buffer (negative control) was added to the wells in triplicates, and the microplates were

incubated at room temperature for 2 h. After rinsing with PBS-T, IgY anti-rPCN antibodies (diluted 1:250 in PBS-T containing 1% gelatin) were added to the wells and incubated for 1 h at 37°C. After rinsing, murine biotinylated IgG anti-IgY (diluted to 1:1000 in PBS-T containing 1% gelatin) was added and incubated for 1 h at 37°C. As the last step, streptavidin-peroxidase conjugate (1:250) was added to each well for 1 h at room temperature. Following rinsing, the reaction was revealed by using the tetramethylbenzidine substrate (Pierce, Thermo Fisher Scientific Inc., Waltham, MA, USA), and color development was detected at a wavelength of 450 nm in a microplate reader (Power Wave X – BioTek Instruments, Inc., Winooski, VT, USA).

Enzymatic Activity of p-rPCN

The *N*-acetyl- β -D-glucosaminidase activity of yeast recombinant paracoccin was assayed as previously described in detail (dos Reis Almeida et al., 2010; Almeida et al., 2015). The substrate *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (100 μ L, 5 mM; Sigma-Aldrich, St Louis, MO, USA) was mixed with 350 μ L of 0.1 M sodium acetate (pH 5.5) and 5 μ g of p-rPCN. As a negative control, we used the vehicle medium. The reaction was incubated for 16–18 h at 37°C, with 1 mL of 0.5 M sodium carbonate added. The enzyme activity values were determined by using a microplate reader at 405 nm (Power Wave X, BioTek Instruments, Inc.).

Culture of Murine Peritoneal Macrophages: Stimulation with p-rPCN

Murine peritoneal macrophages were obtained from WT, TLR2^{-/-}, and TLR4^{-/-} mice after an intraperitoneal injection with 1.0 mL of sterile 3% sodium thioglycollate (Sigma-Aldrich). After 3 days, the peritoneal cavity was washed with 5.0 mL of cold sterile PBS and the cells were harvested and centrifuged at 300 $\times$ g for 10 min. The cell pellet was resuspended in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific Inc.), and the cell counting was performed in a Neubauer chamber. The cells were plated in 48-well culture plates (1 $\times$ 10⁶ cells/mL) and incubated overnight at 37°C in a 5% CO₂ atmosphere. The non-adherent cells were removed by gentle rinsing with PBS, while the adherent macrophages were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Elicited peritoneal macrophages were stimulated with p-rPCN (5.0 μ g/mL). The positive controls were LPS (1 μ g/mL) with IFN- γ (1 ng/mL) added for TLR2^{-/-} macrophages and Pam3CSK4 (100 ng/mL) for TLR4^{-/-} macrophages. The medium alone was used as a negative control. After variable incubation periods (specified in the legends), the culture supernatants were harvested and assessed for the concentration of mediators.

Nitric Oxide Measurement

Nitric oxide concentration was inferred by measuring nitrite levels in the macrophage supernatants by using the Griess reagent system (Green et al., 1982). In short, 50 μ L of

the supernatants were distributed in 96-well microplates and incubated with 50 μ L/well of Griess reagent (1.0% sulfanilamide, 0.1% naphthalenediamine dihydrochloride, and 2.5% H₃PO₄) at room temperature for 10 min. The absorbance at 550 nm was read using a Power Wave-X microplate reader (BioTek Instruments, Inc.). The absorbance was converted to micromolar (μ M) NO on the basis of a standard curve, concomitantly generated by using known concentrations of NaNO₂.

Cytokine Measurement

Supernatants of stimulated macrophages were assessed for their levels of IL-12p40, IL-6, and TNF- α . The cytokines were detected by an enzyme-linked immunosorbent assay (ELISA) using an OptEIA kit (Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions. Standard curves allowed determining cytokine concentrations in pg/mL. The absorbance was read at 450 nm in the Power Wave-X microplate scanning spectrophotometer (BioTek Instruments, Inc.).

Quantitative Reverse Transcription PCR

Macrophages (1 $\times$ 10⁶ cells/mL) from WT, TLR2^{-/-}, and TLR4^{-/-} mice were incubated with rPCN (5 μ g/mL), LPS (1 μ g/mL), Pam3CSK4 (100 ng/mL), IFN- γ (1 ng/mL) plus IL-12p40 (50 ng/mL) (M1 macrophage inducers), IL-10 plus IL-4 (50 ng/mL both) (M2 macrophage inducers), or with medium alone, for 6 h. RNA from macrophages was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The total RNA was reverse-transcribed into cDNA by the ImProm-II Reverse Transcription System (Promega) using oligo(dT). Quantitative real-time PCR was performed using SYBR Green (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) on a 7500 Real-Time PCR System (Applied Biosystems). The relative expression of transcripts was quantified using the $\Delta\Delta$ Ct method, and β -actin was used as endogenous control. The PCR primers used were: β -actin (S-CCTAAGGCCAACCCTGAAAA; AS-GAGGCATACAGGGACAGCACA), Ym1 (S-TCACAGGTCTGGCAATTCTCTCTG; AS-ACTCCCTTCTATTGGCCTGTCC), Arginase-1 (S-GTTC CCAGATGTACCAGGATTC; AS-CGATGTCTTTGGCAGATA TGC), FIZZ1 (S-CCTGAGATTCTGCCCCAGGAT; AS-TTCAC TGGGACCATCAGCTGG), iNOS2 (S-CCGAAGCAAACATCA CATTC; AS-GGTCTAAAGGCTCCGGGCT), STAT1 (S-CAC GCTGCCTATGATGTC; AS-CCTGGAGATTACGCTTGC), ST AT3 (S-GGCACCTTGGATTGAGAG; AS-TGCTGATAGAGG ACATTGG), SOCS1 (S-AGGATGGTAGCACGCAAC; AS-GA AGACGAGGACGAGGAG), and SOCS3 (S-AGGAGAGCGG ATTCTACTG; AS-TCACACTGGATGCGTAGG).

Statistical Analysis

The results are expressed as mean $\pm$ SEM. The statistical analysis was done using the *GraphPad Prism* software (GraphPad Software, San Diego, CA, USA). The homogeneous variance was analyzed, and the difference between means of groups was calculated by the analysis of variance (one-way) and Bonferroni's test thereafter. Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Production of Recombinant Paracoccin in *Pichia pastoris*

Paracoccin purified from *Paracoccidioides brasiliensis* has both lectin and enzymatic properties, which are mimicked by a recombinant form of the protein, expressed in *Escherichia coli* (b-rPCN) (Alegre et al., 2014). To be stricter concerning the required absence of LPS in our preparations, we expressed paracoccin in the *P. pastoris* strain GS115, transformed with the pGAPzαA vector and containing the synthetic transcript of this protein. The secreted protein was purified in a chitin column and called p-rPCN. The electrophoretic profile of the bound fraction showed that it consists of a single 27 kDa band (Figure 1A), which is the expected molecular mass for paracoccin. Our recombinant PCN exhibited the properties of the native protein, which was evident from several facts. First, the fact that its purification was founded on binding to chitin showed that p-rPCN retains the carbohydrate binding capacity of the native protein. Second, it could be detected by binding to laminin (Figure 1B), a property that depends on the lectin domain of paracoccin, as previously reported for PCN and b-rPCN. Finally, using the substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, we demonstrated that p-rPCN exhibits *N*-acetyl-β-D-glucosaminidase activity (Figure 1C). These results validated the use of p-rPCN in the present study aiming to detail the paracoccin effects exerted on macrophages.

Recombinant PCN Induces Macrophages to Produce Proinflammatory Mediators

We have previously demonstrated that native PCN and b-rPCN induce mouse peritoneal macrophages to release high levels of TNF-α and NO (Coltri et al., 2006; Alegre et al., 2014). Then, we examined whether p-rPCN could also stimulate mouse peritoneal macrophages to release inflammatory mediators. Indeed, p-rPCN induced the release of significant levels of NO, IL-12p40, and TNF-α in macrophages (Figures 2A–C, respectively) at 24, 48, and 72 h after the stimulus. The time-course production of IL-12 (Figure 2B) and TNF-α (Figure 2C) was close to that determined by the positive control and consisted of a mixture of LPS and IFN-γ. These findings demonstrate that p-rPCN induces macrophages to release proinflammatory cytokines.

Paracoccin Induces M1 Macrophage Polarization

To advance in the study of the effects of paracoccin on macrophage activation, using real-time PCR, we quantified the expression of M1 (iNOS2) and M2 (Arginase-1, Ym-1, and FIZZ1) polarization markers in response to p-rPCN. Peritoneal macrophages stimulated with p-rPCN showed a more than 200-fold increase in iNOS mRNA, attaining levels that were similar to those in macrophages stimulated by IFN-γ plus IL-12 (Figure 3A). The analysis of M2 markers revealed

that the p-rPCN stimulus did not change Arginase-1, Ym-1, and FIZZ1 expression, whose levels remained close to those found in unstimulated cells and were significantly lower than those measured by the IL-10 plus IL-4 control stimulus (Figures 3B–D). In addition, the expression measurement of key signaling mediators associated with M1/M2 dichotomy showed that the levels of STAT1 and SOCS3 mRNA increased by 10- and 380-fold, respectively, in response to the p-rPCN stimulus (Figures 3E,F). On the other hand, this stimulus did not affect the expression of STAT3 and SOCS1 (Figures 3G,H). Furthermore, the ratio of the expression of SOCS3 and SOCS1 increased 15-fold in response to p-rPCN (Figure 3I). These results indicate that p-rPCN induces the classical activation of macrophages, with the involvement of STAT1 and SOCS3.

TLR4 Is Crucial for the Macrophage Release of Proinflammatory Cytokines Induced by Paracoccin

Given that recombinant paracoccin expressed in *E. coli* interacts with the *N*-glycans of TLR2 and TLR4 on antigen-presenting cells and promotes IL-12 production (Alegre-Maller et al., 2014), we examined the relevance of TLR2 and TLR4 in the production of inflammatory mediators induced by p-rPCN. We stimulated peritoneal macrophages obtained from WT, TLR2^{−/−}, or TLR4^{−/−} mice with p-rPCN for 48 h and quantified the levels of IL-12p40, TNF-α, and IL-6 in the cell supernatants. We found that the lack of TLR2 diminished the IL-12p40 production induced by p-rPCN compared to that of WT macrophages, while the TNF-α and IL-6 levels were unaffected. The absence of TLR4, in turn, abolished the p-rPCN-induced production of IL-12p40 and TNF-α (Figures 4A,B) and severely impaired the IL-6 release (Figure 4C). Interestingly, the cytokine production induced by the control agonists of either receptor (Pam3CSK4 for TLR2 and LPS plus IFN-γ for TLR4) was inhibited by the absence of the respective TLR, but less affected than the production promoted by p-rPCN. We conclude that TLR2 and TLR4 participate in the induction of proinflammatory cytokine production in macrophages stimulated by paracoccin; a more notable role is attributed to TLR4.

TLR4 Is Crucial for the Classical Polarization of Macrophages Induced by Paracoccin

To expand the investigation of the roles of TLR2 and TLR4 on the effects of paracoccin on macrophages, we analyzed whether the M1 polarization of these cells promoted by p-rPCN could be affected by the absence of TLR2 or TLR4. Following the stimulation of peritoneal macrophages harvested from WT, TLR2^{−/−}, or TLR4^{−/−} mice, the cells were assessed for the expression of iNOS, Arginase-1, Ym-1, and FIZZ1. The iNOS expression stimulated by p-rPCN was not affected by the absence of TLR2, but was blocked in TLR4^{−/−} macrophages (Figure 5A). The expression of M2 polarization markers, which did not respond to the p-rPCN

FIGURE 1 | Purification and biological activities of a recombinant form of paracoccin produced using the *Pichia pastoris* expression system.

(A) SDS-PAGE of the fraction of the culture supernatant that was adsorbed to chitin and eluted in acidic buffer; shows a 27 kDa silver stained band, as determined on the basis of the migration index of MM markers (lane MW). (B) Binding of the purified recombinant paracoccin (named p-rPCN, 200 ng) to the laminin coated wells (250 ng) of a microplate. The binding was revealed by reaction with anti-paracoccin IgY. (C) N-acetyl-glucosaminidase activity exerted by the p-rPCN as revealed using the substrate *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (100 μL 5 mM *p*-NPGlcNAc). The results are expressed as mean ± SEM and were compared to the medium through one-way analysis of variance, followed by Bonferroni's test. ****p* < 0.001 and **p* < 0.05.

FIGURE 2 | p-rPCN induces macrophages to release proinflammatory mediators. Peritoneal macrophages (1×10^6 cells/mL) from WT mice were stimulated with p-rPCN (5 μg/mL) for 24, 48, and 72 h. The culture supernatants were assessed for nitrite concentration (μM), measured by the standard Griess reaction (A), IL-12p40 (B) and TNF-α (C) levels (pg/mL) using ELISA. The medium and LPS (1 μg/mL) plus IFN-γ (1 ng/mL) were used as negative and positive controls, respectively. The results are expressed as mean ± SEM and were compared to the medium through one-way analysis of variance, followed by Bonferroni's test. ****p* < 0.001 and ***p* < 0.01.

stimulus, remained in cells from TLR2^{-/-} or TLR4^{-/-} mice at levels as low as those verified in non-stimulated macrophages (medium) (Figures 5B–D). Therefore, we conclude that TLR4 is critical for the paracoccin-induced M1 polarization of macrophages.

DISCUSSION

Considering the previous demonstration that the interaction of paracoccin with TLR2 and TLR4 on macrophages modulates immunity and positively interferes in the PCM course (Alegre

et al., 2014; Alegre-Maller et al., 2014), herein we detailed additional events triggered by paracoccin in those cells. By using a recombinant form expressed in *P. pastoris* (p-rPCN), we showed that paracoccin induces M1 polarization of macrophages, as well

as the production of inflammatory mediators. Notably, TLR4 was critical in the establishment of these responses, suggesting that their triggering was due to the interaction of paracoccin with TLR4 N-glycans, as was previously reported to happen with TLR2 (Alegre-Maller et al., 2014). We postulate that the events reported herein account for the protective effect of paracoccin administration against *P. brasiliensis* infection.

The expression of paracoccin in *Pichia pastoris* was advantageous compared to its expression in *E. coli*, which was utilized in our previous studies. The expression in *P. pastoris* allowed us to avoid several exhaustive chromatographic procedures used to remove bacterial endotoxins during the recombinant protein purification. In addition, it provided substantially higher final yields of homogeneous recombinant paracoccin. Besides, the potential prejudice in protein activity, usually resulting from the hyperglycosylation of proteins expressed in *Pichia pastoris*, did not occur, as demonstrated by the detection of preserved lectin and enzymatic activities and by the fact that there was no shift in the MM of the protein in the SDS-PAGE analysis (Macauley-Patrick et al., 2005; Mattanovich et al., 2012).

Once validated as able to mimic the effects of previously used preparations, p-rPCN was used to investigate additional events implicated in the effects of paracoccin on macrophage activation. As a manifestation of this phenomenon, we found that macrophages produced high levels of inflammatory mediators in response to the p-rPCN stimulus, just as they did in response to the native protein (Coltri et al., 2006). This is an essential activity, considering that we envisage paracoccin to become available for pharmaceutical application as an immunomodulatory agent capable of conferring protection against deep fungal diseases by favoring the development of a balanced Th1 immunity (Alegre et al., 2014; Alegre-Maller et al., 2014). Indeed, the immunomodulatory activity triggered by pathogen lectins is widely studied, and we found strong evidence that *Toxoplasma gondii* microneme proteins, which exhibit lectin properties, confer protection against experimental toxoplasmosis (Pinzan et al., 2015).

We previously verified that the mechanisms of protection conferred by paracoccin against murine PCM require the participation of innate immunity cells (Alegre-Maller et al., 2014). Macrophages and dendritic cells are responsible for the initial response in *P. brasiliensis* infection, and macrophage polarization is known to differ between resistant and susceptible mice infected with the fungus (Calich et al., 2008; Pina et al., 2008; Feriotti et al., 2013). We then investigated the ability of p-rPCN to induce macrophage polarization. The relative expression of M1 and M2 markers showed that p-rPCN stimulated an increase in the expression of iNOS, STAT1, and SOCS3 mRNA, indicating the occurrence of M1 polarization. The activation of STAT1 and STAT3 finely regulates macrophage polarization and activities, and the NF- κ B and STAT1 activation induces M1 macrophage polarization (Sica and Mantovani, 2012). The occurrence of M1 polarization is consistent with the augmented production of proinflammatory cytokines by the p-rPCN-stimulated macrophages. It was already demonstrated

FIGURE 4 | Involvement of TLR2 and TLR4 in the proinflammatory cytokine production induced by paracoccin. Macrophages (1×10^6 cells/mL) obtained from WT, TLR2^{-/-}, and TLR4^{-/-} mice were stimulated for 24 h by p-rPCN (5 μ g/mL). A mixture of LPS (1 μ g/mL) and IFN- γ (1 ng/mL) served as the positive control for WT and TLR2^{-/-} macrophages; Pam3CSK4 (100 ng/mL) was used as the positive control for TLR4^{-/-} macrophages. The culture supernatants were assessed for the levels of IL-12p40 (**A**), TNF- α (**B**), and IL-6 (**C**) using ELISA. The values (pg/mL) were expressed in mean $\pm$ SEM and compared to the negative control (the medium) through one-way analysis of variance, followed by Bonferroni's test. *** $p < 0.001$, ** $p < 0.01$, and non-significant differences (n.s.).

FIGURE 5 | Involvement of TLR4 in the classical activation of macrophages by paracoccin. Macrophages (1×10^6 cells/mL) from WT, TLR2^{-/-}, and TLR4^{-/-} were stimulated for 6 h with p-rPCN (5 μ g/mL). As a positive control for the classical activation, the mixture of IFN- γ (1 ng/mL) and IL-12p40 (50 ng/mL) was used. For alternative activation, a mixture of IL-10 plus IL-4 (50 ng/mL both) was used. The medium alone was used as the negative control. After extraction using TRIzol Reagent®, the RNA was converted into cDNA and the expression of iNOS (**A**), Arginase-1 (**B**), Ym-1 (**C**), and FIZZ1 (**D**) were analyzed by real-time PCR. The relative expressions were determined as described in Section "Materials and Methods" and compared between WT, TLR2^{-/-} and TLR4^{-/-} macrophages. The results are expressed in mean $\pm$ SEM and were compared through one-way analysis of variance, followed by Bonferroni's test. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, and non-significant differences (n.s.).

that M1 polarization favors the innate mechanism of *P. brasiliensis* elimination (Pina et al., 2008; Feriotti et al., 2013), as well as contributing to fungal clearance during experimental infection with *Cryptococcus neoformans* (Hardison et al., 2010; Davis et al., 2013). On the other hand, M2 polarization is related to fungal dissemination. Therefore, we suggest that, in addition to the previously reported effects of paracoccin administration to infected hosts, these include M1 polarization, which contributes to the verified PCM control. This scenario goes in favor of the idea that paracoccin can be applied as an immunotherapeutic agent against PCM. The mechanism behind the mentioned events typically involves TLRs and their ligands. We found that TLR2 participates in cytokine production induced by p-rPCN on macrophages and that TLR4 is required not only to induce the release of proinflammatory cytokines, but also to promote the M1 polarization of macrophages in response to p-rPCN. The interaction of paracoccin with TLR2 and TLR4 N-glycans has already been shown to account for the activation of macrophages and for *P. brasiliensis* elimination. However, there are few studies on the effects of TLR2 and TLR4 activation during the course of this fungal infection. TLR2-deficient mice infected with *P. brasiliensis* showed increased Th17 immunity, reduced regulatory T cells, and unrestrained inflammatory pulmonary reactions (Loures et al., 2009). On the other hand, TLR4-defective mice infected with *P. brasiliensis* exhibited reduced fungal burdens and lower levels of inflammatory mediators compared to TLR4-competent mice (Loures et al., 2010). This observation partially supports our data regarding the critical role of TLR4 in the response to the p-rPCN stimulus.

The ability of b-rPCN to interact with TLR2 and TLR4 on APCs and the therapeutic effects triggered by such interactions on the course of experimental PCM were already established (Alegre et al., 2014; Alegre-Maller et al., 2014). Our current results

reinforce the idea that paracoccin administration can be useful in the therapy of PCM and demonstrate that TLR4 is the main receptor responsible for the biological activities of macrophages triggered by p-rPCN. Therefore, paracoccin can be considered a novel TLR agonist that modulates the host immune response through carbohydrate recognition.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MF, AO, TS, FF, RG, FA, MR-B. Performed the experiments: MF, AO, TS, FF. Analyzed the data: MF, AO, TS, FF. Wrote the paper: MF, AO, TS, FF, FA, MR-B. All authors have read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Considerações sobre o capítulo I

Os resultados apresentados no capítulo I (*Paracoccin induces M1 polarization of macrophages via interaction with TLR4*) corroboram para o atendimento ao primeiro objetivo desta tese, qual seja, o de “Investigar o efeito da paracoccina sobre células da imunidade inata, buscando identificar mecanismos que contribuam para a imunomodulação exercida pela proteína fúngica”, e ao sub-item “Determinar o efeito da paracoccina sobre a polarização de macrófagos murinos”. Destacamos os seguintes aspectos do trabalho: i) PCN recombinante produzida em *P. pastoris* reproduz as funções lectínica e enzimática da lectina nativa e induz macrófagos peritoneais murinos a produzirem mediadores pró-inflamatórios (NO, TNF- α , IL-12 e IL-6); ii) a PCN recombinante induz a polarização M1 de macrófagos, envolvendo os fatores de transcrição STAT1 (*signal transducer and activator of transcription 1*) e SOCS3 (*suppressor of cytokine signaling 3*); e iii) o receptor TLR4 é crucial para a liberação de mediadores pró-inflamatórios induzidos por macrófagos estimulados com PCN recombinante, sendo esse receptor importante para a indução de perfil M1 de macrófagos.

Em suma, PCN pode ser considerada como um novo agonista de TLR, por modular a resposta imune do hospedeiro através do reconhecimento de carboidrato.

2. Capítulo II

Diz respeito a artigo publicado com as especificações que se seguem.

Título: Human neutrophils are targets to paracoccin, a lectin expressed by *Paracoccidioides brasiliensis*.

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Human neutrophils are targets to paracoccin, a lectin expressed by *Paracoccidioides brasiliensis*

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Abstract

Objective and design Paracoccin (PCN), a lectin expressed by *Paracoccidioides brasiliensis* (*Pb*), is known to exert activities on the fungal biology, as well as different immune cells of myeloid origin. The aim of this study was to investigate the direct interaction of the recombinant form of the lectin (rPCN) with neutrophils, a neglected area.

Materials or subjects Freshly isolated human neutrophils from healthy donors were used.

Treatment Neutrophils were incubated with rPCN in vitro.

Methods After the treatment, the production of reactive oxygen species (ROS), DNA release, IL-8, TNF, IFN- γ , IL-10, IL-12p40, TGF- β and IL-1 β production, fungicidal ability, apoptosis and de novo protein synthesis was determined.

Results rPCN was found to induce ROS production as well as DNA release. Using the ROS inhibitor, diphenyleneiodium, both ROS production and DNA release were significantly inhibited. In addition, rPCN was found to induce IL-8 and IL-1 β production, inhibit apoptosis and induce de novo protein synthesis. Addition of cycloheximide, a protein synthesis inhibitor, drastically reversed the antiapoptotic effect of rPCN. Finally, the ability to kill *Pb*

yeasts by human neutrophils was significantly increased after rPCN stimulation.

Conclusions rPCN can alter the biology of human neutrophils increasing their fungicidal ability. Moreover, the ability of rPCN to increase DNA release and to induce suppression of neutrophil apoptosis occurs by a ROS- and de novo protein synthesis-dependent mechanism, respectively.

Keywords Paracoccin · Immunomodulation · Neutrophils · Reactive oxygen species · Apoptosis

Introduction

Neutrophils are particularly known for their roles in innate immunity. However, in addition to their participation in the initial steps of infections, they are also able to communicate with a variety of other cells directly or indirectly via paracrine effects by releasing cytokines, enzymes, DNA, and so on [1–4]. The activation of neutrophils is essential to eliminate microorganisms properly, but deregulation of their activation state can be harmful for the host, promoting tissue lesions. In this respect, modulation of neutrophil functions is certainly a suitable approach to control human diseases.

Some lectins, proteins known to bind sugars selectively, are able to modulate neutrophil functions. For example, plant lectins such as concanavalin-A (Con-A) can attract neutrophils in vivo [5], wheat germ agglutinin (WGA), and phytohemagglutinin (PHA) trigger respiratory burst and H₂O₂ production in neutrophils [6] and *Viscum album* agglutinin-1 (VAA-I) is a very potent inducer of neutrophil apoptosis and is known to act by inhibiting protein synthesis [7, 8]. Mammalian lectins, such as selectins and

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galectins also exert biological activities on neutrophils [9, 10]. For example, galectin-3 was found to induce L-selectin shedding and interleukin-8 production in naïve and primed neutrophils. L-Selectin, in its turn, through its lectin domain, binds to glycoconjugates expressed on endothelium, thus favoring leucocyte migration into tissues [11]. The potential of lectins has been widely explored once they can bind selectively to glycans anchored on the cell surface, triggering on them remarkable responses [12, 13].

Our research group has been working with the immunomodulatory potential of lectins from different sources. ArtinM, isolated from jackfruit seeds, which binds D-mannose, is able to induce IL-12 and IFN- γ secretion by interacting with N-glycans attached to TLR2 into immune cells, such as macrophages and dendritic cells [14, 15]. As result, ArtinM induces a T helper 1 (Th1) immunity, which is favorable to eliminate intracellular pathogens. On neutrophils, it was already shown that the ArtinM-stimulation induces the increased secretion of interleukin-8 (IL-8), IL-1 β , tumor necrosis factor- α (TNF- α), leukotriene B4 (LTB-4), and degranulate elastase (ELA) and myeloperoxidase (MPO) [16, 17]. Ultimately, the ArtinM neutrophil stimulation combines these inflammatory mediators with a phagocytosis increase, and augmented microbicide ability.

Beyond ArtinM, our research group has been working with paracoccin (PCN), a lectin expressed by *Paracoccidioides brasiliensis* (Pb), which binds to N-acetyl glucosamine and chitin [18, 19]. Our efforts became possible to show that PCN, like ArtinM, induces a Th1 immunity. The prophylactic or therapeutic use of the recombinant form of PCN (rPCN) is capable of protecting mice against lethal *P. brasiliensis* infection's challenge [20]. PCN, or rPCN, has been shown to activate macrophages, in a TLR2- and TLR4-dependent way, to produce inflammatory mediators such as IL-12 and TNF- α [18]. In addition to these inflammatory mediators, rPCN also increases IL-10 secretion, a mechanism that we believe could account for the occurrence of an equilibrated Th1 response. To date, there are no data regarding the effects of rPCN on the biology of human neutrophils. Because this type of stimulation, mediated by sugar recognition, presents potential to modulate neutrophil functions, it is greatly important to investigate how rPCN could alter neutrophil functions as reported in this present study.

Materials and methods

Chemicals

Arsenic trioxide (As₂O₃ or ATO), phorbol 12-myristate 13-acetate (PMA), cycloheximide (CHX), the plant lectin *Viscum album* agglutinin-I (VAA-I), the MAPK/ERK

inhibitor PD98059, p38 MAPK inhibitor SB203580, p38 inhibitor CAS 219138-24-6-Calbiochem, and JNK II inhibitor (CAS 129-56-6-Calbiochem) and ROS inhibitor diphenyleneiodonium chloride (DPI) were purchased from Sigma-Aldrich (St-Louis, MO). RPMI-1640, HEPES, penicillin/streptomycin, heat-inactivated fetal bovine serum (FBS), phosphate-buffered saline (PBS) and Hank's balance salt solution (HBSS) were purchased from Life technologies (Grand Island, NY). Recombinant human GM-CSF was purchased from Peprotech Inc. (Rocky Hill, NJ, USA). Ultra-pure lipopolysaccharides (Ultra-pure LPS-EK, from *E. coli* K12) were obtained from InvivoGen (San Diego, CA). The PI3K/Akt inhibitor wortmannin was purchased from EMD Bioscience (San Diego, CA).

Production and purification of recombinant paracoccin (rPCN)

rPCN was produced exactly as previously described [18]. Briefly, the filtered culture supernatant of grown transformed *Pichia pastoris* (GS115 strain) encoding rPCN was dialyzed and concentrated (in PBS; 10 mM, pH 7.2) using centrifugal filtration devices (10,000 m.w. cut-off—Millipore, Darmstadt, Germany) and then applied to a chitin column, manufactured as described previously [21]. After several rinsing steps, the material absorbed into the chitin column was eluted using 0.1% acetic acid. The eluate was dialyzed and concentrated in PBS, and finally sterilized by passing through 0.22- μ m filter. Purity and quality control of rPCN were accessed, respectively, by SDS-PAGE analysis and lectin/enzymatic activity.

Neutrophil isolation

Neutrophils were isolated from venous blood of healthy consenting individuals according to institutionally approved procedures as previously described [22–25]. Briefly, a dextran sedimentation step was performed to remove most of the red blood cells from samples, which were finally added over Ficoll–Hypaque gradient to isolate polymorphonuclear leukocytes by centrifugation. The cellular pellet containing neutrophils was submitted to a quick osmotic shock to remove the residual erythrocytes. Cell viability was monitored by trypan blue exclusion and never exceeded 3% and the purity was > 98% and was verified by cytology from cytocentrifuged preparations colored Hema-3 stain set (Biochemical sciences Inc., Swedesboro, NJ).

ELISA

The cytokine levels were quantified in the supernatants of neutrophils stimulated or not with rPCN or LPS and the

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production of TNF- α , IFN- γ , IL-10, IL-12p40 and TGF- β (BD—Biosciences, USA or R&D Systems, USA) were measured by ELISA according to the manufacturer's instructions.

ROS production

Neutrophils' respiratory burst was tested with 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA, Molecular Probes, Eugene, OR), a cell-permeable indicator for reactive oxygen species (ROS), following the manufacturer's instruction. Briefly, resting or inhibited neutrophils were pre-incubated in HBSS containing H₂DCFDA (10 μ M) for 15 min at 37 °C. The reaction was initiated after stimulating neutrophils with rPCN, PMA (positive control), and unstimulated cells were used as negative control. The H₂DCFDA-enhanced fluorescence was measured in a microplate fluorescence reader (Spectramax M5, Molecular Devices, Sunnyvale, CA) and units of fluorescence were read each 5 min for 20–120 min, at 37 °C. Results were expressed as AUC, the area under the time-course curve, which was used to determine the total amount of measured ROS. In some experiments, cells were pretreated 30 min with the ROS inhibitor DPI (10 μ M).

DNA release measurement

Neutrophils (2×10^5 cells/well in HBSS) were incubated with buffer (Ctrl), 60 nM PMA or the indicated concentrations of rPCN in a 96-well plate for 2 h at 37 °C, in 5% CO₂. DNA was quantified as previously published [17]. In brief, bacterial endonuclease *EcoRI* solution (200 unit/mL) was added (10 μ L/well) for 30 min to partially digest any released neutrophil extracellular traps [26]. The cells and debris were then pelleted (600 $\times$ g for 10 min) and a sample of the supernatant was added to SYTOX green nucleic acid stain (Invitrogen; 1 μ M final concentration) (10 μ L sytox solution + 50 μ L of supernatant) in a black 96-well plate and incubated at 37 °C for 10 min. DNA fragments were quantified using a FLx800 Fluorescence Microplate Reader (BioTek Instruments, USA; excitation 485 nm, emission 528 nm) and the results have been expressed as 'n fold control'. In some experiments, neutrophils were pretreated 30 min with the indicated pharmacological inhibitors prior to the measurements of DNA release.

Assessment of neutrophil apoptosis

Neutrophils (10×10^6 cells/ml in RPMI 1640-HEPES-P/S, supplemented with 10% heat-inactivated autologous serum) were treated with buffer (spontaneous apoptosis), the antiapoptotic cytokine GM-CSF, the proapoptotic plant lectin VAA-I, or the indicated concentrations of rPCN for

24 h. Cytoentrifuged samples of neutrophils were prepared, stained with the Hema-3 stain staining kit according to the manufacturer's instructions and processed as documented previously [24]. In some experiments, neutrophils were treated with buffer (Ctrl), CHX (10 μ g/ml) or with rPCN + CHX to evaluate the importance of de novo protein synthesis during apoptosis. In some experiments, apoptosis was confirmed by flow cytometry. For this procedure, neutrophils were stained with FITC Annexin-V and PI. Ten thousand cells were analyzed by FACScan (Becton–Dickinson, San Jose, CA) using CellQuest program (BD Biosciences, San Jose, CA, USA).

Metabolic labeling and de novo protein synthesis assay

Cells (10×10^6 cells/ml in RPMI-1640 medium supplemented with 10% autologous serum) were metabolically labeled with 4.625 MBq of the Redivue Pro-Mix L-[³⁵S] in vitro cell labeling mix (GE Healthcare, Baie d'Urfé, QC, Canada) in the presence of buffer (Ctrl), GM-CSF, ATO, the indicated concentrations of rPCN with the presence of 5 μ g/ml cycloheximide (CHX) or buffer for 24 h, as previously described [24].

Neutrophil fungicidal ability assay

Paracoccidioides brasiliensis yeasts were maintained at 36 °C, and periodically sub-cultured, in brain–heart infusion (BHI) medium (Duchefa, The Netherlands), supplemented with 1% glucose and 1.5% v/v agar. For assaying the neutrophil fungicidal activity, yeast cells grew in liquid BHI broth at 36 °C on a mechanical shaker at 200 rpm. We only assayed preparations of yeasts with viability higher than 90%, as detected by fluorescein diacetate and ethidium bromide staining [27]. Then, human neutrophils (1×10^5 –250 μ L) were incubated with buffer (Ctrl) or rPCN (2500 ng/mL) at 37 °C for 30 min in a mechanical shaker at 50 rpm. Subsequently, 1×10^4 *Pb* yeast cells (250 μ L) were added to the cultured neutrophils and incubated for 15 min at 37 °C in a mechanical shaker at 50 rpm. The cells were washed three times to remove unbound yeasts. Some samples were reincubated for additional 3 h. After centrifugation, the obtained pellets were resuspended and neutrophils were lysed in sterile water (100 μ L). The resultant materials were plated in agar-BHI and incubated at 37 °C for 1 week. Colony forming units (CFU) were counted and the results for the first 15-min assay were expressed as the “number of ingested yeasts”. For the 3-h assay, results were expressed as “survivors' yeasts number”. Fungicidal ability was determined as “Fungicidal capacity” = (“number of ingested yeasts” – “survivors' yeasts number”).

Statistical analyses

Statistical analyses were performed by Student's *t* test and one-way ANOVA followed by Dunnett's multiple comparison test to detect the differences between groups, using the with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was established at $p < 0.05$, and each experiment was performed and validated at least three times.

Results

rPCN increases ROS production in human neutrophils

Figure 1 illustrates that rPCN increases ROS production in human neutrophils. Indeed, the first set of experiments demonstrates that rPCN increased ROS production in a concentration-dependent manner after 2 h of treatment. The total amount of produced ROS was measured after each 5 min and cumulated for 2 h (area under the curve) as illustrated in Fig. 1a. Of note, the PMA values were high (48023 ± 446.7) and, for clarity, were not included in this graph. Next, we performed experiments to determine the effect of rPCN after 20 min of stimulation and used a pharmacological approach to inhibit rPCN-induced ROS production. As illustrated in Fig. 1b, c, the ability of rPCN to increase ROS production in human neutrophils after 20 min was markedly reversed ($\sim 50\%$ less ROS) with the ROS inhibitor PDI.

rPCN induces DNA release involving ROS

Knowing that rPCN rapidly activates human neutrophils by inducing ROS production, we next determined whether it will also induce DNA release after few hours of treatment. As illustrated in Fig. 2a, rPCN was found to significantly increase DNA release in the supernatant of human neutrophils at levels similar to those obtained in response to the potent inducer PMA, used here as a positive control [17]. Because ROS are known to be involved in NET-DNA release [28], we next used again the ROS inhibitor DPI [29]. While DPI itself did not alter the basal levels of DNA released, it was found to drastically inhibit both rPCN- and PMA-induced DNA release (Fig. 2b). We also studied the possibility that different cell signaling pathways could be involved in NET-DNA release. However, as illustrated in Fig. 2c, pre-treatment with the tested inhibitors indicates that rPCN induces NET-DNA release by a mechanism that is PI3 K/AKT-, p38-, Erk-1/2- and JNK-independent.

rPCN induces IL-8 production and delays apoptosis in human neutrophils

According to the above results, it is clear that short treatments with rPCN can alter the biology of human neutrophils. Therefore, we next determined whether or not rPCN alters neutrophil functions requiring a longer period of time of stimulation such as cytokine production and apoptosis. As illustrated in Fig. 3a, after 24 h of treatment with rPCN, the basal level of IL-8 released into the supernatants increased from 59.9 ± 4.9 pg/ml (mean $\pm$ SEM, $n = 3$) to 565.0 ± 8.6 pg/ml. In these experiments, LPS was used as a positive control, and the concentration of IL-8 detected was 552.9 ± 8.0 pg/ml. Treatment of neutrophils with rPCN for 24 h was also found to significantly suppress spontaneous apoptosis (Fig. 3b). Indeed, the basal level of apoptosis was $65.8 \pm 3.3\%$ (mean $\pm$ SEM, $n = 10$) and decreased to $19.7 \pm 2\%$ after treatment with rPCN, a level that was even lower than that obtained with the well-known antiapoptotic cytokine GM-CSF where the apoptotic rate was $24.3 \pm 3.6\%$. Figure 3c illustrates representative images obtained after the flow cytometry procedure where the apoptotic rate was again decreased by rPCN treatment vs Ctrl cells (arrows). Collectively, these data indicate that rPCN is a potent antiapoptotic agent.

rPCN inhibits neutrophil apoptosis by a de novo protein synthesis-dependent mechanism

Several agents that are known to modulate spontaneous apoptosis in neutrophils can also alter de novo protein synthesis in these cells [24, 25]. Therefore, we next determined whether or not rPCN modulates upward or downward this important biological process. As illustrated in Fig. 4, rPCN was found to increase de novo protein synthesis in human neutrophils. In these experiments, cycloheximide was used to reverse the capacity of rPCN to increase de novo protein synthesis as did the antiapoptotic cytokine GM-CSF and the proapoptotic anticancer drug ATO. Next, we demonstrated the importance of de novo protein synthesis in rPCN-induced suppression of neutrophil apoptosis. Indeed, pre-treatment of cells with cycloheximide was found to markedly reverse the anti-apoptotic effect of rPCN without altering the basal level of spontaneous apoptosis (Fig. 5).

rPCN modulates cytokine production by human neutrophils

Neutrophils are currently known as cells that produce a wide range of cytokines, especially proinflammatory cytokines. Because we detected that rPCN increases both

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Fig. 1 rPCN induces ROS production in human neutrophils. **a** Freshly isolated human neutrophils were treated with buffer (Ctrl) or increasing concentrations of rPCN and the total amount of ROS produced was measured after each 5 min and cumulated for 2 h (AUC, area under curve) and was assessed by flow cytometry using the H₂DCFDA probe as described in “Materials and methods”. **b** Cells were incubated in the presence or absence of the ROS

inhibitor DPI and ROS production was determined after 20 min of treatment with buffer (Ctrl) or 1250 ng/ml rPCN. **c** Cells were treated with buffer (Ctrl) or rPCN (1250 ng/ml) in the presence or absence of DPI and ROS production was determined for the indicated periods of time. Results are mean $\pm$ SEM ($n = 3$ or 4, for **a** and **c**, respectively). RFU relative fluorescence unit. * $p < 0.05$ vs Ctrl; # vs corresponding control

de novo protein synthesis and IL-8 secretion, we ultimately determined the influence of rPCN in modulating the neutrophils production of TNF- α , IFN- γ , IL-10, IL-12p40, TGF- β and IL-1 β production. As illustrated in Fig. 6, while rPCN stimulation increased IL-1 β secretion by human neutrophils, such as LPS, it decreases the TGF- β secretion. No difference was detected between unstimulated (Ctrl) and LPS-stimulated neutrophils concerning the levels of secreted TGF- β (Fig. 6a). The production of TNF- α , IFN- γ , IL-10, IL-12p40 by human neutrophils was not affected by stimulation of either LPS or rPCN (Fig. 6b–e).

rPCN stimulation increases the neutrophil fungicidal ability

Since we have shown that rPCN enhances human neutrophil effector functions that can promote microbicidal activity, we evaluated the capacity of neutrophils to kill *Pb* yeasts. After incubation of rPCN-stimulated or non-stimulated neutrophils with *Pb* yeasts, CFU recovering was analyzed. The number of CFU recovered after 15-min incubation, expressing the *Pb* yeasts uptake, has at least doubled when neutrophils were stimulated with rPCN (Fig. 7a). The number of CFU recovered after 3 h, representing the remaining viable yeasts, did not differ among neutrophils that were stimulated or not with rPCN

Fig. 2 rPCN induces DNA release by a ROS-dependent and PI3K/AKT-, p38-, Erk-1/2- and JNK-independent mechanism. Neutrophils were treated or not with the indicated inhibitors for 30 min and then incubated with buffer (Ctrl), PMA, 625 (a) or 1250 ng/ml (a–c) rPCN

for 2 h and DNA release was determined as described in “Materials and methods”. Results are mean $\pm$ SEM ($n \geq 3$). * $p < 0.05$ vs Ctrl; # vs corresponding control/diluent (dil)

(Fig. 7b). Because yeast uptake was higher in rPCN-stimulated neutrophils, the difference between the CFU number resultant from the up taken yeasts (15 min) and the CFU number resultant from the remaining yeasts (3 h) allowed to conclude that neutrophil fungicidal ability is increased by rPCN stimulation (Fig. 7c).

Discussion

This is the first study investigating the role of the fungal lectin PCN on the human neutrophil biology. The data indicate that both short and long periods of treatments with rPCN can alter the biology of these cells, indicating that rPCN is a human neutrophil activator with a large spectrum of activities. Indeed, when neutrophils were stimulated for few minutes with rPCN, a rapid increase in ROS

production was observed and a DNA release was induced when cells were treated for few hours. In addition, after 24 h of treatment with rPCN, the production of IL-8 was increased, the basal apoptotic rate was decreased and induction of de novo protein synthesis was verified. Recently, a study using low-level laser therapy to stimulate neutrophils for fighting against *Paracoccidioides brasiliensis* (Pb) in vivo in mice reported that this treatment increases ROS production from murine neutrophils [30]. In their approach, the authors used such treatment of the mouse femurs to generate more active neutrophils to fight Pb infection. Although performed with rPCN and human neutrophils, our present data are in agreement with this study. Although the major source of ROS production in human neutrophils is normally via NADPH activation, the use of the DPI inhibitor that reverses rPCN-induced ROS production does not exclude the participation of

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Fig. 3 rPCN induces the production of IL-8 and delays apoptosis in human neutrophils. Freshly isolated human neutrophils were treated with buffer (Ctrl), LPS, GM-CSF (GM) or rPCN for 24 h and the production of IL-8 released in the supernatants was determined by ELISA (**a**) and apoptosis by cytology (**b**) as described in “Materials

and methods”. Results are mean $\pm$ SEM ($n = 3$ or $n = 10$, **a** and **b**, respectively). **c** Representative images obtained after the flow cytometry procedure described in “Materials and methods”. Arrows, the apoptotic rate (% of cells in apoptosis)

mitochondria, since DPI is also known to inhibit mitochondrial ROS production [29]. In one other recent study, two different strains of *Pb* (*Pb18* and *Pb265*) were found to induce NET formation in vitro by human neutrophils [31]. Furthermore, it was observed that the high-virulent (*Pb18*) and lower virulent (*Pb265*) strains were able to induce different patterns of NET formation. Indeed, neutrophils incubated with *Pb18* yeasts showed the presence of NETs in a large coverage area with structures appearing rather loose and scattered, whereas NETs observed in neutrophils incubated with *Pb265* were smaller, denser and compacted. As proposed by the authors, such different NET patterns could be due to an enzyme with DNase-like activity produced by *Pb18* strain as a possible escaping mechanism

avoiding a tight entrapment by NETs [31]. This remains to be determined. In addition, the presence of NETs in vivo was evidenced in their study in tegumentary lesions of patients with paracoccidioidomycosis. Taken together, these observations are in agreement with our results showing that rPCN induces DNA release by human neutrophils.

Interestingly, rPCN was found to reproduce the biological properties of the native protein, in terms of inducing a protective Th1 immune response against *Pb* infection [20]. The induced response included an increased production of TNF- α by murine macrophages. We found here that rPCN also stimulates human neutrophils to increase the production of the proinflammatory cytokine IL-1 β and the

Fig. 4 rPCN induces de novo protein synthesis in human neutrophils. Neutrophils were pre-incubated in the presence or absence (buffer) of cycloheximide for 60 min and then treated with buffer alone (Ctrl), rPCN, GM-CSF or arsenic trioxide (ATO) for 23 h and cell lysates were prepared for electrophoresis as described in “Materials and Methods”. The bottom part of the figure illustrates the corresponding Coomassie blue-stained gel of the autoradiograph illustrated in the upper part (^{35}S). Results are from one representative experiment out of four. mw molecular weight standards

Fig. 5 rPCN delays human neutrophil apoptosis by a de novo protein synthesis-dependent mechanism. Human neutrophils were incubated with buffer (Ctrl), the antiapoptotic cytokine GM-CSF, the proapoptotic plant lectin VAA-I without cycloheximide or with rPCN after a treatment of 30 min with buffer or CHX and apoptosis was determined by cytology. Results are mean $\pm$ SEM ($n = 10$)

potent neutrophil chemoattractant IL-8. Although rodents do not possess IL-8, they have some ‘IL-8-like’ chemokines. Therefore, the ability of rPCN-stimulated human neutrophils to produce a chemokine fits well with the neutrophilic infiltrates that occur in granulomatous lesions of both the experimental murine infection and the human

paracoccidioidomycosis [29, 30]. IL-1 β , in its turn, plays a relevant role in the control and killing of intracellular fungi, namely *Candida albicans* [32] and *P. brasiliensis* [33]. In addition, we found that rPCN decreases the production of TGF- β , which potentially impacts the innate response to intracellular pathogens [34, 35]. Concerning fungal infections, at their early stages, the high production of TGF- β has been associated with increased *Cryptococcus neoformans* pulmonary burden [36]. Here, rPCN modifies the cytokines produced by human neutrophils, a feature among others agreeing with a favorable argument to speculate that neutrophils contribute to the in vivo protection conferred by rPCN against experimental paracoccidioidomycosis.

Although rPCN was found here to induce DNA release, it is curious that, in parallel, it markedly inhibits spontaneous apoptosis, two opposite functions. Even if neutrophil heterogeneity was described several years ago, it is still obscure if such heterogeneity, based on the expression of different markers, could be associated with different biological activities. Recently, the expression of the granule protein olfactomedin 4 (OLFM4) was proposed to define two such neutrophil subsets [37]. However, based on the presence or absence of OLFM4, these two neutrophil subsets were found to undergo apoptosis at similar rates and to exert phagocytosis as same potency. Interestingly, the authors reported that OLFM4 is released during NET formation in OLFM4-positive cells, indicating that different molecules could be released during this process by different neutrophil subsets. Yet, we cannot associate the ability of rPCN to induce DNA release and to delay spontaneous apoptosis by different neutrophil subsets. However, rPCN clearly is a potent antiapoptotic agent, and as GM-CSF, a potent inducer of IL-8 secretion. Therefore, rPCN could act on other cells responsive to IL-8 and/or IL-1 β , indicating its potential large spectrum of biological activities. In line with this, rPCN was found to induce de novo protein synthesis in human neutrophils, which raises the possibility that other released mediators could act on other cells. Identification of the nature of the different proteins neo-synthesized in future will help to understand the rPCN mode of action. Of note, even it is tempting to speculate that the antiapoptotic effect of rPCN could be due to the increased production of IL-8, we previously added in one other study increasing amount of exogenous IL-8 (0–100 ng/ml), including higher amount than the level detected in this study (in terms of pg/ml, Fig. 3); we did not observe any effect on the basal neutrophil’s apoptotic rate [38]. In addition, we previously reported that curcumin increased IL-8 production but induced neutrophil apoptosis

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Fig. 6 rPCN modulates cytokine production in human neutrophils. Freshly isolated human neutrophils were treated with buffer (Ctrl), LPS or rPCN for 24 h. The concentration of TGF- β (a), IL-10 (b),

IFN- γ (c), TNF- α (d), IL-12p40 (e) and IL-1 β (f) in the cells supernatant was measured by ELISA as described in Materials and Methods. Results are means $\pm$ SEM ($n = 3$). * $p < 0.05$ vs Ctrl

[39], while the chemical dieldrin was found to increase IL-8 production without altering the basal apoptotic rate [40]. Works from others also demonstrated that fucoidan delays neutrophil apoptosis and increases IL-8 production (Jin and Yu 2015). Therefore, the fact that rPCN can increase IL-8 production and delay neutrophil apoptosis could not be necessarily correlated.

The results presented here show that rPCN promotes neutrophil activation since beyond stimulating inflammatory cytokine production and NET formation, it also increases the neutrophil fungicidal ability. Nonetheless, activation of neutrophils can also result in host tissue injury. Interestingly, rPCN beneficial effects apparently do not threaten the tissue integrity, since its administration to *P. brasiliensis*-infected mice did not raise inflammatory

lesions. It is consistent with the recent demonstration that the resistance to *Leishmania* spp. infection, conferred by an immunomodulatory plant lectin (ArtinM), receives the contribution of neutrophils, whose activation did not implicate in tissue damage [17]. Therefore, our current results indicate that neutrophils, besides other innate immune cells, contribute importantly to the protection against *P. brasiliensis* infection conferred by the rPCN administration.

In summary, this is the first study demonstrating that PCN can alter the biology of human neutrophils by modulating different functions. In particular, we demonstrated that rPCN increases DNA release and delays apoptosis in neutrophil by a ROS- and a de novo protein synthesis-dependent mechanism, respectively. In addition, we

Fig. 7 rPCN increases the fungicidal ability of human neutrophils. Freshly isolated human neutrophils were treated with buffer (Ctrl) or rPCN for 30 min and then incubated with *P. brasiliensis* yeasts (10:1). After 15 min or 3 h, neutrophils were washed and lysed. The obtained material was plated. **a** The number of CFU recovered from neutrophils lysed 15 min after incubation with yeasts represents the neutrophil-*Pb* uptake. **b** The number of CFU recovered from neutrophils lysed 3 h after incubation with yeasts represents the yeasts resistant to the neutrophil killing activity. **c** Fungicidal capacity is determined by subtracting the number of remaining resistant yeasts from the number of up taken yeasts. Results are means $\pm$ SEM ($n = 3$)

showed that inflammatory cytokine production and fungicidal ability of human neutrophils are increased under rPCN stimulation. The results of this study open new avenues for further research concerning PCN effects on host–fungi interaction.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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Considerações sobre o capítulo II

Os resultados apresentados no capítulo II (*Human neutrophils are targets to paracoccin, a lectin expressed by Paracoccidioides brasiliensis*) corroboram para o atendimento ao primeiro objetivo desta tese, qual seja, o de “Investigar o efeito da paracoccina sobre células da imunidade inata, buscando identificar mecanismos que contribuam para a imunomodulação exercida pela proteína fúngica”, e ao sub-item “Determinar o efeito da paracoccina sobre funções não fagocíticas de neutrófilos humanos”. Destacamos os seguintes aspectos do trabalho: i) a PCN recombinante estimula a produção de ROS e a liberação de redes de DNA por neutrófilos humanos; ii) a PCN recombinante induz a produção de IL-8 e retarda a apoptose em neutrófilos humanos, atuando como molécula antiapoptótica; iii) a PCN recombinante inibe a apoptose através de mecanismo dependente da síntese *de novo* de proteínas; e iv) a PCN recombinante aumenta a atividade fungicida em neutrófilos humanos.

Este é o primeiro estudo que demonstra que PCN pode alterar a biologia de neutrófilos humanos, através da modulação de diversas funções. Além disso, os resultados deste estudo abrem novos caminhos para pesquisas adicionais sobre os efeitos da PCN na interação fungo-hospedeiro.

3. Capítulo III

Diz respeito a artigo publicado com as especificações que se seguem.

Título: Impact of paracoccin gene silencing on *Paracoccidioides brasiliensis* virulence.

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RESEARCH ARTICLE

Impact of Paracoccin Gene Silencing on *Paracoccidioides brasiliensis* Virulence

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ABSTRACT Among the endemic deep mycoses in Latin America, paracoccidioidomycosis (PCM), caused by thermotolerant fungi of the *Paracoccidioides* genus, is a major cause of morbidity. Disease development and its manifestations are associated with both host and fungal factors. Concerning the latter, several recent studies have employed the methodology of gene modulation in *P. brasiliensis* using antisense RNA (AsRNA) and *Agrobacterium tumefaciens*-mediated transformation (ATMT) to identify proteins that influence fungus virulence. Our previous observations suggested that paracoccin (PCN), a multidomain fungal protein with both lectin and enzymatic activities, may be a potential *P. brasiliensis* virulence factor. To explore this, we used AsRNA and ATMT methodology to obtain three independent PCN-silenced *P. brasiliensis* yeast strains (AsPCN1, AsPCN2, and AsPCN3) and characterized them with regard to *P. brasiliensis* biology and pathogenicity. AsPCN1, AsPCN2, and AsPCN3 showed relative PCN expression levels that were 60%, 40%, and 60% of that of the wild-type (WT) strain, respectively. PCN silencing led to the aggregation of fungal cells, blocked the morphological yeast-to-mycelium transition, and rendered the yeast less resistant to macrophage fungicidal activity. In addition, mice infected with AsPCN1, AsPCN2, and AsPCN3 showed a reduction in fungal burden of approximately 96% compared with those inoculated with the WT strain, which displayed a more extensive destruction of lung tissue. Finally, mice infected with the PCN-silenced yeast strains had lower mortality than those infected with the WT strain. These data demonstrate that PCN acts as a *P. brasiliensis* contributory virulence factor directly affecting fungal pathogenesis.

IMPORTANCE The nonexistence of efficient genetic transformation systems has hampered studies in the dimorphic fungus *Paracoccidioides brasiliensis*, the etiological agent of the most frequent systemic mycosis in Latin America. The recent development of a method for gene expression knockdown by antisense RNA technology, associated with an *Agrobacterium tumefaciens*-mediated transformation system, provides new strategies for studying *P. brasiliensis*. Through this technology, we generated yeasts that were silenced for paracoccin (PCN), a *P. brasiliensis* component that has lectin and enzymatic properties. By comparing the phenotypes of PCN-silenced and wild-type strains of *P. brasiliensis*, we identified PCN as a virulence factor whose absence renders the yeasts unable to undergo the transition to mycelium and causes a milder pulmonary disease in mice, with a lower mortality rate. Our report highlights the importance of the technology used for *P. brasiliensis* transformation and demonstrates that paracoccin is a virulence factor acting on fungal biology and pathogenesis.

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KEYWORDS ATMT, fungal virulence, *Paracoccidioides brasiliensis*, paracoccidioidomycosis, paracoccin, paracoccin silencing, chitinase

Among the endemic deep mycoses having impact on public health in Latin America (1), paracoccidioidomycosis (PCM), caused by thermodimorphic fungi of the *Paracoccidioides* genus, is a major source of morbidity (2). About 80% of the reported cases occur in Brazil, with an estimated 3,360 new cases per year; most of the remaining cases occur in Venezuela, Colombia, and Argentina (3, 4). PCM is caused by the thermally dimorphic fungi *Paracoccidioides brasiliensis* and by *P. lutzii* species (5, 6), whose conidia, produced in the mycelial phase, are inhaled by humans. After reaching the pulmonary alveolar epithelium, the propagules transform into the parasitic yeast form (7). Pulmonary lesions can lead to impairment of lung function and permanent interference with the patient's quality of life. The disease can subsequently disseminate to other organs, producing secondary injuries to mucosa, skin, lymphoid tissue, and adrenal glands. Acute and subacute disease develops within weeks to months and causes hypertrophy of the reticuloendothelial system. The chronic disease, which accounts for more than 90% of cases, primarily affects the lungs and progresses slowly, taking months to years to fully develop. In the absence of an effective therapy, it can be lethal (8–10).

Disease manifestation is associated with host factors such as susceptibility and immune status (2, 11) and with fungal factors such as virulence and pathogenicity (7, 12). Several studies that have been conducted have been aimed at identifying protein components from *P. brasiliensis* that are relevant to fungal infection and pathogenicity. Recent studies have employed a new gene modulation technique that allows insertion of a target DNA sequence into the fungal genome of *P. brasiliensis* using *Agrobacterium tumefaciens*-mediated transformation (ATMT) with transfer DNA (T-DNA) binary vectors (13).

Based on our previous data, paracoccin (PCN) might also be considered to be among the *P. brasiliensis* proteins that are candidate virulence factors. PCN is a multidomain protein with both lectin and enzymatic activities that can bind N-acetylglucosamine (14) and chitin but can also act as an N-acetylglucosaminidase (15). These biological activities may potentially explain the role of PCN in fungal cell growth (16). PCN is also able to act as an immunomodulatory agent when injected subcutaneously into *P. brasiliensis*-infected mice. This effect was suggested to be associated with the interaction of PCN with TLR N-glycans and the induction of M1 polarization in macrophages and the subsequent development of Th1 immunity (17–19).

Given the potential relevance of PCN, we aimed to study the role of PCN in *P. brasiliensis* pathogenesis using antisense RNA (AsRNA) and the ATMT methodology. We created PCN-deficient *P. brasiliensis* mutant strains (AsPCN mutants) which were mitotically stable and determined the level of mRNA and protein reduction in these mutants. We showed that PCN silencing did not affect cell viability or growth but instead reduced yeast cell separation. In addition, the morphological transition from yeast to mycelium was blocked in the AsPCN mutants. Our data showed that PCN did not contribute to fungal adherence to lung epithelial cells but instead rendered yeast more susceptible to macrophage fungicidal activity. Furthermore, in mice infected with PCN-silenced yeast, the disease caused by inoculation was milder than that caused by the WT *P. brasiliensis* yeast. In summary, gene silencing of PCN revealed that paracoccin is a *P. brasiliensis* virulence factor and contributes to its pathogenicity.

RESULTS

Generation of *P. brasiliensis* mutant strains. The role of PCN in stimulating *P. brasiliensis* growth was suggested first by the inhibitory effect of anti-paracoccin antibodies in yeast cultures (16). To perform a broader study on the relevance of PCN in fungal biology and virulence, we choose to silence PCN in *P. brasiliensis* using an antisense RNA (AsRNA) methodology. We used transfer DNA (T-DNA) containing two

FIG 1 PCN silencing in *P. brasiliensis* yeasts. (A) The relative PCN expression levels in the transformed clones (AsPCN1, AsPCN2, and AsPCN3) were determined by qRT-PCR and compared to the expression detected in WT *P. brasiliensis*, which was set at 100%. The β -actin and α -tubulin reference genes were used as endogenous controls to normalize the relative PCN expression levels. ***, significant differences compared to WT *P. brasiliensis* ($P < 0.0001$). (B) Crude extracts of yeast cells from WT and AsPCN *P. brasiliensis* strains, cultured in DMEM, were assessed for NAGase (N-acetyl-beta-D-glucosaminidase) activity, detected by the degradation of the pNP-GlcNAc substrate. ***, significantly different compared to WT strain ($P < 0.001$). OD_{405nm}, optical density at 405 nm; Ctrl, control. (C) Graphic representation of Western blotting images captured to determine the percentage of PCN-silencing, after normalization with β -actin. The graphic was generated by analysis offline using the software ImageJ. (D) Western blotting analysis of the PCN content in soluble antigens of yeast cells obtained from WT, EV, and PCN-deficient (AsPCN1, AsPCN2, AsPCN3) *P. brasiliensis* strains.

different AsRNA sequences derived from the PCN gene, under the control of the *RHO2* promoter. *P. brasiliensis* clones (referred to as strains AsPCN1 and AsPCN2) were obtained by transformation with T-DNA containing the AS1 sequence, and strain AsPCN3 was generated with the AS2 sequence. The T-DNA was inserted into *P. brasiliensis* wild-type (WT) strain 18 through *Agrobacterium tumefaciens*-mediated transformation (ATMT). After the mitotic stability process was performed, three independent hygromycin-resistant (HR) clones, AsPCN1, AsPCN2, and AsPCN3, were selected, and hygromycin resistance gene (*HPH*) amplification confirmed the presence of T-DNA. These data demonstrate the efficiency of T-DNA transformation in generating *P. brasiliensis* clones to downregulate PCN expression.

Downregulation of PCN from *P. brasiliensis*. To demonstrate that the AsPCN1, AsPCN2, and AsPCN3 mutant strains have differential levels of expression of the PCN mRNA compared to the WT strain, we investigated the relative levels of expression of PCN mRNA by quantitative real-time PCR (qRT-PCR). The AsPCN1, AsPCN2, and AsPCN3 strains showed reductions of levels of PCN mRNA of approximately 60%, 40%, and 60%, respectively (Fig. 1A). In addition, we analyzed the silencing effect in all three AsPCN mutant strains by measuring total cellular N-acetyl- β -D-glucosaminidase activity, a known enzymatic activity of PCN (15). The enzyme activity of AsPCN cell lysates was significantly lower than that of the lysate from the WT strain (Fig. 1B) and was close to the level seen with the negative control, indicating the PCN silencing. We also evaluated the efficiency of PCN silencing by analyses of Western blotting. Yeast cells were harvested from cultures by centrifugation at $5,000 \times g$ and 25°C for 5 min and were disrupted by sonication, according to a previously reported protocol (20). Through a Western blotting procedure, electrophoresed components of the yeast lysates were transferred to a membrane and reacted with the anti-PCN antibody. A labeled second-

FIG 2 Growth of *P. brasiliensis* yeasts from WT, EV, and PCN-deficient strains. Yeasts from WT, EV, and PCN-deficient (AsPCN1, AsPCN2, and AsPCN3) strains were cultivated in DMEM at 37°C for 9 days. Growth curve profiles were determined by counting of yeasts by optical microscopy, using Neubauer chambers.

ary antibody developed the reactions. There was reduced staining of the PCN 27-kDa band in the material from all three mutated yeasts compared to the WT and EV (empty vector) strains (Fig. 1C and D). In summary, all three of these analyses confirmed the efficiency of PCN knockdown in the AsPCN1, AsPCN2, and AsPCN3 mutant strains.

PCN silencing alters the growth characteristics of *P. brasiliensis*. To better understand the effect of PCN on *P. brasiliensis* yeast growth, AsPCN1, AsPCN2, AsPCN3, WT, and EV strains were grown in Dulbecco's modified Eagle's medium (DMEM) at 37°C for 9 days. Since all cultures reached the stationary-growth phase by 216 h, as monitored by cell counting in optical microscopy, we conclude that the growth of the AsPCN mutant strains is similar to the WT strain growth (Fig. 2). To evaluate levels of cell viability among the different strains, cells were stained with diacetate fluorescein-ethidium bromide at the beginning of growth (0 h) and after 192 h of growth; all strains had similar levels of cell viability. In spite of these similarities, the growth patterns differed between the AsPCN mutant strains and the WT strain since the mutant cells were more agglomerated than the WT cells. From this, we conclude that the PCN silencing did not affect the growth of *P. brasiliensis* but led to agglomeration of the fungal cells.

PCN silencing inhibits the yeast-to-mycelium transition. We evaluated morphologically the yeast-to-mycelium (Y → M) transition of the AsPCN1, AsPCN2, and AsPCN3 mutant strains compared to the EV strain. For this purpose, strains were grown at 25°C in liquid medium for the microscopic analysis of fungal cell morphology. The cells grown were analyzed by fluorescence microscopy after calcofluor white staining. At all time points in the observation period, the AsPCN1, AsPCN2, and AsPCN3 strains grew similarly; the cells assumed the elongated morphology of pseudohyphal forms (Fig. 3). In contrast, the EV strain underwent complete Y → M conversion, exhibiting conidia in the apical regions of hyphae 10 days after inoculation. From this, we conclude that PCN is important in the mycelium conversion process.

PCN silencing favors *P. brasiliensis* susceptibility to killing by macrophages. To assess PCN involvement in the interaction of *P. brasiliensis* with host cells, the capacity of AsPCN mutant strains to adhere to A549 lung epithelial cells was compared with ability of the WT strain to do the same. After 2 h of cell contact, the efficiencies of yeast cell adherence to epithelial cells, as estimated by CFU counting, did not differ between the AsPCN mutant strains and the WT strain (Fig. 4A). The involvement of PCN in *P. brasiliensis* interactions with phagocytes was evaluated by coculturing AsPCN1, AsPCN2, AsPCN3, EV, or WT strains with murine peritoneal macrophages. Using optical microscopy to counting yeast cells in a Neubauer chamber, after disruption of macrophages, we observed that all yeast strains were similarly phagocytosed by macrophages after 4 h of coculture (Fig. 4B). We conclude that PCN does not play a role in macrophage phagocytosis of *P. brasiliensis*. To examine whether PCN is relevant in *P. brasiliensis* sensitivity to macrophage killing, we measured yeast cell recovery using

FIG 3 Effect of PCN silencing on the morphological transition of *P. brasiliensis* cells. EV, AsPCN1, AsPCN2, and AsPCN3 strains were grown in solid BHI medium or liquid DMEM at 25°C. The images represent the results of fluorescence microscopy of calcofluor white-stained cells from the various *P. brasiliensis* strains cultured for 1 and 10 days in DMEM. Bars correspond to 50 μ m.

a CFU assay of lysates from murine macrophages obtained after 48 h of incubation with AsPCN1, AsPCN2, AsPCN3, or the WT strain. We found that a reduced number of colonies were recovered from macrophages infected with the AsPCN mutant strains compared to the WT strain (Fig. 4C). Taken together, these results demonstrate that PCN silencing does not affect the early phases of infection of host cells by *P. brasiliensis* but instead is important in resistance to macrophage killing. This observation suggests that PCN may act as a *P. brasiliensis* virulence factor.

Murine infection with PCN-silenced mutant yeasts causes reduced tissue fungal burden and pulmonary injury. We then evaluated whether the course of experimental paracoccidioidomycosis could be influenced by PCN silencing in the infectious yeast strain. We inoculated BALB/c mice with AsPCN1, AsPCN2, or AsPCN3 or with the WT yeast strain via the intranasal route. At 30 days postinfection, CFU analysis of the lung revealed a decreased fungal burden in the mice that were infected with AsPCN1, AsPCN2, or AsPCN3 compared to those mice infected with WT yeast. The lungs of the mice that were infected with AsPCN mutant strains exhibited a lower number of granulomas than those infected with the WT strain (Fig. 5). A similar distribution of lesions was observed when mice were infected intravenously (date not shown). Therefore, PCN silencing diminishes the ability of *P. brasiliensis* to cause disease. The fact that PCN enhances fungal pathogenicity suggests that PCN is a *P. brasiliensis* virulence factor.

PCN is a contributory virulence factor in *P. brasiliensis* yeasts. Since PCN silencing reduces *P. brasiliensis* resistance to macrophage killing and decreases fungal pathogenicity, we evaluated whether PCN is important in *P. brasiliensis* virulence by comparing the mortality curves, obtained over an 80-day period, of mice infected with

FIG 4 Effect of PCN silencing on *P. brasiliensis* interaction with host cells. WT, EV, AsPCN1, AsPCN2, and AsPCN3 *P. brasiliensis* yeasts were grown in DMEM at 37°C for 5 days. (A) The yeast cells were incubated for 2 h with A549 human lung epithelial cells; after washing, the number of adhering yeasts was estimated by CFU count. (B) The yeasts were incubated for 4 h with murine peritoneal macrophages; after macrophage lysis, the recovered yeast cells were counted by optical microscopy, using Neubauer chambers. (C) CFU recovered from the lysate of murine peritoneal macrophages after 48 h of coculture at 37°C with WT, AsPCN1, AsPCN2, or AsPCN3 *P. brasiliensis* cells. The CFU count gives an estimate of the resistance of the yeast strains to macrophage killing. ***, significant difference from the WT strain ($P < 0.0001$).

PCN-silenced or WT yeasts. Figure 6 shows that, at 50 days postinfection, all animals infected with the WT strain had died. In sharp contrast, the survival rates in mice infected with AsPCN2, AsPCN1, and AsPCN3 were 60%, 75%, and 85%, respectively, and these survival rates were stable from day 50 to day 80 of observation. These data show that PCN silencing affects *P. brasiliensis* pathogenesis and that PCN acts as a contributory virulence factor.

DISCUSSION

The development of a paracoccidioidomycosis infection is closely related to fungus virulence and pathogenicity (7, 12), as well as to host susceptibility and immune status (2, 11). Several studies have shown that *P. brasiliensis* genes may be associated with fungal virulence (21–25) and thereby influence the host immune response (18–20, 26–31). In addition to participating in fungal morphogenesis, an important role was recently attributed to PCN in *P. brasiliensis* infection (15, 16). The results of that study encouraged us to generate yeast strains in which the PCN gene was silenced and to use

FIG 5 Fungal burden and tissue injury in mice infected intranasally with PCN-silenced *P. brasiliensis* yeast strains. BALB/c mice were inoculated intranasally with 2×10^6 WT, AsPCN1, AsPCN2, or AsPCN3 *P. brasiliensis* cells. Analyses were performed at 30 days postinfection. (A) Number of recovered CFU/g pulmonary tissue. ***, significant difference from WT strain-infected animals ($P < 0.0001$). ND, not detected. (B) The lung was fixed in formalin, embedded in paraffin, cut into 5- μ m-thick sections, and stained with hematoxylin and eosin (H&E) for light-microscopy analysis. The black line scale bar corresponds to 400 μ m.

these strains to define the role of PCN in *P. brasiliensis* biology and pathogenesis. In comparison to WT yeast, the PCN-silenced yeast strains formed cellular aggregates and had an inability to make the yeast-to-mycelium transition as well as greater susceptibility to the fungicidal activity of macrophages. Moreover, infection of mice with the PCN-silenced yeast strains resulted in a mild infection and low mortality rates. These results clearly show that PCN is involved in the pathogenicity and virulence of *P. brasiliensis*.

Cultures of the PCN-silenced yeast strains, as well as the WT yeast strain, exhibited similar cell viability and growth results; however, in spite of this, the PCN-silenced yeast cells were very noticeably agglomerated compared to their WT counterparts. These data are consistent with other reported PCN features: (i) PCN in *P. brasiliensis* is colocalized with the β -1,4-GlcNAc homopolymer in the yeast budding sites; and (ii) PCN has N-acetyl- β -D-glucosaminidase activity, which is known to be important in the mechanism of cell wall remodeling (15). The yeast aggregation noted in the AsPCN strains is similar to that reported for fungi that had a deletion of the gene encoding chitinase, e.g., *CST1* in *Saccharomyces cerevisiae* (32) and *CTH3* in *Candida albicans* (33).

Because the fungal morphological transition requires coordinated cell wall remodeling (34) and this process appeared to be disrupted in the PCN-silenced yeast strains, we investigated the transition process in the mutant yeast strains. As predicted, the process was blocked in the PCN-silenced yeast strains, whose morphology became more compatible with that of pseudohyphae, a fungal form that is unusual for *P. brasiliensis*. Because we did not obtain the PCN-silent yeast strains in mycelium form, we could not verify the effect of PCN deficiency on the transition to the parasite form

FIG 6 Survival curves of mice infected intravenously with PCN-silenced *P. brasiliensis* yeast strains. Male BALB/c mice were infected with 1×10^7 WT, AsPCN1, AsPCN2, or AsPCN3 *P. brasiliensis* cells. The mice were monitored daily for survival over an 80-day period following infection. Mouse survival is expressed as a percentage. *, significant difference from mice infected with the WT strain ($P < 0.05$).

(yeast), a process that is important in the virulence of dimorphic fungi (35). These results suggest that PCN plays an important role in *P. brasiliensis* cell morphology.

The adherence of PCN-silenced yeast strains to lung epithelial cells was not affected. However, PCN silencing did enhance the yeast's susceptibility to macrophage effector function. This is relevant because macrophages constitute fungal replication niches and provide the chief mechanism of defense against *P. brasiliensis* infection (36). Taken together, these effects of PCN favoring fungal growth support the concept of PCN being a *P. brasiliensis* virulence factor. Furthermore, mice infected with PCN-silenced yeast strains had a lower pulmonary fungal burden, milder tissue lesions, and lower mortality rates than mice infected with a WT strain of *P. brasiliensis*. Since the disease outcomes were similar in mice inoculated either intravenously or intranasally with PCN-silenced yeast strains, it can be concluded that the infection route does not explain the milder disease caused by the *AsPCN* mutant strains. Taken together, these data obtained in the experimental PCM model strongly support the concept that PCN is involved in fungal virulence.

Virulence factors may be targets in the immune response (37), drawing attention to the fact that virulence influences host immunity (38). Consistent with this, the work of Alegre et al. (18) showed that recombinant PCN, administered prophylactically to mice, induced a host protective immunity manifested by a decrease in both tissue fungal load and granuloma occurrence. Success in the use of virulence factors as vaccine antigens is consistent with the principle that the immune response can be altered by reducing or nullifying the virulence of certain microbes (39). Because we have previously reported that the subcutaneous injection of recombinant PCN in mice exerts a prophylactic or therapeutic effect against *P. brasiliensis* infection (18, 19), it might be expected that the PCN-silenced fungi would cause a more severe manifestation of experimental disease. We observed the opposite, which led us to suppose that the slow endogenous PCN release by the infecting yeast was not sufficient to maintain an immunomodulatory effect; conversely, this effect was attained when a concentrated aliquot of exogenous PCN was subcutaneously administered to the host. It is possible that, whereas endogenous PCN would act directly on the infecting fungi, positively influencing virulence and pathogenicity, injected PCN would interact directly with host immune cells, causing their activation, production of inflammatory mediators, and the development of a protective response to *P. brasiliensis*.

Another relevant issue for the concept of virulence factors is that they may function as essential (requisite) or relative (contributory) molecules. Requisite virulence factors confer pathogenicity and the ability to cause disease, whereas contributory virulence factors modify disease magnitude and extension (38). On the basis of the effects of gene silencing of PCN in *P. brasiliensis*, including increased susceptibility to the fungicidal activity of macrophages, reduced disease severity in mice, reduced tissue fungal load and injury, and a lower mouse mortality rate, we conclude that PCN is a contributory virulence factor for the establishment of *P. brasiliensis* infection.

MATERIALS AND METHODS

Mice and ethics statement. Male BALB/c mice that were 6 to 8 weeks of age, obtained from the Animal Facility of Ribeirão Preto Campus, were maintained in the animal facility of the Molecular and Cellular Biology Department, Ribeirão Preto School of Medicine, University of São Paulo. All animals were acclimated to the facility for 1 week before the study start. Animals were housed in individually ventilated cages in light-tight cabinets and were maintained at 20 to 22°C using a 12-h light–12-h dark cycle, under optimized hygienic conditions, and with *ad libitum* access to chow and water. Animal procedures were conducted in accordance with the ethical principles of animal research adopted by the Brazilian Society of Laboratory Animal Science and approved by the Ethical Committee for Ethics in Animal Research (CETEA) of the Ribeirão Preto School of Medicine, University of São Paulo, under protocol 60/2016.

Microorganism strains. *P. brasiliensis* strain 18 (WT) and PCN-downregulated strains (*AsPCN*) were maintained in brain heart infusion (BHI) solid or liquid media (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1% glucose and ampicillin (100 µg/ml) or Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) at 37°C on a mechanical shaker (200 rpm). Bacterial cells were maintained in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics or left unsupplemented. *Escherichia coli* DH5α was used as the host for plasmid amplification and cloning. *Agrobacterium*

tumefaciens strain LBA1100 (40) was used as the recipient for the binary vectors. The time period and temperature of the incubation and the use of antibiotics are described below.

Construction of *P. brasiliensis* PCN antisense RNA (AsPCN) strains. To obtain a PCN-silenced *P. brasiliensis* strain, we used the antisense RNA (AsRNA) strategy and *Agrobacterium tumefaciens*-mediated transformation (ATMT), as described previously (13, 21, 41). Briefly, two regions within the second exon of the *PCN* gene (18) which comprise nucleotides between positions 159 and 527 (AS1) and positions 288 and 527 (AS2) were amplified using a high-fidelity proofreading DNA polymerase (NZYTech, Lisbon, Portugal) and AsPCNF1-AsPCNR and AsPCNF2-AsPCNR primers (Sigma-Aldrich) (see Table S1 in the supplemental material), respectively. Both AS1 and AS2 were inserted individually into the pCR35-RHO2 vector under the control of the Rho (Ras homology) GTPase 2 (*RHO2*) promoter region from *P. brasiliensis*. The pCR35-RHO2 vectors carrying AS1 or AS2 were used for AsRNA cassette amplification and were inserted individually within the transfer DNA (T-DNA) of pUR5750 parental binary vector. These vectors were used to transform *A. tumefaciens* LBA1100. Transformants were selected in LB medium containing 100 µg/ml kanamycin and were used thereafter for transformation of *P. brasiliensis*. To achieve this, *A. tumefaciens* carrying the pUR5750 binary vector containing AsRNA cassettes was cocultured with *P. brasiliensis* yeast at a 1:10 ratio on sterile Hybond N filters (GE Healthcare Life Science, Pittsburgh, PA) in a solid medium IM (induction medium) at 28°C for 3 days. After cocultivation, the membranes were transferred to liquid BHI medium containing cefotaxime (200 µg/ml). The cells in suspension were incubated for 48 h at 200 rpm at 37°C before being transferred to the selective BHI medium containing hygromycin B (100 µg/ml) and cefotaxime (100 µg/ml) at 37°C for 15 days.

Mitotic stability and detection of the hygromycin resistance gene (*HPH*). Hygromycin B-resistant transformants were randomly selected and serially transferred to nonselective BHI solid medium for three final times. Then, transformants were serially inoculated on plates with nonselective and selective medium (100 µg/ml) for three consecutive rounds to examine mitotic stability. In order to confirm the presence of T-DNA in the selected AsPCN mutant strains, these transformants were evaluated by PCR analysis by amplifying the hygromycin B resistance gene (*HPH*) with HPHF and HPHR primers (Sigma-Aldrich) (Table S1), according to the procedure described by Torres et al. (23). The WT strain was used as the negative control, and pUR5750 parental binary vector was used as the positive control. The reaction products were analyzed on a 1% agarose gel stained with SYBR Safe DNA gel stain (Thermo Fisher Scientific Inc., Waltham, MA) and visualized with UV light.

RNA extraction and cDNA synthesis. Total RNA was extracted using TRIzol (Thermo Fisher Scientific) from WT, AsPCN1, AsPCN2, and AsPCN3 yeast cells grown in DMEM (Sigma-Aldrich) at 37°C for 96 h during the early exponential phase. Yeast cells were collected by centrifugation at $3,000 \times g$ at 25°C for 5 min and washed with 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]) mixed in diethyl pyrocarbonate (DEPC) ultrapure water (Sigma-Aldrich). Pelleted yeast cells were transferred to a mortar, liquid nitrogen was added, and cells were disrupted with a pestle. After this process, the RNA was purified according to the TRIzol protocol. The quality of the purified RNA was assessed by electrophoretic analysis on a 2% agarose gel stained with SYBR Safe DNA gel stain and by the use of a NanoDrop 1000 instrument (Thermo Fisher Scientific). The absence of contaminating chromosomal DNA was confirmed, after the treatment of the RNA with DNase I (Thermo Fisher Scientific), by the absence of the α -tubulin gene as shown by PCR. The cDNA was synthesized from 1 µg RNA using Moloney murine leukemia virus (MMLV) reverse transcriptase, according to the instructions of the manufacturer (Promega, Fitchburg, WI).

Quantitative real-time PCR (qRT-PCR). Real-time PCR was carried out by mixing the cDNA derived from each sample with platinum SYBR green pPCR SuperMix-UDG with ROX, according to the instructions of the manufacturer (Thermo Fisher Scientific). PCR amplification was performed using a CFX96 real-time PCR detection system (Bio-Rad, Hercules, USA). The sequences of the RT-PCNF and RT-PCNR primers used to detect the PCN mRNA expression levels are shown in Table S1. α -Tubulin (42) and β -actin (sequence identifier [ID] reference XM_010763641.1) genes were used as references, and the relevant primer sequences are also shown in Table S1. Melting curve analysis was carried out after the amplification phase to eliminate the possibility of nonspecific amplification or primer-dimer formation. The PCN gene relative expression levels were quantified using the threshold cycle ($\Delta\Delta C_T$) method ($2^{-\Delta\Delta C_T}$) and normalized to β -actin and α -tubulin as follows: $\Delta C_T = C_{T-PCN} \text{ value} - \text{average of } C_{T-\alpha\text{-tubulin}} \text{ and } C_{T-\beta\text{-actin}}$ values. All experiments were performed using biological and experimental triplicates.

N-Acetyl- β -D-glucosaminidase activity assay. N-Acetyl- β -D-glucosaminidase activity was assayed through reaction with *p*-nitrophenol-N-acetylglucosamine (*p*NPGlcNAc) (Sigma-Aldrich) and detection of *p*-nitrophenol formation (15). Briefly, 50 µl of cell lysate (5 µg of total protein) derived from the WT, AsPCN1, AsPCN2, and AsPCN3 strains were prepared according to the method reported by Fernandes et al. (26) and was added individually to 350 µl of 0.1 M sodium acetate (pH 5.5) and 100 µl of 5 mM *p*NPGlcNAc. After incubation for 18 h at 37°C, the mixture was added to 0.5 M sodium carbonate (1 ml). The amount of released *p*-nitrophenol was determined by measuring the solution's absorbance at 405 nm. As a negative control, cell culture medium was used.

Western blotting. The Western blot analysis was performed by the use of 12% SDS-PAGE to separate the soluble components of *P. brasiliensis* yeasts from strains WT, EV, AsPCN1, AsPCN2, and AsPCN3, grown in DMEM (Sigma-Aldrich) at 37°C for 96 h (exponential phase). The components were electrottransferred to a polyvinylidene difluoride (PVDF) membrane (Hybond membranes [Amersham; GE Healthcare]) (pore size, 0.45 µm). Thereafter, membranes were blocked with Tris-buffered saline-Tween 20 (TBS-T) (20 mM Tris-HCl, 150 mM NaCl, 0.1% [vol/vol] Tween 20 [pH 7.6]) containing 3% bovine serum albumin (BSA) for 4 h at 25°C. The membrane was then incubated with anti-PCN IgY polyclonal antibody diluted 1:3,000 in TBS-T or with anti- β -actin mouse monoclonal antibody IgG (Santa Cruz Biotechnology Inc., Dallas, TX, USA), also diluted 1:5,000 in TBS-T. The membrane was washed five times with TBS-T and incubated for

1 h at room temperature with the anti-IgY secondary antibody conjugated to peroxidase (Sigma-Aldrich) diluted 1:1,000 or with anti-IgG secondary antibody conjugated to peroxidase (Sigma-Aldrich) diluted 1:3,000 in TBS-T containing 1% BSA. After five washes with TBS-T were performed, the membrane was immersed in a fresh mixture of DAB peroxidase substrate kit SK4100 (Vector Laboratories, Burlingame, CA). Distilled water was used to stop the reaction. The images were analyzed offline using the software ImageJ (W. S. Rasband, National Institutes of Health, Bethesda, MD; <https://imagej.nih.gov/ij/>), which generated graphic representations of the staining intensity of the PCN band (expressed in percentages), after normalization with β -actin.

Growth curve and cell viability. WT, *AsPCN1*, *AsPCN2*, and *AsPCN3* yeasts were cultured in DMEM (Sigma-Aldrich) at 37°C with aeration on a mechanical shaker (200 rpm). Growth of the organisms was assessed by cell counting using optical microscopy and a Neubauer chamber at 0, 36, 72, 96, 120, 144, 168, 192, and 216 h until the stationary-growth phase was reached. All cultures were performed with an initial concentration of 1×10^5 cells/ml. At the time points of 0 and 192 h, a sample was harvested for cell viability analysis by staining with diacetate fluorescein-ethidium bromide, as described by Calich et al. (43).

Morphological transition. WT, *AsPCN1*, *AsPCN2*, and *AsPCN3* yeasts were cultured at 25°C in DMEM (Sigma-Aldrich). Aliquots of the DMEM cultures were harvested at 1 and 10 days, fixed in 3.7% formaldehyde at 25°C for 45 min, and stained with calcofluor white (50 μ g/ml) for observation with a fluorescence microscope.

Assay of adherence of *P. brasiliensis* yeasts to lung epithelial cells. The assay to assess adherence of yeast to host cells was carried out as described by Hernández et al. (22), with small modifications. The monolayers of A549 cells (1×10^6 cells/well) were cocultured for 2 h with WT, *AsPCN1*, *AsPCN2*, and *AsPCN3* strains (2×10^5 cells) at 37°C in a 5% CO₂ atmosphere. The fungal inoculum suspension was obtained from the supernatant of liquid cultures that were allowed to settle, without shaking, for 10 min at room temperature. This process decants the fungal aggregates, providing a nonaggregated inoculum. The supernatants from the cultures, containing nonadherent yeast cells, were discarded, the monolayers were lysed, and the resulting suspension of adherent yeast cells was plated onto solid BHI medium supplemented with 1% glucose. The percentage of adherent yeast cells was estimated from the ratio of the number of CFU obtained from each well to the number of CFU obtained from the control wells, which contained only yeast cells. This assay was performed in triplicate in three independent experiments.

Assay of fungal phagocytosis and killing by macrophages. Yeast cells from WT, *AsPCN1*, *AsPCN2*, and *AsPCN3* strains, grown in DMEM (Sigma-Aldrich) at 37°C for 96 h (exponential phase), were cocultured with macrophages obtained from the peritoneal cavity of male BALB/c mice which had been previously injected with 1.0 ml of 3% sterile sodium thioglycollate (Sigma-Aldrich), according to a protocol adopted by Freitas et al. (17). The fungal inoculum was prepared as described for the previous item. To assess phagocytic activity, macrophages (1×10^6 /well) were plated into a 24-well microplate (BD Biosciences) and incubated with yeast cells (2×10^5 cells/well) for 1 h at 25°C (yeast-to-macrophage ratio of 1:5). After 4 h at 37°C in a 5% CO₂ atmosphere, the coculture supernatant was discarded. The macrophage monolayers were lysed with sterile ultrapure water, and yeast cells were harvested for counting using a Neubauer chamber in optical microscopy. To estimate the macrophage fungicidal activity, viable yeast cells were cocultured with macrophages at 37°C in a 5% CO₂ atmosphere for 4 h. The culture supernatant was discarded, and the cells were gently rinsed with phosphate-buffered saline (PBS) and incubated in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; HyClone) at 37°C in a 5% CO₂ atmosphere. After 48 h of incubation, the culture supernatant was discarded and macrophages were lysed using sterile ultrapure water. The cell lysate was serially diluted and cultured for 10 days at 37°C in solid BHI medium supplemented with 1% glucose. Analyses were performed by determining the number of CFU.

Tissue fungal burden and lung histopathology. Male BALB/c mice were infected with yeast cells from WT, *AsPCN1*, *AsPCN2*, and *AsPCN3* strains (5 animals per group) grown in DMEM (Sigma-Aldrich) supplemented with 4% fetal bovine serum (FBS) at 37°C for 96 h (exponential phase). Infections were performed by either intranasal (i.n.) or intravenous (i.v.) delivery of 2×10^6 or 1×10^6 yeast cells per animal, respectively. At 30 days postinfection, the lung, liver, and spleen fungal loads were determined by CFU counting, as described previously (26, 44, 45). The histopathological analysis of the lungs was performed according to a protocol adopted by Fernandes et al. (26).

Generation of the survival curve. Male BALB/c mice (eight animals/group) were intravenously inoculated with 1×10^7 viable yeast cells from WT, *AsPCN1*, *AsPCN2*, and *AsPCN3* strains grown in DMEM (Sigma-Aldrich) for 96 h (exponential phase) at 37°C. The mice were monitored daily for survival over an 80-day period following infection.

Statistical analysis. GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis. The results are expressed as means $\pm$ standard errors of the means (SEM). All data are representative of results from at least three independent assays. Statistical differences among the means of data from the different experimental groups were determined using one-way analysis of variance followed by Bonferroni's posttest. Differences with *P* values of <0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.00537-17>.

TABLE S1, DOCX file, 0.01 MB.

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Considerações sobre o capítulo III

Os resultados apresentados no capítulo III (*Impact of paracoccin gene silencing of Paracoccidioides brasiliensis virulence*) corroboram para o atendimento ao segundo objetivo desta tese, qual seja o de “*Estudar a relevância da paracoccina para a biologia de leveduras de P. brasiliensis e patogênese da doença por elas causada, através da utilização de transformantes do fungo cujos genes codificadores da paracoccina foram silenciados ou superexpressos*” e ao sub-item “*Promover o silenciamento e a superexpressão do gene que codifica a paracoccina em leveduras de P. brasiliensis*” e “*Avaliar os efeitos do silenciamento e da superexpressão da paracoccina sobre a patobiologia de leveduras de P. brasiliensis*”. Destacamos os seguintes aspectos do trabalho: i) a partir do silenciamento gênico de PCN através da tecnologia ATMT em *P. brasiliensis*, observamos redução *down-regulada* de PCN por mRNA, redução da atividade N-acetilglicosaminidase e *western blot*; ii) o silenciamento de PCN não afetou o crescimento de *P. brasiliensis*, mas levou ao aglomeramento das células fúngicas, culminando na inibição da transição levedura-hifa; iii) o silenciamento da PCN favoreceu a suscetibilidade de *P. brasiliensis* à morte por macrófagos, sugerindo que PCN seja um fator de virulência nesse fungo; iv) a infecção murina com leveduras silenciadas para PCN reduziu a recuperação fúngica e a injúria pulmonar nesses animais; e v) o silenciamento de PCN afetou a patogenicidade, corroborando com a ideia de que essa quitinase é um importante fator de virulência em *P. brasiliensis*.

Concluimos que PCN contribui para a virulência e estabelecimento da infecção por *P. brasiliensis*.

4. Capítulo IV

Título: Paracoccin overexpression in *Paracoccidioides brasiliensis* reveals the influence of chitin hydrolysis on fungal virulence and host immune response.

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Periódico: Manuscrito em fase final de preparação.

Paracoccin overexpression in *Paracoccidioides brasiliensis* reveals the influence of chitin hydrolysis on fungal virulence and host immune response

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ABSTRACT

Paracoccidioides genus includes the thermo-dimorphic species *P. brasiliensis* and *P. lutzii*, which are the etiological agents of paracoccidioidomycosis (PCM), a systemic mycosis endemic in several Latin America countries. These thermo-dimorphic fungi develop as filamentous at 25 °C to 30 °C and as yeast at 35 °C to 37 °C. Few *Paracoccidioides spp* proteins are well characterized. We have been studying one of these fungal components, Paracoccin (PCN) from *P. brasiliensis*. The relevant roles played by PCN in the biology of the fungus and its relationship with host innate immune cells have encouraged us to perform studies that revealed the PCN gene as annotated as PADG_03347, which encodes a 218 amino acids multi-domain translated protein. Its cloning and heterologous expression allowed identifying its lectin, enzymatic and immunomodulatory properties. Recently, we employed the system based on *Agrobacterium tumefaciens*-mediated transformation (ATMT) to manipulate *P. brasiliensis* yeast genes and obtain PCN-knocked clones, which are unable to transit from the yeast to mycelium forms and causes a mild pulmonary disease. Herein we report the development of *P. brasiliensis* clones that overexpress PCN and the use of these transformed organisms to investigate the role played by PCN in the fungal biology and pathogenesis, as well as the mechanisms accounting for the PCN-overexpression effects on the infection course. Augmented detection of PCN mRNA, NAGase activity, and PCN protein on the yeast cells wall and the supernatant of yeasts culture confirmed the occurrence of PCN-overexpression

(ov-PCN) in *P. brasiliensis* yeasts. The transformation did not affect the growth nor the viability of the yeasts and favored their separation in culture. The ov-PCN clones transitioned faster to mycelium than the WT yeasts. When infected with ov-PCN *P. brasiliensis*, mice displayed increased fungal burden and mortality. Among the infected mice with WT-yeasts, 90 % survived throughout 70 days post-inoculation, while 100 % of the mice infected with ov-PCN yeasts died from the 27th to the 35th-day post-inoculation. The investigation on the mechanisms accounting for augmented virulence and pathogenesis of ov-PCN revealed that they are more resistant to phagocytosis and killing by host macrophages. Moreover, a second mechanism concerns the size of the chitin particles resultant from the enzymatic cleavage. The very small chitin particles isolated from the ov-PCN yeasts cultures induced host macrophages to augment the IL-10 production, whereas the particles with a wider range of sizes harvested from WT-yeasts cultures induced augmented TNF- α and IL-1 β production. The anti-inflammatory microenvironment resultant from the macrophages stimulation with small chitin particles disfavors the development of a protective immune response against the fungus. Then, we postulate that the high grade of chitin cleavage, provided by augmented PCN expression, engages a relevant mechanism of augmenting pathogenesis following *P. brasiliensis* infection and reinforces the notion that PCN is a relevant virulence fungal factor.

Keywords: ATMT; fungal virulence; paracoccin overexpression; paracoccin; *Paracoccidioides brasiliensis*; paracoccidioidomycosis

INTRODUCTION

Paracoccidioidomycosis (PCM) is a severe mycosis widespread in Latin America (San-Blas, Niño-Vega et al. 2002). The fungi that cause PCM belong to the genus *Paracoccidioides*, which include two species of thermo-dimorphic fungi developing as filamentous at 25-30°C, mainly in soil, and assuming the yeast form at 35-37°C (Rappleye and Goldman 2006). *Paracoccidioides lutzii* is endemic in the Midwest and Southeast regions of Brazil, whereas

57 *Paracoccidioides brasiliensis* founded in the southeast and north of the country (Teixeira,
58 Theodoro et al. 2009; Theodoro, Teixeira Mde et al. 2012; Mendes, Klafke et al. 2017).
59 The virulence mechanisms of *Paracoccidioides spp* are poorly known; it is related to the fact
60 that few fungal components have been characterized. Among them, we highlight the
61 glycoprotein **gp43** (Puccia, Schenkman et al. 1986; De Camargo, Unterkircher et al. 1988;
62 Giannini, Bueno et al. 1990; Travassos, Puccia et al. 1995; Cisalpino, Puccia et al. 1996), **Hsp60**
63 (Izacc, Gomez et al. 2001; Cunha, Zancope-Oliveira et al. 2002; Fernandes, Oliveira et al.
64 2016), **Pb40** (Fernandes, Martins et al. 2012), **Pb27** (Fernandes, Martins et al. 2011), and
65 **Paracoccin** (PCN) (Fernandes, Oliveira et al. 2017). We described PCN from *P. brasiliensis*
66 as a major protein component of a GlcNAc-binding fraction of yeast extracts (Coltri, Casabona-
67 Fortunato et al. 2006; dos Reis Almeida, de Oliveira et al. 2010). The preparations obtained by
68 affinity to immobilized GlcNAc or chitin allowed us to identify several properties of PCN: (i)
69 contributes to the fungal adhesion to extracellular matrix (ECM) components, such as laminin,
70 in a carbohydrate recognition- and concentration-dependent manner (Coltri, Casabona-
71 Fortunato et al. 2006); (ii) induces macrophages to produce TNF- α and nitric oxide (NO)
72 (Coltri, Casabona-Fortunato et al. 2006); (iii) exerts N-acetylglucosaminidase (NAGase)
73 activity, which accounts for the yeast growth and cell wall biogenesis (Ganiko, Puccia et al.
74 2007; Dos Reis Almeida, Carvalho et al. 2011).

75 The only known molecular system of *Paracoccidioides spp* genetic manipulation is based on
76 *Agrobacterium tumefaciens*-mediated transformation (ATMT). It was adopted by the few
77 research groups working on transformation of these fungi (Almeida, Carmona et al. 2007;
78 Almeida, Cunha et al. 2009; Hernandez, Almeida et al. 2010; Menino, Almeida et al. 2012;
79 Menino, Saraiva et al. 2013; Torres, Hernandez et al. 2013; Torres, Hernandez et al. 2014;
80 Marcos, Silva Jde et al. 2016; Tamayo, Munoz et al. 2016). The method uses T-DNA binary
81 vectors harboring user-defined genetic constructs, which have already allowed the expression

of an exogenous marker, the green fluorescent protein (GFP) (Almeida, Carmona et al. 2007) or to down-regulate gene expression by antisense RNA (*aRNA*) (Menino, Almeida et al. 2012). The publication of the complete genome sequence of three isolates (Pb18, Pb03 and Pb01) from the *Paracoccidioides* genus (Desjardins, Champion et al. 2011; Munoz, Gallo et al. 2014) permitted further improvement of the mentioned methodology. In recent studies (Marcos, Silva Jde et al. 2016; Tamayo, Munoz et al. 2016), strains with downregulation of Pb14-3-3 and PbSOD3 genes were developed by using *aRNA* through ATMT technology. It was shown that the Pb14-3-3 contributes for the fungal attachment to ECM/pneumocytes and fungal virulence (Marcos, Silva Jde et al. 2016), whereas PbSOD3 is essential for events underlying host-pathogen interaction (Tamayo, Munoz et al. 2016).

Recently, our group achieved to knockdown the PCN expression. Working with the obtained *P. brasiliensis* transformed yeasts, we verified that PCN is essential for the fungal morphological transition and virulence (Fernandes, Oliveira et al. 2017). To date, overexpression of endogenous components has not been explored in *Paracoccidioides spp.* In this work, we developed overexpression of PCN in *P. brasiliensis* yeasts, which were used to investigate the PCN role in the fungal biology and fungus-host interaction, revealing also mechanisms accounting for the severe pulmonary disease caused by the mice infection with the transformed yeasts.

100

101 MATERIAL AND METHODS

102 Ethics statement

103 The in vivo experiments detailed protocols were approved by the Committee for Ethics in
104 Animal Research (CETEA) of the Ribeirão Preto Medical School, University of São Paulo. All
105 efforts were done to minimize suffering, and the animal experiments were conducted in

106 accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of
107 Animal Experimentation (COBEA) (Protocol 61/2016-1).

108 **Microorganisms and culture medium**

109 All strains used in this study are listed in Table 1. *P. brasiliensis* yeast cells were maintained at
110 36 °C by periodic subculturing on brain heart infusion (BHI) medium (Duchefa, The
111 Netherlands), supplemented with 1 % glucose and 1.5 % v/v agar or Dulbecco's modified
112 Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA). For the assays performed in
113 this study, yeast cells grew in liquid BHI broth at 36 °C with aeration on a mechanical shaker
114 at 200 rpm. Their viability was evaluated by fluorescein diacetate and ethidium bromide
115 staining (Calich, Purchio et al. 1979) and only yeasts suspensions with viability higher than 90
116 % were included in the study. The LBA1100 strain of *A. tumefaciens* was used as the recipient
117 of the binary vectors herein constructed (Menino, Almeida et al. 2012). Bacterial cells were
118 maintained at 28 °C in Luria Bertani (LB) broth (Kasvi, Italy), containing kanamycin (50
119 µg/ml), spectinomycin (250 µg/ml) and rifampicin (20 µg/ml). *Escherichia coli* DH5α
120 competent cells, grown at 37 °C in LB broth supplemented with antibiotics (kanamycin 50
121 µg/ml; ampicillin 100 µg/ml) were used as hosts for plasmid amplification and cloning.

122 **Construction of ov-PCN cassettes**

123 Plasmids and genomic DNA extraction, recombinant DNA manipulations, and *E. coli*
124 transformation procedures were performed as described elsewhere (Almeida, Carmona et al.
125 2007). DNA from *P. brasiliensis* strain Pb18 was extracted from yeast cultures during
126 exponential growth, and a high-fidelity proofreading DNA polymerase (NZYTech, Portugal)
127 was employed to amplify 1025 bp with exon and intron sequences corresponding to
128 PADG_03347 (Gene ID: 22582669) sequence. The primer sequences were synthesized by
129 Sigma-Aldrich (USA), and the sequence is gParacoccin forward (5'-
130 GGCGCGCCATGGCCTTCGAAAATCAG-3') and gParacoccin reverse (5'-

CTCGAGTTACCATGAACTCGTCGA-3'). PCN cassette was inserted in a pCR35-RHO2 vector under control of the Rho (Ras homology) GTPase 2 (*RHO2*) promoter region from *P. brasiliensis*. The pCR35 plasmid was digested with the restriction enzymes *AscI* and *XhoI*. The amplified *P. brasiliensis* PADG_03347 sequences were digested with the same enzymes and cloned into *AscI-XhoI* digested pCR35. Genomic PCN expression cassettes were amplified with the primers gParacoccin forward and reverse, and digested with the *KpnI* restriction enzyme. Subsequently, they were cloned into the T-DNA region of the binary vector pUR5750, previously digested with *KpnI*. Resulting vectors were introduced into *A. tumefaciens* LBA1100 ultracompetent cells by electroporation, as previously described (Almeida, Carmona et al. 2007). Transformants were isolated by selection on kanamycin at 100 µg/ml.

ATMT of *P. brasiliensis*

Insertion of recombinant T-DNA harboring the ov-PCN cassettes and a hygromycin B resistance marker into the genome of *P. brasiliensis* yeast cells was accomplished by ATMT (Almeida, Carmona et al. 2007; Michielse, Hooykaas et al. 2008). Briefly, *A. tumefaciens* strains carrying the binary vector pUR5750 harboring the ov-PCN cassettes were grown in LB broth containing kanamycin (100 µg/ml), rifampicin (20 µg/ml), and spectinomycin (250 µg/ml) at 29 °C with aeration in a mechanical shaker at 150 rpm overnight. Bacterial cells were spun-down, washed with induction medium (IM) (Almeida, Carmona et al. 2007; Michielse, Hooykaas et al. 2008) and resuspended in 10 ml of IM added of 0.2 M acetosyringone (AS) (Sigma-Aldrich, USA) and antibiotics. Cells grew until providing an optical density (OD₆₀₀) of approximately 0.8. Then, yeast cells were grown in BHI broth and harvested during the exponential growth phase. The cells were washed with IM and adjusted to a final concentration of 1×10⁸ cells/mL, estimated by using direct microscopic counts in a Neubauer chamber. For co-cultivation, 1:1 and 10:1 ratios of *A. tumefaciens* and *P. brasiliensis* cells were spotted on sterile Hybond N filters (Amersham Biosciences, USA), in solid IM with AS and antibiotics,

dried in a safety cabinet for 30 min in the dark and incubated for 3 days at 25 °C. Membranes were then transferred to BHI broth containing cefotaxime (200 µg/ml), and cells were dislodged with the aid of a spatula before incubated for 48 hours at 37 °C in a shaker (220 rpm). *P. brasiliensis* cells were then plated on BHI broth, supplemented with the appropriate antibiotic (hygromycin 75 µg/ml), and grown at 37 °C for 15 days. The obtained transformed yeasts were kept for further analysis. We used *P. brasiliensis* yeasts transformed with an empty vector (EV) pUR5750 as a control in assays carried out in this study.

Gene and ov-PCN analysis

Yeast cells of *P. brasiliensis* obtained from a single colony were inoculated and cultured in BHI broth at 37 °C (200 rpm), until the exponential growth phase. The culture medium was refreshed once, after 4 days. Total RNA obtained from ov-PCN and WT yeasts and isolated according to the Trizol protocol (NZYTech, Portugal). The RNA samples were subsequently incubated for 20 min at 37 °C with 2 U of DNase I (Roche, Germany). The absence of DNA contamination in the samples was checked by the lack in the isolated RNA of conventional PCR amplification of the *GP43* gene. Total RNA (1 µg) was reversely transcribed using the NZYTech Reverse Transcriptase cDNA Synthesis kit (NZYTech, Portugal) according to the manufacturer's instructions. As a template for real-time quantification, cDNA (1 µL) of was used in the SoFast EvaGreen SuperMix (Bio-Rad, USA) according to the manufacturer's instructions. Quantitative Real-time was carried out on a CFX96 Real-Time System (Bio-Rad, USA), using threshold cycle (Ct) values for β -tubulin (*TUB2*) and ribosomal protein (*L34*) transcripts as endogenous references. The primer sequences were synthesized by (NZYTech, Portugal) and the sequence is β -tubulin forward (ACGCTTGCGTCGGAACATAG), β -tubulin reverse (ACCTCCATCCAGGAAGCTCTTCA), *L34* forward (CGGCAACCTCAGATACCTTC) and *L34* reverse (GGAGCCTGGGAGTATTCACG). mRNA differential ov-PCN was evaluated by

normalizing gPCN Ct values with the reference and comparing the ratio amongst the tested samples. All the measurements were performed in triplicate.

Molecular Detection of the Hygromycin Resistance Gene (HPH)

Genomic DNA from ov-PCN yeasts and controls yeast cells were isolated according to the glass beads protocol described by Van Burik (van Burik, Schreckhise et al. 1998). In order to confirm the presence of the hygromycin B resistance cassette, PCR analysis was carried out to detect an HPH amplification product (1000-bp) using primers hph forward (AACTCACCGCGACGTCTGTCTGA) and hph reverse (CTACACAGCCATCGGTCCAGA) (data not shown). PCR amplification included 30 cycles of 5 min at 94 °C, for denaturing, 40 s at 55 °C for annealing, and 1 min 10 s at 72 °C for an extension. The reaction products were analyzed on 1 % agarose gel and visualized with SYBR Green, under UV light.

Enzymatic activity of ov-PCN yeasts

The N-acetyl- β -D-glucosaminidase activity of ov-PCN yeasts was assayed as previously described (dos Reis Almeida, de Oliveira et al. 2010; Almeida, Sardinha-Silva et al. 2015). Briefly, the substrate p-nitrophenyl-N-acetyl- β -D-glucosaminidase (100 μ L, 5 mM; Sigma–Aldrich) was mixed with of 0.1 M sodium acetate, pH 5.5 (350 μ L) and a sample of the supernatant cultures (50 μ L). As a negative control, we used the vehicle medium. The reaction was incubated for 16–18 h at 37 °C, in medium to which we have added 0.5 M sodium carbonate (1 mL). The enzyme activity values were estimated by using a microplate reader at 405 nm (Power Wave X, BioTek Instruments, Inc.). The quantitation of NAGase activity in the supernatant of WT and transformed yeasts cultures was considered as expressing the relative concentration of PCN produced by each *P. brasiliensis* strain.

Growth curve and viability assay

Growth curves were performed in DMEM medium (10 mL) by inoculating, at 48 h, washed fungal cells (1×10^6); the DO_{600nm} was adjusted to reach the value 0.2. Cellular density was

measured in triplicates in a spectrophotometer (Ultrospec[®]3000pro, GE), at 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 hours of growth, for WT and ov-PCN yeasts of the strain Pb18, as previously described (Torres, Hernandez et al. 2013). The viability of WT and ov-PCN yeasts was determined by fluorescein diacetate-ethidium bromide staining (Calich, Purchio et al. 1979) and detected by fluorescence microscopy (excitation bands: blue-BP480/40; green-BP515-560) (DMI6000B, Leica). Only suspensions containing at least 90 % of viable yeasts were included in the studies. The experiments were performed in triplicate.

Fungal phagocytosis and killing by murine macrophages

Yeast cells from ov-PCN and WT strains, grown in BHI broth at 37 °C for 72 hours (exponential phase), were co-cultured with RAW 264.7 murine macrophages (obtained from the American Type Culture Collection [ATCC]) were cultivated in complete RPMI (Sigma-Aldrich®), supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine and 100 µg/mL streptomycin/ampicillin. The fungal inoculum was prepared as described for the previous item. To assess their phagocytosis, the macrophages were distributed (5×10^5 cells/well) in a 24-well microplate (Costar, Corning Inc., Corning, NY, USA) and incubated with yeast cells (5×10^4 yeasts/well) for 4 h at 37 °C (yeast-to-macrophage ratio of 1:10) in a 5 % CO₂ atmosphere, the co-culture supernatant was discarded. The macrophage monolayers were lysed with ice-cold water, and the cell lysate was plated on BHI agar broth supplemented with 1 % glucose, for 7 days at 37 °C.

To estimate the macrophage fungicidal activity, viable yeast cells were co-cultured with macrophages at 37 °C in a 5 % CO₂ atmosphere for 4 hours. The cultures supernatant was discarded, and the cells were gently rinsed with PBS and incubated in RPMI (Sigma-Aldrich®) supplemented with 10 % FBS (HyClone) at 37 °C in a 5 % CO₂ atmosphere. After 48 hours of incubation, the culture supernatant was discarded, macrophages were lysed using ice-cold water, and the cell lysate was serially diluted and cultured, for 7 days at 37 °C, in solid BHI

230 broth supplemented with 1% glucose. The analysis was performed by counting the colony
231 forming units (CFU).

232 **Mice infection**

233 We used male BALB/c mice, 6–8 weeks aged, obtained from the animal facility of the
234 University of São Paulo at Ribeirão Preto, São Paulo, Brazil. They were maintained in the
235 animal facility of the Cell and Molecular Biology Department, Ribeirão Preto Medical School,
236 University of São Paulo, under optimized hygienic and temperatures conditions. We performed
237 the infection through intranasal on intravenous inoculation of yeast cells, collected from
238 exponentially growing batch cultures in BHI broth, and counted in Neubauer chamber. Animals
239 of each group (n=5) were inoculated with 2×10^6 yeast cells from the WT or ov-PCN *P.*
240 *brasiliensis* strains, contained in 40 μ L PBS, as specified in Table 2. Uninfected control mice
241 were inoculated with vehicle alone, under the same conditions as the infected group. At 30 days
242 post-infection, mice were euthanized and their lungs were harvested for analysis. For the
243 survival studies, each mouse of a group (n=10) was intravenously inoculated with 1×10^6 yeast
244 cells of WT or ov-PCN strain, contained in 15 μ L PBS (Tab. 3). Mice were monitored daily for
245 death occurrence during a 70 days period post-infection.

246 **Pulmonary fungal burden in ov-PCN infected mice**

247 The pulmonary fungal burden was evaluated by counting the colony-forming units (CFU)
248 recovered from a similar lung fragment of each infected-mice. The fragment was aseptically
249 removed, weighed, and homogenized in 1.0 mL sterile PBS in a tissue homogenizer (Ultra-
250 Turrax T25 Basic; IKA Works, Inc., Wilmington, USA). The supernatant was plated on solid
251 BHI medium supplemented with 4 % (v/v) heat-inactivated fetal bovine serum (Invitrogen, Life
252 Technologies, Camarillo, CA, USA) and gentamicin at 96 μ g/mL (Gibco, Grand Island, USA).
253 The plates were incubated at 36 °C for 7 days, when the colonies were counted.

254

255 Lung histopathological analysis

256 An excised lung of each mouse was fixed in 10 % para-formaldehyde for 24 hours, dehydrated
257 in ethanol, diaphanized in xylene, and embedded in paraffin. Histological sections of 6 μm
258 thickness were hematoxylin-eosin stained for histological analysis. Images were acquired in a
259 ScanScope multi-user scanner Lab, using an optical microscope (Olympus VS120 in a BX 61
260 microscope, Axiophot Photo microscope; Carl Zeiss GmbH, Germany) and a camera (JVC TK-
261 1270; Victor Company of Japan Ltd., Japan).

262 Fluorescence labeling multifoton microscopy

263 Multiphoton microscopy was performed on yeasts that were harvested from WT, empty vector
264 and ov-PCN yeasts, grown in BHI (Kasvi) at 37 °C for 72 h (exponential phase) and separated
265 by centrifugation at $4,000 \times g$ at 25 °C for 6 min. Yeast cells were fixed using 3.7 % para-
266 formaldehyde in phosphate-buffered saline (PBS) pH7.2 at 25 °C. After 1 h, yeasts were washed
267 with PBS containing 1 % Glycine. For the labeling, blocked samples with 1 ml PBS containing
268 1 % BSA, at 25 °C for 1 h, were incubated with biotin-conjugated anti-PCN IgY (1:50) for 1 h
269 at 25 °C. Then, the yeast cells were incubated with streptavidin Alexa 594-conjugated (Thermo
270 Fisher Scientific), for 1 h at 25 °C. The cells images were acquired in the LSM 780
271 AxioObserve Inverted Microscope (Carl Zeiss, Jena, Germany), available in a multi-user
272 institutional facility, installed in the Cell and Molecular Biology Department. For all samples
273 obtained from WT, EV and ov-PCN yeasts, identical photomultiplier gain and laser power were
274 employed. Images were recorded and analyzed offline with the aid of the software ImageJ [(W.
275 S. Rasband/National Institute of Health, Bethesda, MD, USA (<http://rsb.info.nih.gov/ij/>)).

276 Chitin capture from supernatants of yeasts cultures

277 Wheat Germ Agglutinin (WGA) (Sigma Aldrich®- Saint Louis, MO 63103, USA) was coupled
278 to CNBr-Activated Sepharose 4B beads (GE Healthcare), according to the manufacturer's
279 instructions. The conjugation yield was about 90 % (4.5 mg protein per ml of resin). The lectin-

resin coupling was stabilized through crosslinking with glutaraldehyde (Scher, Resneck et al. 1989). Two columns of 1mL resin were packaged, each one dedicated to a kind of sample (chitin derived from WT or transformed yeasts, respectively). The yeasts cultures (ov-PCN or WT yeasts) were grown in BHI broth, for seven days, 37 °C, 180 rpm. After of centrifugation of the cultures at 3300 x g for 10 min, at room temperature, the cultures supernatants were loaded (10 mL) in the columns. After incubation for 90 min, under slow rotation at room temperature, the unbound material was eluted with PBS while the bound material was subsequently eluted with 0.5 M NaCl in PBS. The PBS-eluate material was centrifuged and re-chromatographed (same protocol) to overcome an eventual overloading of the column in the first procedure. The NaCl 0.5 M-eluate (WGA-bound material) was ultradialyzed against water (OS20LX) in a 0.05 µm VM membrane (Millipore®). The resultant preparations derived from WT- and ov-PCN yeasts were stored at 4°C. They were named WT-chitin and ov-PCN chitin.

Size of chitin particles and thickness cell wall determined by transmission microscopy

The column containing immobilized WGA was used for purification of chitin particles ov-PCN and WT yeasts (fixed in glutaraldehyde 2% and para-formaldehyde 4%, and prepared on SPURR resin as according to the manufacturer's instructions). Chitin particles and yeasts were analyzed by transmission electron microscopy (*Jeol JEM-100 CXII equipped with Hamamatsu digital camera ORCA-HR*), (Magnification 20,000, 80,000 and 200,000 x). The images were analyzed in ImageJ software (Image-adjust-threshold- (select the area) -analyze particles) and the thickness of the cell wall in the ov-PCN and WT yeasts was determined.

Bone marrow-derived macrophages (BMDMs) activation by chitin particles

BMDMs were prepared from BABL/c mice femurs and tibias after flushing with RPMI medium to release the bone marrow cells. These cells were cultured in 100 mm x 20 mm suspension culture dish (Corning, Corning, NY, USA) with 10 mL of RPMI 1640 broth supplemented with

305 supernatant from L929 cell cultures (20% FCS and 30% supernatant from L929 cell cultures,
306 LCCM, L929-cell conditioned medium). In the fourth day, 10 mL of RPMI medium
307 supplemented with supernatant purified from L929 cell cultures were added. In the seventh day,
308 the non-adherent cells were removed, and the dishes were washed with PBS. After that, the
309 adherent cells (macrophages) were removed by heat shock with cold PBS (for 10 min) and
310 washed for twice with PBS. The concentration of the cells was determined in a Neubauer
311 chamber, and the BMDMs (1×10^6 /mL) were cultured in 96-well microplates for 24, 48 and 72
312 hours at 37 °C in the presence with a pool of particles (0.5, 1.5, 5, 10 and 20 μ g in 200 μ L) or
313 medium alone. The supernatant was used for quantification of cytokines.

314 **Quantification of cytokines**

315 Supernatants of stimulated of BMDMs macrophages were assessed for their levels of TNF- α ,
316 IL-1 β , and IL-10. The cytokines were detected by an enzyme-linked immune sorbent assay
317 (ELISA) using an OptEIA kit (Pharmingen, SanDiego, CA, USA), according to the
318 manufacturer's instructions. Standard curves allowed determining cytokine concentrations in
319 pg/mL. The absorbance was read at the 450nm filter in the Power Wave X microplate scanning
320 spectrophotometer (BioTek Instruments, Inc.).

321 **Western Blot**

322 The *P. brasiliensis* wild type (WT) yeasts grown in BHI broth at 37 °C for 72 hours (exponential
323 phase). After, the WT yeasts were co-cultured of THP-1 human lineage cells during 24 hours.
324 The western blot analyzes were performed by the use of 12 % SDS-PAGE to separate the
325 soluble elements in supernatants of co-cultures. The components were electrophoretic run the
326 proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-
327 PTM, Amersham GE Healthcare) at 50 V and current of 90 mA overnight using transfer buffer
328 (1.9 % tris base, 9.1 % glycine). The non-specific interactions were blocked by incubating the
329 membranes with TBS-T 1 x (20 mM Tris-HCl, 150 mM NaCl, 0.05 % Tween 20 [pH 7.6])

330 containing 5 % skim milk powder (Molico®) for 1 h under slow stirring at room temperature.
331 Subsequently, the membrane was incubated with anti-PCN IgY polyclonal antibody diluted
332 1:3,000 in TBS-T. The membrane was washed five times with TBS-T and incubated for 1 hour
333 at room temperature with the anti-IgY secondary antibody conjugated to peroxidase (Sigma-
334 Aldrich®) diluted 1:1,000. After five washes with TBS-T were performed the membrane was
335 immersed in a fresh mixture of DAB peroxidase substrate kit SK4100 (Vector Laboratories,
336 Burlingame, CA). Distilled water was used to stop the reaction. The images were analyzed
337 using the chemiDocTMMP Imagin System Bio-Rad instruments.

338 **Carbohydrate quantification**

339 The samples' carbohydrate content was determined through the modified Dubois Colorimetric
340 Method (Dubois, Gilles et al. 1951), which was especially useful to analyze the chitin purified
341 preparations. The standard curve concentration was constructed by serially diluting in water a
342 starting solution of glucose (5 mg/mL). The reaction was performed in 2 mL microtubes by
343 using 500 µL samples, concentrated sulfuric acid (800 µL) and an 80 % phenol solution (50
344 µL). Optical density (490 nm) was read in a spectrophotometer Power Wave X microplate
345 scanning spectrophotometer (BioTek Instruments, Inc.). All reactions were done in triplicates.
346 The sugar concentration was expressed as micrograms.

347 **Statistical analysis**

348 All statistical analysis was performed by using the GraphPad Prism Software version 6.0. Data
349 were reported as the mean $\pm$ standard error of the mean (SEM). All the results of *in vivo* and *in*
350 *vitro* assays are representative of three independent assays. Five or ten animals constituted
351 each control or experimental group. For all data analysis, statistical significance was considered
352 at the level of followed by Bonferroni's test or Mann-Whitney test. Differences with
353 **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.1$ were considered statistically significant.

354

355 RESULTS

356 Increased Paracoccin expression by co-culturing *P. brasiliensis* yeasts with macrophages

357 In a recent study, we compared the PCN transcripts in different morphotypes of WT *P.*
358 *brasiliensis*. Although detected in all fungal forms, the PCN mRNA expression was higher in
359 hypha and yeast-to-hypha transition forms (Oliveira, Fernandes et al. 2017). Herein we
360 investigated the PCN expression in yeasts that were co-cultured for 24 hours with THP-1
361 human macrophages. The PCN expression was 9,000 fold higher than the expression exhibited
362 by yeasts that were cultured in the absence of macrophages (Fig. 1A). In addition, as evaluated
363 by Western blot, the paracoccin protein content was not detectable in the supernatant of yeasts
364 cultured alone and well visualized in the supernatant of yeast/macrophage co-culture (Fig. 1B).
365 These results motivated us to investigate the implications of PCN overexpression in the fungal
366 biology and host immunity.

367 Characterization of PCN-overexpressing yeasts generated by the *Agrobacterium* 368 *tumefaciens*-mediated transformation system

369 PCN is structurally related mostly to chitinases from *Histoplasma capsulatum* (83 % similarity),
370 *Blastomyces dermatitidis* (81 % similarity) and class III ChiA1 of *A. nidulans* (83 % similarity)
371 (NCBI – National Center for Biotechnology Information). To generate PCN-overexpressing *P.*
372 *brasiliensis* yeasts, we used the *Agrobacterium tumefaciens*-mediated transformation (ATMT)
373 methodology, after cloning the genomic sequence of a hypothetical sequence PADG_03347
374 (gPCN) under the RHO2 promoter control in transfer DNA (T-DNA). The yeast cells of Pb18
375 wild type strain were transformed with T-DNA containing gPCN (Fig. 2A) or empty vector.
376 The obtained transformants were verified for the T-DNA integration of the hygromycin
377 phosphotransferase (HPH) cassette in the genomic DNA of six different transformants (data not
378 shown). Positive clones were proved to have mitotic stability in serial subcultures grown in
379 selective BHI broth containing hygromycin B (HygB) (data not shown). We demonstrated, by

comparing PCN mRNA levels determined by qRT-PCR, that the transformed yeasts expressed 1.5 to 7 fold more PCN than the WT yeasts (Fig. 2B). The ones with high PCN expression were individually named according to the fold changes in PCN expression they presented in comparison with WT yeasts (gPCN7 > gPCN6 > gPCN4) and selected to be used in the subsequent experiments. We estimated their level of PCN protein detection by quantifying the N-Acetyl- β -D-glucosaminidase activity (dos Reis Almeida, de Oliveira et al. 2010) in the supernatant of the yeasts cultures. As shown in Figure 2C, the supernatants of the cultures of the gPCN yeasts provided significantly higher enzymatic activity than the detected in the supernatant of WT control yeasts. Once substantiated the functionality of the PCN protein expressed in the transformed yeasts, we studied the growth, viability, and size of the gPCN yeasts.

To compare the growth curve of the gPCN and WT yeasts, the cellular density of cultures (37°C, for 10 days) was estimated by regular readings of the optical density at DO_{600nm} of the cultures. It resulted in overlapped time-course curves for all the transformed and WT yeasts, in a demonstration that PCN overexpression did not affect the fungal growth. We also examined the cultured fungi cells for their viability by fluorescence microscopy, after stained with diacetate fluorescein and ethidium bromide, which revealed viability superior to 90 % for the gPCNs or WT yeasts (Fig. 2D). From this point of the study, because we verified no significant difference among the three clones of transformed yeasts regarding their general characteristics, we started working only with gPCN7, which became identified simply as ov-PCN yeast. The measurement of the total area occupied by the mother plus daughter yeast cells showed that ov-PCN yeasts had a smaller size than the WT or EV-transformant yeasts (Fig. 2 E and F). We inferred that the low size of the ov-PCN mother and daughter cells did not affect the yeast's growth or viability, which reproduced the WT yeasts patterns (Fig. 2D).

PCN was quantified in ov-PCN and WT yeasts by multiphoton microscopy. After incubation with IgY anti-PCN conjugated to Alexafluor 294, the yeasts samples had PCN quantified by measuring the fluorescence intensity, with the aid of ImageJ software. As expected, fluorescence was highest in the ov-PCN yeasts, compared to WT and EV control yeasts (Fig. 2G). The yeasts samples were also analyzed for the PCN distribution, as well as for its binding to chitin, a previously reported PCN property (dos Reis Almeida, de Oliveira et al. 2010) that accounts for its localization in the fungal cell wall (Oliveira, Fernandes et al. 2017). In both, ov-PCN and WT yeasts, PCN was faintly detected along the yeast cell wall, and primarily concentrated in the budding regions (Fig. 2H). These fungal structures were more intensely fluorescent in ov-PCN than in WT yeasts.

Recently, we have shown that PCN-silenced yeasts of *P. brasiliensis* are unable to perform the morphological transition from yeast to mycelium (Fernandes, Oliveira et al. 2017). Herein we studied the transition of ov-PCN yeasts by quantitative optical microscopy, during a 7 days (168 h) period following the shift of cultures temperature from 37 to 25° C. We observed that, at 24 h, more than 20 % of the ov-PCN yeasts showed morphological signs of differentiation, which increased to 50 % at 72 h. The yeasts transition to mycelium was complete during the experimental period of seven days for 90 % of the ov-PCN fungal cells. Meanwhile, the control cultures of WT-yeasts or EV-transformed yeasts required 96 to 120h to have 50 % of fungal cells with morphological signs of differentiation; furthermore, during the total experimental period of one-week, differentiation had not even started for 10 to 20 % WT yeasts (Fig. 2I). These results show that ov-PCN yeasts transitioned quicker than the WT or EV-transformed yeasts. Taken together with the knowledge derived from the study on PCN-silenced yeasts (Fernandes, Oliveira et al. 2017), we demonstrate that PCN is critical for the occurrence of *P. brasiliensis* morphological transition from yeast to mycelium.

The results presented herein are consistent with those of previous and still incipient studies on the PCN contribution for the *P. brasiliensis* yeasts growth and cell wall biogenesis (Ganiko, Puccia et al. 2007; Dos Reis Almeida, Carvalho et al. 2011; Oliveira, Fernandes et al. 2017).

Pathogenic features of the mice infection with PCN-overexpressing yeasts

Because the *P. brasiliensis* PCN-knocked yeasts are less virulent than the WT-yeasts, we thought whether ov-PCN yeasts could promote a murine infection with a distinct course of the caused by WT-yeasts. The lungs of BALB/c mice, inoculated through the intranasal via with 2×10^6 cells of ov-PCN or WT yeasts were microscopically examined, thirty days after infection, regarding the extension of the granulomatous lesions. In mice that were infected with WT yeasts, small and circumscribed granulomas were focally distributed in the pulmonary tissue, whereas in mice infected with ov-PCN yeasts the granulomas were large and coalescent, occupying an extended area of the lungs (Fig. 3A). Methenamine/silver-staining of the pulmonary sections revealed few and focally distributed viable yeasts, more centrally localized in the small and compact granuloma of WT yeasts-infected mice, while abundant viable yeasts were dispersed in all the area of coalescent granulomatous lesions of the infected-mice with ov-PCN yeasts (Fig. 3B). Morphometric analysis of pulmonary tissue injury showed that the area occupied by lesions was 60 % larger in mice that were infected with ov-PCN yeasts than in WT- yeasts infected-mice (Fig. 3C). Pulmonary CFU counting has quantitatively validated the results obtained by optical microscopy: the number of colonies provided by the ov-PCN yeasts infected-mice was at least one order of magnitude higher than the given by WT yeasts infected mice (Fig. 3D). Concerning the mice survival to the fungal infection, we observed that all animals infected with WT yeasts survived throughout a 40 days post-infection period, whereas the infected with ov-PCN yeasts started dying at day 27 post-infection and no one has survived to a 35 days post-infection period (Fig. 3E). We concluded that PCN-overexpression aggravates

the disease caused by *P. brasiliensis* yeasts, reinforcing the notion that PCN acts as a *P. brasiliensis* virulence factor (Fernandes, Oliveira et al. 2017).

The effect of PCN overexpression on the chitin content of the yeast cells wall

Defined the augmented detection of PCN protein in ov-PCN yeasts (Fig. 2) and considering the already known PCN properties of binding to chitin and exerting chitinase activity (dos Reis Almeida, de Oliveira et al. 2010), we looked for evaluating the chitin content on the cell wall of transformed *P. brasiliensis* yeasts. The staining of ov-PCN, WT, and EV yeasts with calcofluor white allowed detecting chitin by fluorescent multiphoton microscopy (Fig. 4A). With the aid of the ImageJ software, we could verify that chitin detection was reduced by 40 % in ov-PCN yeasts, in comparison to WT-yeasts (Fig. 4B). We also detected the chitin content of yeasts cells by using a TexasRed conjugate to WGA, a highly specific chitin-binding lectin (Itakura, Nakamura-Tsuruta et al. 2017). The analysis by cell cytometry also showed a significant lowering (by 30 %) of the chitin content in ov-PCN yeasts, compared to the detected in WT yeasts, at 72 hours (Fig. 4C). Consistently, the cell wall of WT yeasts, as examined by electron microscopy, was about 6 fold thicker than that of ov-PCN yeasts (Fig. 4D and 4E). Then, the size of chitin particles contained in the supernatant of yeasts cultures and captured by immobilized WGA was analyzed by electron microscopy. The isolated chitin particles had variable sizes, as denoted by measuring their area (nm^2). We detected only particles with less than 60 nm^2 in the material derived from the supernatants of ov-PCN yeasts. This low size range was also prominent in the material obtained from the WT-yeasts; nevertheless, this preparation included a wider size distribution, which included particles with areas as large as 240 nm^2 (Fig. 4F). It is reflected by the median of the particles areas: 2 nm^2 for those derived from ov-PCN yeasts and 6 nm^2 for the WT yeasts particles (Fig. 4F). A second analysis of the chitin particles size was performed by cytometry of the captured fragments from the supernatants of cultures of ov-PCN and WT yeasts. They shared the presence of similar amounts of very small chitin

particles (diameter inferior to 4 μm), which were accompanied, in the case of WT yeasts, by larger chitin particles. A more precise determination of the particles size by cytometry was prejudiced by the unavailability of calibrating commercial beads, whose diameters fit in the low size range of the chitin particles, as directly measured through electron microscopy. In spite of this methodological restriction, the set of results we obtained is appropriate to support the idea that the PCN overexpression optimizes the hydrolysis of the cell wall chitin, favoring the exclusive presence of very small chitin particles into the supernatant of ov-PCN yeasts cultures. Finally, the obtained results consolidate the notion that PCN hydrolyses chitin of the *P. brasiliensis* cell wall, which become reduced in terms of chitin content and width. In addition, the process triggered by PCN promotes the releasing of very small chitin particles in the extracellular milieu.

Macrophages activation by chitin particles from ov-PCN yeasts

It was previously reported that the size of chitin fragments, which results from hydrolysis of whole chitin molecules by chitinase, correlates with the particles property of stimulating macrophages to produce inflammatory or anti-inflammatory cytokines (Da Silva, Hartl et al. 2008; Da Silva, Chalouni et al. 2009; Mora-Montes, Netea et al. 2011; Dubey, Moeller et al. 2014; Wagener, Malireddi et al. 2014; Becker, Aimaniananda et al. 2016; Dong, Tong et al. 2018). Based on these still contradictory studies, we examined whether the chitin fragments we captured from the supernatants of ov-PCN (containing only small chitin fragments) or WT yeasts (containing a large spectrum of small and larger chitin fragments) (Fig. 4) could stimulate distinct responses from murine bone marrow-derived macrophages (BMDMs). Consequently, it could explain the distinct course of the experimental infection caused by *P. brasiliensis* ov-PCN and WT-yeasts. Then, isolated chitin particles of WT and ov-PCN yeasts were assayed for the ability of inducing cytokines production by BMDMs. Dose-response and time-course curves (shown in supplementary data. S. 1A, 1B and 1C) were drawn for TNF- α , IL-1 β and IL-10 cytokines, whose production was tested toward five different chitin concentrations (from 0.5

to 20 μg) during the periods of 24, 48, and 72 hours. The dose-response curves for the levels of TNF- α and IL-10, representing inflammatory and anti-inflammatory cytokines, respectively, are summarized in figure 5D, which shows that the two curves are inverted: lower chitin concentrations correlate with higher IL-10 levels, whereas higher chitin concentrations determine higher TNF- α production (Fig. 5D). The production of the pro-inflammatory cytokines TNF- α (Fig. 5A) and IL-1 β (Fig. 5B), measured in the BMDMs supernatants harvested 72 h after stimulation, were significantly higher when stimulated by chitin particles derived from WT than ov-PCN yeasts. Otherwise, the IL-10 levels measured in the supernatant of BMDMs (Fig. 5C), also harvested 48 hours after stimulation, were significantly higher when stimulated with chitin particles derived from ov-PCN than WT-yeasts. Taken together, these results demonstrate that all the chitin captured from the supernatant of cultures of *P. brasiliensis* WT yeasts, consisted by low and high size particles, used at concentrations higher than 5 μg induce BMDMs to produce the pro-inflammatory cytokines TNF- α and IL-1 β . Inversely, the chitin particles captured from the supernatant of ov-PCN yeasts cultures, consisted exclusively of low size chitin particles, used at concentrations as low as 0.5 to 5 μg , sustained the BMDMs' basal production of the anti-inflammatory cytokine IL-10. Because high levels of anti-inflammatory cytokines in the initial phase of *P. brasiliensis* infection induce the non-protective Th2 immune response (Calich and Kashino 1998), our findings explain, at least partially, the high severity of the pulmonary disease developed in mice that were infected with *P. brasiliensis* yeasts overexpressing the chitinase PCN.

Resistance of PCN-overexpressing yeasts to macrophages effector functions

Searching for mechanisms accounting for the severe course of mice infection with ov-PCN yeasts, compared to WT yeasts, we considered the fact that the *P. brasiliensis* ability of causing disease largely depends on the yeasts resistance to the defense mechanisms provided by host phagocytes (Cano, Singer-Vermes et al. 1995). Then, we assayed *in vitro* the sensitivity of ov-

PCN and WT yeasts to macrophages effector functions. Phagocytosis was examined 4 hours after incubating macrophages with ov-PCN or WT yeasts. We lysed the infected macrophages and plated the lysates to obtain the number of intracellular-CFU recovering. Counts were significantly higher in lysates of infected-macrophages with ov-PCN yeasts than in the infected with WT yeasts (Fig. 6A), indicating that phagocytosis of ov-PCN yeasts is more effective than of WT-yeasts. The fungal killing by macrophages was investigated by obtaining the lysate of macrophages that were 48 hours incubated with yeasts. We recovered a significantly higher CFU number from the cells infected with ov-PCN yeasts than with WT yeasts (Fig. 6B), showing that the transformed yeasts are more resistant to the killing by macrophages. We concluded that macrophages have internalized more efficiently the ov-PCN yeasts, whereas a higher number of *P. brasiliensis* WT yeasts were killed by macrophages. It demonstrates that ov-PCN yeasts survive to the macrophages fungicidal activity. The facilitated phagocytosis of ov-PCN yeasts and their enhanced resistance to the fungicidal activity of macrophages are probably implicated in the worst evolution of mice infection with the *P. brasiliensis* transformed-yeasts.

A model for the mechanism by which PCN acts as a virulence factor of *P. brasiliensis* yeasts

DISCUSSION

This is the first report of a gene overexpression in fungal species of the genus *Paracoccidioides*. The gene (annotated as PADG_03347) codes for Paracoccin (PCN), whose previous cloning and heterologous expression have allowed identifying the protein as multidomain, exerting biological activities of lectin, which binds to GlcNAc and chitin (Coltri, Casabona-Fortunato et al. 2006), enzyme, which hydrolyses chitin (dos Reis Almeida, de Oliveira et al. 2010), and immunomodulatory agent (Alegre-Maller, Mendonca et al. 2014; Alegre, Oliveira et al. 2014).

The yeasts transformation was mediated by *Agrobacterium tumefaciens* (ATMT) (Almeida, Carmona et al. 2007; Michielse, Hooykaas et al. 2008), a system reported as successful when employed to obtain *P. brasiliensis* knocked-down clones for proteins playing relevant roles in the fungal virulence or pathogenesis. This is the case of CDC42 (Almeida, Cunha et al. 2009), PbHAD32 (Hernandez, Almeida et al. 2010), asSconC (Menino, Saraiva et al. 2013), PbGP43 (Torres, Hernandez et al. 2013), PbP27 (Torres, Hernandez et al. 2014), Pb14-3-3 (Marcos, Silva Jde et al. 2016), and PbSOD3 (Tamayo, Munoz et al. 2016). By applying the ATMT methodology, we recently get PCN-silenced *P. brasiliensis* yeasts, which made possible identifying PCN as a fungal virulence factor (Fernandes, Oliveira et al. 2017). Finally, in the present study, the ATMT system successfully provided PCN-overexpressing *P. brasiliensis* yeasts, which allowed confirming that PCN is a virulence factor that affects fungal pathogenesis and demonstrating mechanisms accounting for the PCN played roles. The ATMT methodology was also successful in terms of the mitotic stability of the generated ov-PCN yeasts. The verified variability of the PCN expression levels among the *P. brasiliensis* transformed clones (around 7%) has not a clear explanation; possibly, it is due to a variable number of insertions in the fungal genome. Apropos, in the study we used the fungal genomic sequence to perform the gene cloning because, at the time we initiated the fungal manipulation, we knew only a partial PCN sequence.

Our interest on the PCN gene manipulation came from demonstrations that the subcutaneous administration of recombinant PCN (rPCN) to infected mice with *P. brasiliensis* promotes modulation of the host immune response and confers protection against the fungal disease (Alegre-Maller, Mendonca et al. 2014; Alegre, Oliveira et al. 2014). The response is triggered by the PCN lectin domain interaction with N-glycans of TLR2 and TLR4 (Alegre-Maller, Mendonca et al. 2014; Ricci-Azevedo, Roque-Barreira et al. 2017). Both receptors are expressed on the surface of macrophages, which undergo M1 polarization followed by high

578 production of proinflammatory mediators, such as the Th1 polarizing cytokine IL-12 (Freitas,
579 Oliveira et al. 2016). We demonstrated that the developed Th1 immune response accounts for
580 the host resistance to the *P. brasiliensis* infection conferred by the PCN administration (Alegre-
581 Maller, Mendonca et al. 2014). A subsequent study, focused on the PCN distribution in *P.*
582 *brasiliensis*, revealed its association with chitin and prominent localization in structures related
583 to fungal growth, such as hyphae tips and budding regions of yeasts cell wall (Oliveira,
584 Fernandes et al. 2017). The relevant biological activities and peculiar localization of PCN
585 motivated us to investigate the role the endogenous PCN could play in fungal biology, as well
586 as its effect on the host immune response.

587 The chosen approach to obtain ov-PCN yeasts and evaluate their biological characteristics
588 demonstrate that PCN is a virulence factor that strongly influences the fungal transition and the
589 course of the murine *P. brasiliensis* infection. There are no previous data on the influence of
590 the overexpression of any *P. brasiliensis* protein on the fungal biology or pathogenesis, which
591 could propitiate a better discussion of our results. Nonetheless, a significant compensation is
592 given by the detection of opposite biological effects between ov-PCN and PCN-silenced fungi,
593 verified in our present and previous studies, respectively, on PCN gene manipulation
594 (Fernandes, Oliveira et al. 2017), as shown in Table A. The observation that fungal growth was
595 not affected by PCN gene manipulation was surprising, since we had two reasons to expect the
596 contrary. First, in a previous work, we verified yeasts growth inhibition when anti-PCN
597 antibodies were added to the cultures (Ganiko, Puccia et al. 2007). Second, the prominence of
598 PCN immunolocalization in structures related to fungal growth, especially in yeasts budding
599 regions (Oliveira, Fernandes et al. 2017). It was considered suggestive that the PCN chitinase
600 activity would be required for hydrolyzing chitin, allowing the budding of the daughter cells.
601 In spite of these contradictory results, we ponder that PCN-silencing and overexpression would
602 provide more reliable data on the PCN role in yeast cells.

We found that ov-PCN yeasts had an accelerated transition to hypha, compared to WT-yeasts. Furthermore, PCN-silenced yeasts underwent transition blockage (Fernandes, Oliveira et al. 2017). Delayed inhibition of yeast-to-hyphae transition was previously associated to other *P. brasiliensis* components silencing, such as PbSconC (Menino, Saraiva et al. 2013), and Pb14-3-3 (Marcos, Silva Jde et al. 2016). Our current results, in a first view, are not consistent with the previous demonstrations that administration of exogenous rPCN to mice confers resistance against PCM (Alegre-Maller, Mendonca et al. 2014; Alegre, Oliveira et al. 2014). Indeed, augmentation of endogenous PCN, to which ov-PCN yeasts infected mice were exposed, brought no benefic effect to the host; at contrary, animals were severely illness, with high fungal burden, serious pulmonary lesions, and high mortality score (see model shown at Figure A). The reasons for this finding are under investigation in our laboratory; we hypothesize that the subcutaneous PCN injection in bolus (2 doses of 0.5 µg PCN) is efficient in terms of promoting immunomodulation, whereas the slow release promoted by the yeast itself, is not. In addition the systemic PCN distribution may facilitate the contact with a greater number of macrophages, especially in the lung, which will be activated and provide a more diffuse inflammatory environment. Furthermore, as shown in this report, the endogenous PCN releasing engages another chain of biological events that may overcome a perhaps more timid immunomodulatory effect.

The finding that mice infection with transformed yeasts with PCN scarcity or abundance cause mild or severe pulmonary disease, respectively, was surprising, once opposite to the results that demonstrated the PCN immunomodulatory ability. Anyway, we had only investigate possible mechanisms accounting for the new discovery. Literature has provided information that raised the suspicion that there would be involvement of the PCN chitinase activity in the sought mechanism; indeed, the involvement of the substrate of the PCN chitinase, which is chitin of the yeast cell wall and the fragments generated by the chitin hydrolysis. Several studies,

published in the last decade, have explored particles derived from chitin hydrolysis concerning their property of stimulating macrophages to produce cytokines. The release of IL-10 and TNF- α was regularly examined as markers of the anti-inflammatory or inflammatory microenvironment, known as generated by the stimulation of macrophages with chitin particles. The response variability is dependent on the size and concentration of the stimulating chitin particles, as well as on the innate immunity receptor(s) targeted by the chitin particles. As well known, an anti-inflammatory microenvironment, marked by a high IL-10 detection (Da Silva, Hartl et al. 2008), promotes the development of Th2 immune response, which results in severe pulmonary damage in *P. brasiliensis* infected hosts. In our studies, chitin particles obtained from the supernatant of ov-PCN yeasts were the smallest measured fragments and induced the production of IL-10 when stimulating macrophages. Otherwise, chitin particles derived from the WT-yeasts had a broader size distribution and has stimulated macrophages to a prominent TNF- α production. The availability of high PCN amounts, as occurs in ov-PCN yeasts, leads to a more effective chitin cleavage and stimulation of IL-10 release by macrophages. Then, it is comprehensible the worse course of the mice infection with ov-PCN *P. brasiliensis* yeasts, as well as the more severe pulmonary damage, compared to the disease caused by WT-yeasts. In summary, the severe pulmonary disease caused by ov-PCN yeasts is mechanistically related to the macrophages anti-inflammatory activity toward the well-hydrolyzed chitin and the consequent Th2 immune response against the fungus (see model at Figure A). Our conclusion is consistent with literature data showing that small chitin particles induce IL-10 production by macrophages (Lee, Da Silva et al. 2008; Da Silva, Chalouni et al. 2009), whereas higher chitin particles induce TNF- α release (Da Silva, Hartl et al. 2008; Lee, Da Silva et al. 2008; Da Silva, Chalouni et al. 2009; Dubey, Moeller et al. 2014; Dong, Tong et al. 2018). Our data demonstrate that the macrophage response of producing cytokines depends also on the concentration of chitin particles used to stimulate macrophages. When the particles were used at 5 to 10 μ g there

was a better discrimination between the levels of IL-10 and TNF- α produced by macrophages stimulated with chitin particles derived from ov-PCN and WT-yeasts. This observation is consistent with those of Wagener et al. (2014), when working with chitin particles derived from *C. albicans* wall. Besides the mechanism involving the macrophages activation by chitin particles, the severity of the pulmonary disease caused by ov-PCN yeasts is also favored by the resistance of these yeasts to the macrophages fungicidal activity. Compared to WT-yeasts, a higher number of ov-PCN yeasts is phagocytized by macrophages, but the cell fungicidal activity is effective only against a low number of internalized yeasts.

In conclusion, by overexpressing PCN in yeasts, we demonstrated that Paracoccin is a virulence factor of *P. brasiliensis*, able to cause a severe pulmonary disease. The mechanisms involved in the severe course of the murine infection with the PCN overexpressing yeasts is linked to its augmented resistance to the fungicidal activity of macrophages, as well as its ability of hydrolyzing chitin of the cell wall in very small particles. They induce macrophages to generate an anti-inflammatory environment, able to prone the adaptative immune response to a Th2 axis, unable to combat the fungal pathogen (see model at Figure A). Although competent and supported by literature data, the mechanisms herein proposed may be insufficient to explain all the aspects of the severe pulmonary disease caused by ov-PCN yeasts. Further investigation is required to a complete understanding of the Paracoccin role in *P. brasiliensis* infection.

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Table 1 – Strains and plasmids used in this study.

Strains	Genotype	Source
<i>A. tumefaciens</i> LBA1100	C58 chromosomal background Rifampicin ^R , pectinomycin ^R ; pAL1100 or pTiB6 ΔTL, ΔTR, Δtra, Δocc	(Beijersbergen, Dulk-Ras et al. 1992)
<i>E. coli</i> DH5α	F ⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoRnupGΦ80dlacZΔM 15 Δ(<i>lacZYA-argF</i>) U169, hsdR17(rK- mK+), λ-	(Meselson and Yuan 1968)
<i>P. brasiliensis</i>	Pb18	USP-RP, Brazil
Plasmids	Selection/genetic Markers	
pUR5750	Hygromycin ^R	(de Groot, Bundock et al. 1998)
pCR35::P _{RHO2} -gPCN	Kanamycin ^R	This study
pUR5750::P _{RHO2} -gPCN	Hygromycin ^R	This study

Table 2. ov-PCN groups intranasal infection.

Groups	ov-PCN and WT yeasts
G1	<i>P. brasiliensis</i> (Wild Type)
G2	<i>P. brasiliensis</i> (gPCN ₇)
Control	PBS*

*Mice received PBS in a regimen similar to that of groups.

Table 3. ov-PCN groups intravenously infection.

Groups	ov-PCN and WT yeasts
G1	<i>P. brasiliensis</i> (Wild Type)
G2	<i>P. brasiliensis</i> (gPCN ₇)

Figure 1. *P. brasiliensis* yeasts co-cultured with human macrophages augment PCN expression. (A) Augmented PCN expression in *P. brasiliensis* yeasts co-cultured for 24 hours with THP1 human macrophages. The levels of PCN mRNA expression were determined by qRT-PCR, normalized to the expression level of the internal references TUB2 and L34. Bars depict the mean $\pm$ SEM fold change in PCN expression found in co-cultured yeasts with macrophages in relation to the PCN expression verified in yeasts cultured alone. The comparison was done through the Bonferroni's test. * $p < 0.1$. (B) Western blot analysis of a recombinant PCN protein, expressed in *Pichia pastoris* (Freitas, Oliveira et al., 2016) and the PCN content in the supernatant of yeast cells that were co-cultured with THP-1 macrophages. Reactions were developed by IgY anti-PCN conjugated to HRPO. MW, Protein marker.

Figure 2. Characterization of ov-PCN yeasts generated by using the pUR5750 plasmid and a transformation system mediated by *Agrobacterium tumefaciens*. (A) PCN-overexpression cassette. The genomic paracoccin sequence (PADG_03347) was produced based on Pb339 (PbWT) genomic sequence, as detailed in the Material and Methods section, and cloned under the control of the Rho2 ATPase promoter from *P. brasiliensis* (RHO2). The constructs were sub-cloned into the T-DNA region of the binary vector pUR5750 harboring the *E. coli* hygromycin B phosphotransferase (HPH) resistance gene driven by the glyceraldehyde 3-phosphate promoter from *A. nidulans* (PGPDA) and bearing

transcriptional terminators (T) from cat-B and TRPC; LB: left border; RB: right border. **(B)** Gene expression levels of gPCN yeasts and controls WT and empty vector after subculture for 3 days (gene expression levels obtained by qRT-PCR were normalized to the level of expression of the internal reference, TUB2, and L34; $*P < 0.01$ compared with WT strains). **(C)** Supernatants of gPCN yeasts and WT strains, cultured in DMEM medium, were assessed for NAGase activity, detected by the degradation of the p-nitrophenyl-N-acetyl- β -D-glucosaminide substrate. **(D)** gPCN yeasts and WT controls had their growing evaluated by culture turbidity, as determined by 600 nm O.D. reading; they were also examined for yeast cells viability by diacetate fluorescein and ethidium bromide (Calich, Purchio et al. 1979). **(E and F)** The area (in nm^2) of each mother and daughter yeast cells harvested in the 3th day culture (exponential growth) was determined by using the ImageJ software (<http://rsb.info.nih.gov/ij/>). A total of 50 cells were examined per slide, by multiphoton microscopy. **(G)** Fluorescence intensity of yeasts as detected by multiphoton microscopy and analyzed with the aid of the ImageJ software. **(H)** The ov-PCN yeasts and the respective control yeasts (WT and EV) grew in solid BHI medium supplemented with 4 % FBS for 72 hours (exponential phase). The images were obtained in a multiphoton fluorescence microscope after the samples incubation with IgY anti-PCN conjugated to Alexafluor 294 fluorophore. Bars correspond to 200 μm . **(I)** Frequency of transition forms for either transformants or control yeasts was determined as previously reported (Nunes, Costa de Oliveira et al. 2005; Menino, Saraiva et al. 2013) through quantitative optical microscopy (40 X magnification). Results were expressed as the percent of total counted cells ($n=300$). Graphical representation of the morphological signs of (Y→M) transition occurred during the 7 days cultures, at 25 °C. The assay was performed in triplicate with three replications and utilized previous established patterns to drive the fungal forms classification (Nunes, Costa de Oliveira et al. 2005; Menino, Saraiva et al. 2013). The assays were performed in triplicate. Bars depict the mean $\pm$ SEM. Results provided by the ov-PCN and WT yeasts were compared through the Bonferroni's test. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.1$.

Figure 3. Pulmonary fungal burden, granulomatous lesions, and mortality of infected mice with ov-PCN *P. brasiliensis* yeasts. BALB/c mice were infected by intranasal via with 2×10^6 ov-PCN or WT *P. brasiliensis* yeasts. On day 30 after infection, a pulmonary lobe of each animal was assessed for the recovery of pulmonary CFU. **(A)** Each group was consisted of five mice. A non-infected control group (mice inoculated with a vehicle, PBS) was included. The sections were stained with hematoxylin and eosin (H&E) or **(B)** with Gomori's methenamine silver to visualize the viable yeasts. Images were captured using a Carl Zeiss Axiophot microscope coupled to a JVC TK1270 camera. Magnification bars: 500 nm for images of the whole organ sections and 200 nm for partial images of lung sections. **(C)** The area (%) of lung sections occupied by granulomatous lesions, was quantitated with the aid of the ImageJ software. **(D)** CFU recovery from the lung homogenates of infected mice with ov-PCN or WT-yeasts. **(E)** Survival curve of male BALB/c infected-mice with ov-PCN or WT-yeasts (1×10^7 yeasts per animal) that were monitored daily for death occurrence over a 160° day post-infection period. Mice survival is expressed in percentage. The assays were carried out in triplicates. Bars depict the mean $\pm$ SEM and the values provided by infected mice with ov-PCN yeasts were compared to values obtained from WT yeasts infected-mice by the Bonferroni's test. **** $p < 0.0001$ and * $p < 0.1$.

Figure 4. The PCN overexpression in *P. brasiliensis* yeasts correlates with diminished chitin content, narrowed cell wall, and detection of small chitin particles in the supernatant of yeasts cultures. (A) ov-PCN yeasts and the controls WT and EV yeasts, grown in solid BHI broth for 72 hours (exponential phase) and stained with calcofluor white (10 mg/mL) were assessed for their chitin content in the cells wall by fluorescence multiphoton microscopy. Bars correspond to 200 μ m. The mean fluorescence intensity (MFI) and the distances (micron) of the fluorescence pics from the nucleus were determined with the aid of the ImageJ software and graphically represented (at the right side of panel

A). (B) The MFI of the yeasts was determined with the aid of the ImageJ software. (C) To assess the amount of chitin present in the yeast cell wall, 500 μ L samples of cultures supernatants of ov-PCN or WT yeasts were harvested at 72 h (exponential growth). The samples were incubated for 30 min with WGA conjugated with a Texas-Red fluorophore. Analysis was performed by flow cytometry. Bars depict the mean $\pm$ SEM and were compared WT marker by Mann-Whitney's test, ** p <0.0022. (D) Electron microscopy of the cell wall of WT and ov-PCN yeasts. Bars correspond to 300 nm. (E) The cell wall area (nm^2) was analyzed with the aid of ImageJ software. The results provided by the empty vector and WT yeast cells were similar. (F) The diameter of chitin fragments isolated from the ov-PCN and WT cultures by using a column with WGA and the area size (nm^2) and integrated density (pixels) was analyzed by ImageJ software. Bars depict the mean $\pm$ SEM and were compared WT fragments by Mann-Whitney's test, **** p <0.0001.

Figure 5. Chitin particles isolated from supernatants of ov-PCN and WT *P. brasiliensis* yeasts induce macrophages activation, manifested by cytokines release. BMDMs (1×10^6 cells/mL) were stimulated by chitin particles captured from the supernatant of ov-PCN or WT *P. brasiliensis* cultures. Following 24, 48, and 72 h stimulation, the supernatants of BMDM cultures were assessed by ELISA for cytokines levels (pg/mL). (A) TNF- α (72 h). (B) IL-1 β (72 h). (C) IL-10 (48 h). (D) Dose-response curves of TNF- α and IL-10 production by BMDMs that were 48 h stimulated with chitin (0.5 to 20 μ g) derived from ov-PCN (continuous lines) or WT (dashed lines). Unstimulated and LPS stimulated BMDMs were used as negative and positive controls, respectively. The results are expressed as mean $\pm$ SEM and compared to the response to medium alone through one-way analysis of variance, followed by Bonferroni's test. *** p < 0.001, ** p <0.01 and * p <0.1.

Figure 6. Sensitivity of PCN-overexpressing yeasts to phagocytosis and killing by macrophages. Murine macrophages of the RAW 264.7 cell lineage (1×10^6 cells/mL) were infected with ov-PCN or WT *P. brasiliensis* yeasts (MOI=1:10). At 4 and 48 h post-infection, macrophages were washed and lysed. The lysates were assessed for CFU recovering, whose results allowed to estimate the yeasts sensitivity to the macrophages effector functions, phagocytosis (A, 4h) and killing (B, 48 h), respectively. The results are expressed as mean $\pm$ SEM and compared between ov-PCN and WT yeasts through two-tailed t-test analysis of variance. * $p < 0.1$.

Figure A. Paracoccin acts as a virulence factor of *P. brasiliensis*, as demonstrated by comparing the performance of PCN-overexpressing and WT-yeasts in *in vitro* and *in vivo* assays. The study propitiates a model for the PCN role in *P. brasiliensis* infection. PCN confers yeasts resistance to the macrophages fungicidal mechanisms, propitiates chitinase activity, promotes an efficient hydrolysis of chitin of the yeast cells wall and generates very small chitin particles. Once released in the extracellular milieu, the small particles activate macrophages to produce anti-inflammatory cytokines. The generated microenvironment induces a non-protective Th2 immune response against the fungus. Such chain of events is likely responsible, at least in part, for the severe pulmonary disease installed following the mice infection with *P. brasiliensis* yeasts that overexpress paracoccin.

Table A. General characteristics of PCN-silenced and ov-PCN *P. brasiliensis*. The analyzed parameters were: detection of PCN mRNA or protein; effects on the *in vitro* yeasts growth; effects on the transition from yeast to mycelium; sensitivity to killing by macrophages; effects on the infection pathogenesis, and grade of virulence. The virulence was inferred from the set of analyzed parameters.

Features Transformed yeast	Detection	Yeast Growth	Transition (Y→M)	Killing by Mφ	Pathogenesis	Virulence
PCN-silencing	↓ mRNA ↓ NAGase	Not affected	Blocked	Susceptible	↓ Fungal burden ↓ Lung lesions	Reduced
PCN-overexpressing	↑ mRNA ↑ NAGase	Not affected	Accelerated	Resistance	↑ Fungal burden ↑ Lung lesions	Augmented

Supplementary data

Figure S1. The chitin isolated from supernatants of ov-PCN and WT yeasts induces macrophages to release cytokines in a dose-dependent manner. BMDMs (1x10⁶ cells/mL) were stimulated for 24, 48, and 72 h with chitin particles captured from the supernatants of cultures of yeasts (ov-PCN or WT-yeasts). The macrophages supernatants were assessed by ELISA for cytokines levels (pg/mL). (A) TNF-α. (B) IL-1β, and (C) IL-10. The supernatants of unstimulated macrophages or LPS-stimulated macrophages (1 mg/mL) were used as negative and positive controls, respectively. The results are expressed as mean ± SEM and were compared to the given by the negative control (cells) through one-way analysis of variance, followed by Bonferroni's test. **** p< 0.0001, *** p< 0.001, ** p<0.01 and * p<0.1.

Considerações sobre o capítulo IV

Os resultados apresentados no capítulo IV (*Paracoccin overexpression in Paracoccidioides brasiliensis reveals the influence of chitin hydrolysis on fungal virulence and host immune response*) corroboram para o atendimento ao segundo objetivo desta tese, qual seja, o de “*Estudar a relevância da paracoccina para a biologia de leveduras de P. brasiliensis e patogênese da doença por elas causada, através da utilização de transformantes do fungo cujos genes codificadores da paracoccina foram silenciados ou superexpressos*” e ao sub-item: “*Promover o silenciamento e a superexpressão do gene que codifica a paracoccina em leveduras de P. brasiliensis*”, “*Avaliar os efeitos do silenciamento e da superexpressão da paracoccina sobre a patobiologia de leveduras de P. brasiliensis*” e “*Investigar os mecanismos responsáveis pelas alterações no curso da doença causada pela inoculação de leveduras transformantes, em comparação à causada por levedura selvagem de P. brasiliensis*”. Destacamos as seguintes considerações: i) a superexpressão da PCN foi controlada pelo promotor RhoII e a validação se deu através da quantificação de mRNA, aumento da atividade NAGase, transição acelerada de levedura para hifa, redução do tamanho das células mãe e filhas e não houve alteração no crescimento dos transformantes em comparação a linhagem selvagem; ii) a infecção em camundongos levou a inflamação pulmonar grave com maior área de lesão granulomatosa, maior recuperação fúngica e virulência aumentada em comparação ao tipo selvagem; iii) quanto às características da parede celular fúngica, constatamos redução da quantidade de quitina através de marcação com *calcofluor white* e WGA conjugado a *texasRed*; iv) análises de microscopia eletrônica de transmissão indicam redução do tamanho da parede celular das leveduras que superexpressam PCN; e v) os fragmentos de quitina liberados no sobrenadante das culturas dos transformantes são menores do que das culturas do tipo selvagem.

Concluimos que doença pulmonar fúngica de maior gravidade é causada pela infecção com leveduras superexpressoras de PCN e identificamos que contribuem para esse fato os seguintes

mecanismos: 1º) maior número de leveduras superexpressoras de PCN são internalizadas por macrófagos, mas elas resistem mais aos mecanismos fungicidas que essas células dispõem; o que contribui para a ocorrência de maior carga fúngica pulmonar; e 2º) o maior grau de hidrólise de quitina desencadeado pela quitinase PCN expressa em maior quantidade leva à liberação de fragmentos menores de quitina, em comparação com os derivados de leveduras da linhagem selvagem; partículas menores de quitina, como as verificadas no sobrenadante de culturas de leveduras que superexpressam PCN estimulam macrófagos a produzirem altas concentrações de IL-10, fato que culmina em resposta imune ineficiente pelo hospedeiro. Em oposição, o estímulo de macrófagos com partículas maiores de quitina, liberadas na cultura de leveduras selvagens, induz maior produção de TNF- α e a consequente geração de resposta imune mais eficiente por parte do hospedeiro.

Considerações Finais

A identificação de PCN como uma lectina fúngica capaz de atuar como um agente imunomodulador foi previamente estabelecida por nosso grupo, através de estudos *in vivo* e *in vitro* (Alegre-Maller, Mendonca et al. 2014; Alegre, Oliveira et al. 2014), que atenderam também à necessidade de elucidar os mecanismos através dos quais PCN modula a resposta imune do hospedeiro e confere proteção contra a infecção por *P. brasiliensis*.

Os primeiros estudos sobre PCN foram realizados com preparações da proteína nativa, obtidas de leveduras de *P. brasiliensis*. Essas preparações foram enriquecidas da lectina através de cromatografia de afinidade em coluna de N-acetilglicosamina (Coltri, Casabona-Fortunato et al. 2006) ou de quitina (dos Reis Almeida, de Oliveira et al. 2010). Estudos subsequentes utilizaram uma forma recombinante, denominada p-rPCN, que induziu macrófagos peritoneais murinos a produzirem mediadores pró-inflamatórios (IL-12p40, TNF- α , IL-6 e NO) e assumirem um perfil M1 de polarização. Tais efeitos de p-rPCN foram mediados por ligação da lectina a N-glicanos ligados ao exodomínio do receptor TLR-4, mas não de TLR2 (publicação inserida no capítulo I) (Freitas, Oliveira et al. 2016). Além disso, p-rPCN induziu neutrófilos humanos a formarem NETs e liberarem IL-8 (publicação inserida no capítulo II) (Ricci-Azevedo, Goncales et al. 2017).

O investimento seguinte referiu-se ao estudo do papel da PCN na biologia do próprio fungo. Nesse contexto, um primeiro estudo havia sido realizado em colaboração com o grupo da Profa Dra Rosana Puccia, utilizando a lectina obtida de leveduras de *P. brasiliensis*. Apesar da limitação imposta pelo baixo rendimento do processo de obtenção de PCN nativa, anticorpos específicos foram produzidos e serviram para localizar PCN na parede de leveduras e revelar a interação de PCN com quitina da parede celular, especialmente nas regiões de brotamento. Além disso, o estudo evidenciou o papel no crescimento fúngico de PCN, já que seu bloqueio com anticorpos específicos decresceu o número de CFU obtido da cultura de leveduras e alterou a morfologia dessas formas de *P. brasiliensis* (Ganiko, Puccia et al. 2007).

O estudo do papel de PCN na biologia do fungo foi recentemente ampliado por sua detecção, através de microscopia confocal, em micélios, leveduras e formas de transição para micélios. O trabalho revelou que PCN existe em maior quantidade em micélios do que em leveduras, com predominância em estruturas relacionadas ao crescimento fúngico, como as pontas das hifas, clamidoconídeos em formas de transição e regiões de brotamento em leveduras (Oliveira, Fernandes et al. 2017). Entretanto, os passos mais importantes e claros, referentes a elucidação do papel de PCN na biologia fúngica, foram proporcionados pelos procedimentos de silenciamento e superexpressão do gene codificador de PCN. O silenciamento gênico deixou claro que PCN é um importante fator de virulência em *P. brasiliensis*, como revelado por ensaios de transição morfológica e pelas amenas manifestações da infecção em camundongos. Os fungos tornaram-se mais suscetíveis aos mecanismos de eliminação fúngica e menor número de leveduras foi recuperado do pulmão de animais infectados, que mostraram discretas lesões granulomatosas (Fernandes, Oliveira et al. 2017). Tais resultados são detalhadamente descritos, documentados e discutidos na publicação anexada ao capítulo III desta Tese.

Esses fatos nos motivaram a investigar o mecanismo pelo qual PCN atua como um fator de virulência em *P. brasiliensis*. Para tanto, propusémo-nos a superexpressar PCN em leveduras de *P. brasiliensis*, tarefa atemorizante, uma vez que não havia relatos prévios de superexpressão de qualquer proteína nesse fungo. O sucesso do procedimento permitiu que constatássemos o papel essencial de PCN no remodelamento da parede celular. A superexpressão, como relatado nesta Tese, associou-se à redução do tamanho das células mães e filhas, da espessura da parede celular e da quantidade de quitina na parede, bem como a maior hidrólise de quitina, em comparação ao observado na linhagem selvagem (Gonçales et al., em fase final de preparação). Preparações de quitina foram obtidas do sobrenadante das culturas de leveduras superexpressoras de PCN e de selvagens, submetidas à purificação em coluna contendo WGA. A utilização desse método de isolamento fundamentou-se no conhecimento de

que quito-oligômeros fúngicos são reconhecidos com alta afinidade por WGA, lectina comumente usada em estudos de distribuição celular e estudos funcionais de quitinas e moléculas semelhantes a quitinas (Orgad, Alroy et al. 1984). Estudo recente, publicado por Liedke e colaboradores (2017), mostrou que a quimera WGA-Fc, por eles construída, pode corresponder a alternativa terapêutica contra doenças fúngicas invasivas (Liedke, Miranda et al. 2017). Oligômeros derivados de quitina têm sido implicados na divisão celular em diferentes modelos (Chen and Contreras 2004). Quito-oligômeros alvejados por ligantes específicos podem levar ao desenho de nova alternativa terapêutica para o controle da criptococose. Em estudo realizado por Fonseca e colaboradores (2009), mostrou que a presença do inibidor da síntese de glicosamina 6-fosfato resultou na expressão alterada de quitina na parede celular fúngica. Em adição, essas células formaram cápsulas que estavam apenas frouxamente conectadas à parede e que continham fibrilas com diâmetros diminuídos e com alteração da composição de monossacarídeos (Fonseca, Nimrichter et al. 2009). Esses resultados têm contribuído para a compreensão do papel exercido por quitina e quito-oligossacarídeos na estrutura capsular de *C. neoformans*, ampliando o espectro de funções atribuídas a estruturas constituídas de GlcNAc (Fonseca, Nimrichter et al. 2009). Além disso, quito-oligômeros solúveis (GlcNAc)₃ interagem com *C. neoformans* e diminuem a associação com macrófagos em até 25% (Fonseca, Guimaraes et al. 2013). No que concerne a *P. brasiliensis*, não há relatos de estudos sobre o papel de quito-oligômeros liberados no sobrenadante das culturas de *P. brasiliensis* e nem a sua purificação em resina com WGA imobilizada.

A rede de microfibrilas de quitina associa-se covalentemente a β -(1,3)-glucanos, uma estrutura de polissacarídeos abundantes na parede celular de fungos, na camada mais externa, que recobre a camada constituída de quitina (Klis, Boorsma et al. 2006). Parte da quitina fúngica é sintetizada e subsequentemente deacetilada pela ação de uma ou mais quitina-deacetilases com geração de quitosana. Curiosamente, a quitina sintase Chs3 e o regulador Csr2 podem

formar um complexo com quitina deacetilases convertendo quitina em quitosana (Banks, Specht et al. 2005; Baker, Specht et al. 2007). A deacetilação de quitina torna o polímero mais flexível e protege o micro-organismo contra a ação de quitinases, incluídas no arsenal de defesas anti-fúngicas de diversos organismos. Em *C. albicans*, menos de 5% da quitina é deacetilada, ao passo que a maioria dos zigomicetos e *C. neoformans* possuem mais de dois terços de quitosana deacetilada (Bartnicki-Garcia 1968; Baker, Specht et al. 2007).

O reconhecimento de componentes fúngicos constitui-se num passo essencial para que ocorram a fagocitose da levedura e a ativação de macrófagos. Nesse sentido, moléculas de superfície denominadas MAMPs (*microbe-associated molecular patterns*) (Didierlaurent, Sirard et al. 2002; Klemptner, Sherwood et al. 2014) desempenham papel importante. O MAMP melhor caracterizado é a β -(1,3)-glucana, reconhecida em mamíferos pelo receptor dectina-1 (Brown and Gordon 2001). Outro receptor que reconhece quitina é FIBCD1 (*Fibrinogen C domain-containing protein 1*); estruturalmente, é uma proteína transmembrana homotetramérica do tipo II de 55 kDa, expressa no trato gastrointestinal (Schlosser, Thomsen et al. 2009).

Em nosso trabalho, a partir do isolamento de fragmentos de quitina contidos no sobrenadante de culturas de *P. brasiliensis*, partimos para a sua caracterização quanto ao tamanho, através de microscopia eletrônica, e quanto ao seu papel na resposta imune do hospedeiro. Estudos sobre quitinas fúngicas foram realizados por diversos grupos, a partir de preparações obtidas da parede celular de leveduras, como *C. albicans* e *A. fumigatus*, ou de quitinas comerciais, tratadas ou não com quitinases, sendo AMCase (*acidic mammalian chitinase*) humana a mais frequentemente utilizada.

A resposta imune frente à quitina e a ação exercida pelo receptor que a reconhece ainda são temas de controvérsias na literatura. Mora-Montes e colaboradores (2011) mostraram que quitina é reconhecida pelo receptor dectina-1, mas não por Mincle (*Macrophage inducible*

Ca²⁺-dependent lectin receptor), TLR2 e TLR4. Nesse estudo, constataram que quitina fúngica purificada da parede celular não estimula PBMCs (*peripheral blood mononuclear cell*) a produzirem citocinas (Mora-Montes, Netea et al. 2011). Em adição, quitinas isoladas da parede celular de *A. fumigatus*, cujo tamanho é inferior a 0.5 µm em 70% das moléculas, não induzem PBMCs a liberarem citocinas pró-inflamatórias, tanto na presença como na ausência de soro humano no meio de cultivo (Becker, Aimagani et al. 2016). Diferenças na resposta inflamatória elicitada por fragmentos de quitina de diferentes tamanhos podem ser atribuídas a associações distintas com TLR2 e dectina-1 e, em menor grau, com o reconhecimento por receptor de manose e à atividade fagocítica, desencadeando sinalização de vias distintas, que levam à produção de TNF-α ou de IL-10, respectivamente (Da Silva, Chalouni et al. 2009). Explicitamente, fragmentos de quitina entre 40-70 µm e de tamanho inferior a 40 µm (e entre 2-10 µm) induzem macrófagos a produzirem TNF-α, enquanto apenas os fragmentos menores a 40 µm induzem a produção de IL-10 (Da Silva, Chalouni et al. 2009). Nesse trabalho, mono-, di-acetil glicosamina e fragmentos entre 70-100 µm não elicitam macrófagos e, portanto, têm sido considerados inertes em termos da ativação dessas células. Sob a ação catalítica de AMCase, pequenos fragmentos de quitina são gerados; eles induzem a produção de IL-10 e YKL-40/BRP-39 (Lee, Da Silva et al. 2008). Estudo realizado por Dubey e colaboradores (2014), utilizando preparações insolúveis da parede de *A. fumigatus* (enriquecidos de quitina e β-glucanos), mostraram que partículas menores que 40 µm induzem macrófagos a liberarem TNF-α. Outros estudos relatam que partículas de quitina estimulam a produção de IL-12, IL-10, TNF-α e IFN-γ por diversos tipos celulares (Shibata, Foster et al. 1997; Shibata, Metzger et al. 1997; Cash, Whitham et al. 2006). Em estudo realizado por Wagener e colaboradores (2014), foi verificado que partículas entre 1-10 µm induzem a expressão de IL-10 e TNF-α, dependendo da concentração utilizada. No que concerne ao reconhecimento fúngico e à indução de ativação celular, os receptores NOD2, TLR9 e o receptor de manose desempenham papéis

essenciais. Foi observado ainda que polimorfismos em receptores de quitina, NOD2 e TLR9, predis põem indivíduos a inflamação e desregulação na expressão de quitinases, importantes para gerar partículas de quitinas fúngicas indutoras de IL-10 *in vitro*, fato relacionado a doenças inflamatórias e asma (Wagener, Malireddi et al. 2014). Em nosso estudo, constatamos que fragmentos menores de quitina, liberados subsequentemente à hidrólise por quitinase no sobrenadante de culturas de leveduras que superexpressam a quitinase PCN, induzem macrófagos a produzirem maiores concentrações de IL-10 do que o induzido por fragmentos de quitina liberados em culturas de fungos selvagens. Em contrapartida, fragmentos isolados do sobrenadante de linhagens selvagens induzem maiores quantidades de TNF- α do que os fragmentos isolados a partir das culturas que superexpressam PCN (Gonçales et al., em fase final de preparação) (publicação inserida no capítulo IV).

A partir dos achados de que *C. albicans* utiliza N-acetilglicosamina para manipular o pH ambiental (Naseem, Araya et al. 2015; Sherrington, Sorsby et al. 2017) foi possível caracterizar o metabolismo de GlcNAc como o terceiro mecanismo geneticamente independente utilizado por esses fungos para combater a rápida acidificação do fagolisossomo, permitindo que as células escapem e propaguem a infecção (Vesely, Williams et al. 2017). Outro estudo mostrou um novo mecanismo de indução de células Th2 durante infecção fúngica. Células dendríticas CD11b⁺ reconhecem quitina via Chit1 (*chitotriosidase*) e impulsionam a geração de células Th2 em resposta à infecção fúngica pulmonar (Wiesner, Specht et al. 2015). Dong e colaboradores (2018) verificaram que células escleróticas de *Fonsecae pedrosoi*, após digestão da parede celular com AMCase, não reverte completamente a inibição sobre essas células e nem a produção de IFN- γ . Nesse sentido, o acúmulo de quitina pode contribuir para inibir a polarização M1 de macrófagos e, conseqüentemente a resposta Th1 (Dong, Tong et al. 2018) frente à infecção fúngica. Em nosso estudo, observamos que pequenos fragmentos de quitina foram gerados por leveduras superexpressoras de PCN, fragmentos esses que induziram

um perfil de macrófagos predominantemente M2, enquanto fragmentos maiores relacionaram-se à polarização M1.

A parede celular fúngica tem sido amplamente estudada como possível alvo para combate ao desenvolvimento de fungos filamentosos como *A. fumigatus* (Bruneau, Magnin et al. 2001). Algumas variações físicas incluindo tamanho, complexidade, fonte e método de purificação relacionam-se com o reconhecimento imune, perfil de citocinas, recrutamento de células e processo inflamatório gerado (Alvarez 2014; Amarsaikhan and Templeton 2015). Salientamos que a metodologia de superexpressão da proteína PCN em *P. brasiliensis*, bem como a purificação dos fragmentos de quitina do sobrenadante das culturas de leveduras de *P. brasiliensis* são pioneiras na área e foram importantes para que viabilizássemos o estudo de um importante mecanismo de virulência fúngica, dependente do tamanho das partículas de quitina geradas, que pode favorecer a sobrevivência do fungo, o remodelamento da parede celular durante o processo de infecção e o estabelecimento de forma grave de paracoccidioidomicose.

Conclusões

A propriedade imunomoduladora de PCN já foi bem estudada e caracterizada por nosso grupo, bem como divulgada num conjunto de publicações que demonstraram o efeito protetor contra a infecção por *P. brasiliensis* exercido pela administração de PCN. O efeito imunomodulador é disparado pela interação de PCN com N-glicanas de TLR4, seguida de alta produção de IL-12 e diferenciação de linfócitos Th1, com consequente produção de IFN- γ , sequência de eventos que se constitui, sabidamente, em resposta protetora contra o fungo. Ademais, essa resposta é favorecida pela atividade de PCN de induzir a polarização M1 dos macrófagos por ela ativados. PCN interage também com neutrófilos, aumentando a produção de IL-8, a formação de NETs e a liberação de ROS. O silenciamento e a superexpressão de PCN tornaram patentes sua ação como um importante fator de virulência de leveduras de *P. brasiliensis*. Revelaram ainda que a maior hidrólise de quitina, induzida pelo aumento da expressão de PCN, induz a expressão aumentada de IL-10 e menor de TNF- α , favorecendo a montagem de respostas adaptativas Th2, ineficientes no combate ao fungo. Esse estudo representa um importante avanço no conhecimento sobre o papel exercido pela quitinase PCN em *P. brasiliensis*.

- i)** Isolar quitina da parede celular fúngica de linhagens de *P. brasiliensis* silenciadas (kd-PCN) ou superexpressoras de PCN (ov-PCN) e selvagem (wt-PCN);
- ii)** Isolar quitina do sobrenadante das culturas de *P. brasiliensis* kd-PCN, ov-PCN e wt-PCN;
- iii)** Caracterizar através de microscopia eletrônica de transmissão e de varredura os fragmentos isolados e purificados em coluna de WGA;
- iv)** Através da Espectroscopia de Energia Dispersiva (EDS) verificar os constituintes químicos das preparações de quitina ou de partículas de quitina purificados em coluna de WGA;
- v)** Relacionar os tamanhos dos fragmentos de quitina obtidos do sobrenadante das culturas e da parede celular fúngica com o perfil de ativação de macrófagos;
- vi)** Relacionar o papel da IL-17 em macrófagos estimulados com fragmentos de quitina liberados no sobrenadante das culturas e da parede celular fúngica de kd-PCN, ov-PCN e wt-PCN;
- vii)** Estudar o (s) possível (is) receptor (es) de quitina em macrófagos que possam relacionar-se aos processos de ativação e sinalização celulares.

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1. Anexo I

Aprovação Comitê de Ética em Pesquisa Animal

Protocolo: 61/2016

**UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS**

C E R T I F I C A D O

Certificamos que o Protocolo intitulado “*Superexpressão da paracoccina: fenótipo e virulência de linhagens de Paracoccidioides brasiliensis*”, registrado com o número **061/2016**, sob a responsabilidade da **Profa. Dra. Maria Cristina Roque Antunes Barreira**, envolvendo a produção, manutenção ou utilização de animais pertencentes ao *filo Chordata, subfilo Vertebrata* (exceto humanos) para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794 de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009 e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi **APROVADO** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo em reunião de 23 de maio de 2016.

Este Protocolo prevê a utilização de 123 camundongos Balb/C machos pesando 25g, oriundos do Serviço de Biotério da Prefeitura do *Campus* de Ribeirão Preto da Universidade de São Paulo. Vigência da autorização: 23/05/2016 a 27/06/2018.

We certify that the Protocol *nº 061/2016*, entitled “*Overexpression of paracoccin: phenotype and virulence from P. brasiliensis strains*”, is in accordance with the Ethical Principles in Animal Research adopted by the National Council for the Control of Animal Experimentation (CONCEA) and was approved by the Local Animal Ethical Committee from Ribeirão Preto Medical School of the University of São Paulo in *05/23/2016*. This protocol involves the production, maintenance or use of animals from *phylum Chordata, subphylum Vertebrata* (except humans) for research purposes, and includes the use of 123 male Balb/C mice weighing 25g from the Central Animal House of Ribeirão Preto Medical School, University of São Paulo. This certificate is valid until *06/27/2018*.

Ribeirão Preto, 23 de maio de 2016

Prof. Dr. Fernando Silva Ramalho
 Presidente da CEUA – FMRP – USP