Universidade do Minho

Escola de Ciências da Saúde

João Filipe Marques de Almeida Ferreira Menino

Paracoccidioides brasiliensis: virulence factors and host susceptibility

Paracoccidioides brasiliensis: factores de virulência e susceptibilidade do hospedeiro

Tese de Doutoramento

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Trabalho efectuado sob a orientação de:

Doutor Fernando José dos Santos Rodrigues

Professor Associado da Escola de Ciências da Saúde,

Universidade do Minho, Braga, Portugal

Doutor António Gil Pereira Castro

Professor Associado da Escola de Ciências da Saúde,

Universidade do Minho, Braga, Portugal

Prof. Doutor Gustavo Henrique Goldman

Professor titular da Faculdade de Ciências Farmacêuticas de Ribeirão Preto

Universidade de São Paulo, Brasil

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DECLARAÇÃO

Nome: João Filipe Marques de Almeida Ferreira Menino

Endereço Electrónico: joaomenino@ecsaude.uminho.pt

Telefone: +351 936714989

Número do Bilhete de Identidade: 12032705

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Orientadores:

Doutor Fernando Rodrigues

Doutor António Gil Castro

Prof. Doutor Gustavo H. Goldman

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PARACOCCIDIOIDES BRASILIENSIS: VIRULENCE FACTORS AND HOST SUSCEPTIBILITY

ABSTRACT

In the last decades, significant advances were accomplished on the study of neglected fungal diseases, mainly in defining the pathogen genetics and host risk factors. Paracoccidioidomycosis, a mycosis caused by *Paracoccidioides* species, is one of these diseases, and it is estimated to affect 10 million individuals in countries from Latin America. Over the last years, genomic and transcriptome studies on this fungus raised important questions, especially on the consequences that the multinucleated nature and morphological heterogeneity of *Paracoccidioides brasiliensis* can have on triggering host defence mechanisms. However, these aspects were never addressed. Moreover, the lack of efficient molecular tools to further explore *P. brasiliensis* has been hindering the identification of genetic factors that govern virulence and the polymorphic nature of the yeast pathogenic phase, as well as the mechanisms underlying its dimorphic behaviour.

The highly heterogeneous yeast morphology and budding patterns are considered a hallmark of this fungus, and previous genetic studies have highlighted the relevance of the cell division cycle 42 (*CDC42*) expression on such phenotype. Nevertheless, the morphological trends followed by each *Paracoccidioides* isolate were never evaluated. Similarly, a possible association between genetic factors determining the fungus morphology and the morphological pattern of each isolate was never addressed.

To address these issues, a detailed morphogenetic evaluation was carried out in the yeast-form of 11 clinical and environmental *Paracoccidioides* isolates from the different groups of *P. brasiliensis* and *Paracoccidioides lutzii* species. We found that each phylogenetic group does not follow any characteristic morphologic profile, whereas bud area and shape of each isolate reveals to be highly dependent on the mother cell, indicating a high level of conservation of these traits throughout cell progeny. Importantly, we also found strong correlations between *PbCDC42* expression (a molecule known to control the morphological behavior of the yeast phase) and both the shape of mother and bud cells and the area of the buds. Altogether, these findings further explore the polymorphic nature of *P. brasiliensis*, providing information on the trends followed by *P. brasiliensis*.

During the conidia/mycelium-to–yeast transition, a known requirement for the pathogenesis of this fungus, several morphological and phenotypical alterations occur. Among these alterations, the auxotrophy to organic sulfur compounds that is associated to the yeast phase of *P.*

brasiliensis is particularly striking. Although this yeast-phase related nutritional requirement is shared with other dimorphic fungi, the molecular bases underlying it are yet to be uncovered. In this sense, we addressed the role of SconCp, the negative regulator of the inorganic sulfur assimilation pathway, in the fungus dimorphism and virulence. By means of genetic down-regulation of *SCONC*, we show that *P. brasiliensis* can overcome its yeast-auxotrophy to organic sulfur compounds, being able to assimilate inorganic sulfur. However, this revealed to drastically reduce the ATP and NADPH cellular levels, leading to increased oxidative cellular stress. This redox imbalance, consequence of the activation of the inorganic sulfur assimilatory pathway, probably led to the decreased virulence of the knock-down isolates, as we show using a mouse model of infection. Our data provides relevant insights on the mechanisms controlling *P. brasiliensis* dimorphism, revealing SconCp as a novel virulence determinant.

P. brasiliensis yeast form is also characterized by its multinucleated nature. Thus, knowing that during the infection fungal cell death is likely to result in the release of large amounts of DNA, one could expect the triggering of innate immune mechanisms of the host via Toll-Like Receptor 9 (TLR9). This molecule is the member of the TLR family known to recognize unmethylated CpG sequences in DNA molecules. Nevertheless, TLR9 role during *P. brasiliensis* infections was never assessed. We herein demonstrate that activation of this receptor upon recognition of *P. brasiliensis* yeast cells is an event that seems to be crucial in early-times of infection. Lack of this receptor caused the premature death of the hosts (in a mouse model of infection with *P. brasiliensis* yeast cells), associated with signs of organ-pathology and high production of pro-inflammatory cytokines. One possible explanation for this profile can be the abnormal neutrophilia observed in TLR9-depleted infected mice. Overall, we show that TLR9 activation is immuno-protective in early stages of *P. brasiliensis* infections, playing an important role on the development of a controlled cell-mediated response.

The work developed throughout this thesis provides new data on the morphological traits followed by the pathogenic fungus *P. brasiliensis,* and further establishes the relevance of *CDC42* on the heterogeneity of cell shape. Moreover, we present new data on the yeast-phase metabolism, further clarifying how dimorphism impacts sulfur metabolism and its relevance for pathogenesis. We also provide relevant data for the elucidation of the mechanisms prompted by the host for the development of an appropriate immune response against *P. brasiliensis* infections, bringing

together the multinucleated nature of this pathogen with the protective activation of the pattern recognition receptor TLR9.

PARACOCCIDIOIDES BRASILIENSIS: FACTORES DE VIRULÊNCIA E SUSCEPTIBILIDADE DO HOSPEDEIRO

RESUMO

Nas últimas décadas obtiveram-se avanços significativos no estudo de doenças negligenciadas, nomeadamente no que concerne à elucidação da genética do organismo patogénico e de factores de risco do hospedeiro. A paracoccidioidomicose, uma micose causada por espécies de *Paracoccidioides*, é um desses casos, estimando-se afectar cerca de 10 milhões de indivíduos em países da América Latina. Diversos estudos, nomeadamente aqueles focados na genómica e transcriptómica de isolados de *Paracoccidioides brasiliensis*, têm levantado questões importantes, particularmente no que respeita ao efeito que a sua natureza multinucleada e variabilidade morfológica possa ter na activação de mecanismos de defesa do hospedeiro, um aspecto nunca abordado até à data. Além destes aspectos, a ausência de ferramentas moleculares é um factor que tem vindo a dificultar tanto a identificação de factores genéticos envolvidos na virulência e natureza polimórfica da fase leveduriforme do fungo, bem como a elucidação dos mecanismos subjacentes ao seu comportamento dimórfico.

Uma particularidade da fase leveduriforme destas espécies é a sua heterogeneidade morfológica, assim como os seus padrões de gemulação. Estudos anteriores salientam a importância do gene *CDC42* (cell division cycle 42) na definição destas características, no entanto, estas nunca foram determinadas individualmente para cada isolado de *Paracoccidioides*. Outro aspecto nunca abordado é a identificação de possíveis associações entre factores genéticos que determinem a morfologia do fungo e o padrão morfológico de cada isolado.

De forma a elucidar estes aspectos, realizou-se uma detalhada análise morfogenética da fase leveduriforme de 11 isolados clínicos e ambientais de *Paracoccidioides* pertencentes a espécies de *P. brasiliensis* e *Paracoccidioides lutzii*. Os nossos resultados demonstram a ausência de um perfil morfológico característico para cada uma das linhagens destas espécies. No entanto, a área e forma das gémulas de cada isolado revelou-se altamente dependente da célula-mãe, sugerindo um elevado nível de conservação destas características ao longo das várias gerações. De referir também as fortes correlações encontradas entre a expressão do gene *PbCDC42*, uma peça importante no controlo do comportamento morfológico da fase leveduriforme, e a forma das células-mãe e gémulas, assim como com a área das gémulas. Estes dados permitem

ampliar o conhecimento actual referente à natureza polimórfica de *P. brasiliensis*, nomeadamente no que respeita à clarificação das suas características morfológicas.

Em *P. brasiliensis*, diversas alterações morfológicas e fenotípicas ocorrem durante o processo de transição da fase de conídeo/micélio para a fase leveduriforme, um processo fundamental inerente à patogenicidade do fungo. Para além destas alterações, a auxotrofia da fase leveduriforme para compostos de enxofre orgânico é também uma característica marcante. Embora este requisito nutricional seja comum a outros fungos dimórficos, as suas bases moleculares nunca foram clarificadas. Neste contexto, procedeu-se à avaliação do papel da proteína SconC, um regulador negativo da via de assimilação do enxofre inorgânico, no dimorfismo e virulência do fungo. Através da redução de expressão do gene *SCONC* por manipulação genética demonstrou-se que, nestas circunstâncias, a fase leveduriforme das células de *P. brasiliensis* consegue ultrapassar a sua auxotrofia a compostos de enxofre orgânico, utilizando fontes de enxofre inorgânico. No entanto, esta alteração metabólica levou a uma redução drástica dos níveis celulares de ATP e NADPH, provocando um aumento significativo do stress oxidativo. Este facto pode explicar a diminuição de virulência observada nas estirpes de *P. brasiliensis* reduzidas na expressão do gene *SCONC*, como verificado experimentalmente em ensaios com ratinhos. Os resultados aqui apresentados fornecem informações relevantes relativas ao controlo dos mecanismos subjacentes ao processo dimórfico de *P. brasiliensis*, evidenciando SconCp como uma molécula determinante no processo de virulência.

A forma leveduriforme de *P. brasiliensis* é também caracterizada pela sua natureza multinucleada. Durante o processo de infecção, a morte celular do fungo pode resultar na libertação de elevadas quantidades de DNA, levando eventualmente à activação de mecanismo de imunidade inata via receptores Toll-like 9 (TLR9) por parte do hospedeiro. O TLR9 é a molécula da família dos receptores Toll-like responsável pelo reconhecimento de sequências CpG não-metiladas em moléculas de DNA. No entanto, o papel deste receptor no contexto de infecções por *P. brasiliensis* nunca foi explorado. Neste trabalho demostrou-se que a activação deste receptor é um processo que parece ser crucial durante as fases iniciais de infecção com *P. brasiliensis*. Utilizando como modelo ratinhos deletados em TLR9, verificou-se uma morte prematura do hospedeiro aquando da sua infecção com células de *P. brasiliensis*, associada a sinais de patologia em vários órgãos e elevada produção de citocinas pró-inflamatórias. Este perfil pode estar associado ao elevado recrutamento de neutrófilos para o local de infecção. Em

suma, demonstrou-se que a activação de TLR9 é imuno-protectora em fases iniciais de infecções por *P. brasiliensis*, tendo um papel preponderante no controlo do desenvolvimento de uma resposta imune mediada por células.

O trabalho desenvolvido no âmbito desta tese acrescenta novos conhecimentos relativamente à morfologia de *P. brasiliensis*, relevando-se a importância dos níveis de expressão do gene *CDC42* no perfil morfológico da fase leveduriforme do fungo. São também apresentados novos dados sobre o metabolismo da fase leveduriforme de *P. brasiliensis*, permitindo esclarecer o impacto do dimorfismo celular no metabolismo de enxofre e sua relevância na patogenicidade do fungo. São também apresentados dados essenciais que permitem novos conhecimentos sobre os mecanismos desenvolvidos pelo hospedeiro numa resposta imune apropriada a infecções por *P. brasiliensis*, associando-se a natureza multinucleada do fungo com a activação de mecanismos de defesa via TLR9.

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OBJECTIVES AND OUTLINE OF THE THESIS

The work presented throughout this thesis was developed in the context of the project **Unravelling the specific involvement of the small Rho-like GTPase Cdc42 in the highly polymorphic nature of *Paracoccidioides brasiliensis* yeast cells**, funded by *Fundação para a Ciência e a Tecnologia* (Grant Number: PTDC/BIA-MIC/108309/2008) and coordinated by Doutor Fernando Rodrigues. All the work was performed in the Microbiology and Infection research domain of the Life and Health Sciences Research Institute (ICVS/3B's), School of Health Sciences, University of Minho, Braga, Portugal.

The main objectives of the research conducted in the scope of this thesis aimed to (i) characterize *Paracoccidioides* isolates morphologically and explore possible associations between each isolate morphological trends and genetic factors (ii) understand the molecular basis underlying *P. brasiliensis* dimorphism and its association with the fungus auxotrophy to organic sulfur compounds (iii) provide new data on the role of TLRs in host responses against *P. brasiliensis*.

Chapter 1 consists of a general introduction, presenting a review of the current knowledge and up-to-date literature on *P. brasiliensis* biology and paracoccidioidomycosis, with special emphasis on the genetics underlying the fungus morphology and dimorphism, the basis of sulfur metabolism, studies on fungus virulence factors and host-immune responses to *P. brasiliensis*.

Chapter 2 focuses on the morphological characterization of the different clades of *Paracoccidioides* species. To achieve this objective, a detailed morphometric evaluation of different clinical and environmental *P. brasiliensis* isolates comprising all the phylogenetic lineages, including a strain from *Paracoccidioides lutzii* species (Pb01), was performed. Moreover, correlations between the analyzed morphological parameters and *PbCDC42* expression levels were estimated. We have therefore explored the highly polymorphic nature of *P. brasiliensis*, providing additional evidences on how the morphology of this fungus is controlled, corroborating the key role of *PbCDC42* in defining *P. brasiliensis* yeast cell area and shape.

In chapter 3, *P. brasiliensis* dimorphic traits are discussed. The role of sulfur metabolism during the dimorphic processes is explored, taking into special consideration the yeast-phase auxotrophy to organic sulfur. The data presented in this chapter highlights the key role of SconC, the negative regulator of the inorganic sulfur assimilatory pathway, during mycelium-to-yeast transition, providing valuable information on the fungus metabolism and genetics. Moreover,

important data was obtained for the establishment of a new virulence determinant, a relevant achievement for the future development/design of new therapeutic strategies against paracoccidioidomycosis.

Chapter 4 centers on the study of immune responses developed by the host upon *P. brasiliensis* infection, specifically on the role played by TLR9. An *in vivo* approach was employed to determine the outcome of infection in TLR9-depleted mice, and *in vitro* studies were performed to dissect the immunological profile followed by the host in early-times of infection. Data presented during this chapter adds important knowledge on host-immunity against *P. brasiliensis*, suggesting TLR9-driven responses essential for the early development of an appropriate protective immune response.

In chapter 5, concluding remarks are presented, bringing together Chapter 2, 3 and 4 in the context of the initially proposed objectives. Furthermore, future prospects for the investigation of *P. brasiliensis* are depicted, highlighting the further elucidation of other virulence determinants and how host-immune responses are governed during infection.

ABBREVIATIONS

4-HPPD	4-hydroxyl-phenyl pyruvate dioxygenase
aRNA	antisense-RNA
ATMT	*Agrobacterium-tumefaciens* mediated transformation
ATP	Adenosine-5'-triphosphate
BMDM	Bone marrow-derived macrophage
CLR	C-type lectin receptor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DHE	Dihydroethidium
DHR 123	Dihydrorhodamine 123
ESTs	Expressed sequence tag
FCM	Flow cytometry
HIV	Human immunodeficiency virus
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PCM	Paracoccidioidomycosis
PCR	Polymerase chain-reaction
PFGE	Pulse field gel electrophoresis
PLB	Phospholipase B
PMN	Polymorphonuclear neutrophil
RDA	Representational difference analysis
RNA	Ribonucleic acid
SSH	Subtractive suppression hybridization
TLR	Toll-like receptor

CHAPTER 1

GENERAL INTRODUCTION

PARACOCCIDIOIDES BRASILIENSIS BIOLOGY

PHYLOGENY AND CRYPTIC SPECIATION

P. brasiliensis was first described in 1908 by Adolf Lutz, who reported the disease caused by this fungus as a new "American hyphoblastomycosis" (Lutz 1908). In 1911, Alfonse Splendore proposed the denomination of *Zymonema brasiliense* to the fungus, which was altered to *Paracoccidioides brasiliensis* in 1930 by Floriano de Almeida (Palmeiro 2005). *P. brasiliensis* is a dimorphic ascomycete fungus taxonomically related to the family Ajellomycetaceae (order Onygenales, phylum Ascomycota) (Bagagli, Theodoro et al. 2008). Together with *Blastomyces dermatitidis*, *Coccidioides immitis*, *Coccidioides posadasii* and *Histoplasma capsulatum*, these species belong to a fungal group that is common to most agents of endemic systemic mycoses (Bagagli, Theodoro et al. 2008). Due to the fact that a teleomorphic form of the fungus has not yet been revealed, *P. brasiliensis* was for long time considered to be a single species, despite the high genetic variability found among different isolates (Soares, Madlun et al. 1995; Montoya, Moreno et al. 1997; Nino-Vega, Calcagno et al. 2000). The inability to determine the sexual stage in fungi such as *P. brasiliensis* led to the development of phylogenetic analysis of molecular markers as a technique for species recognition and determination of the reproductive mode (Tibayrenc 1996). In this sense, Matute and co-workers were the first to define three distinct and unrecognized species within the *P. brasiliensis* taxa, based on sequence analysis of eight regions of five nuclear loci (Matute, McEwen et al. 2006). Two of these phylogenetic species were described as monophyletic (PS2, comprising Brazilian and Venezuelan isolates, and PS3, comprising Colombian isolates), while the third one as paraphyletic (S1, comprising isolates from Brazil, Argentina, Peru, Venezuela and Paraguay) (Fig. 1). Isolates belonging to S1 and PS2 clades were found to be sympatric and recombinant, whereas those from PS3 allopatric and clonal (Matute, McEwen et al. 2006). Recently, a genetically different population comprising 17 isolates, including Pb01, was assembled as a new species, named *Paracoccidioides lutzii*. Isolates from the *P. lutzii* species are endemic to the Brazilian Central-Western region (Fig. 1), and tend to present phenotypic characteristics that differentiate them from *P. brasiliensis* (e.g. virulence, resistance to fungicides) as well as different responses to the host (Teixeira, Theodoro et al. 2009).

The isolates belonging to this new cluster were found to be genotypically similar among them, but distinct from the S1/PS2/PS3 clade. As reported by Teixeira and co-workers, isolates from these

Paracoccidioides species exhibit significant morphological and genetic differences compared to the isolates from the *P. brasiliensis* species. Additionally, recombination analysis revealed independent events inside *P. lutzii* and S1/PS2/PS3 clades, suggesting reproductive isolation (Teixeira, Theodoro et al. 2009).

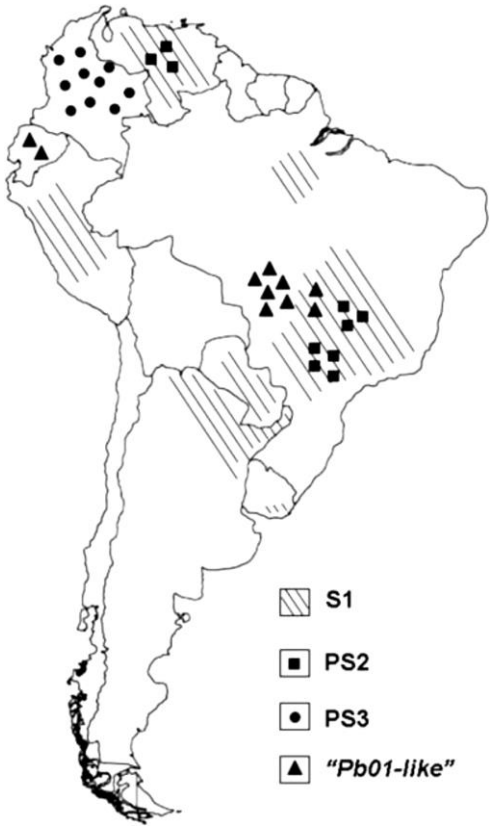

Figure 1. Geographic distribution of the phylogenetic species of *Paracoccidioides* genus (adapted from Teixeira *et al*, 2009).

ECOLOGY

Although the natural habitat of *Paracoccidioides* species has not yet been precisely determined, epidemiological data indicates that the natural reservoir for this fungus lies in a humid environment (Brummer, Castaneda et al. 1993) mainly in geographic areas of South and Central America (Simoes, Marques et al. 2004). Although the fungus has been sporadically isolated from commercial dog chow (Ferreira, Freitas et al. 1990), penguin excreta from Antarctica (Gimenez, Tausk et al. 1987) and feces from bats (Grose and Tamsitt 1965), several studies indicate that it can be found with high incidence in the nine-banded armadillo, *Dasypus novemcinctus*, a wild

mammal usually found in South America and that lives in the soil (Bagagli, Sano et al. 1998; Corredor, Castano et al. 1999; Silva-Vergara, Martinez et al. 2000; Bagagli, Franco et al. 2003). In these animals unusual granulomas were found, containing fungal cells (Bagagli, Sano et al. 1998; Silva-Vergara, Martinez et al. 2000). Their low body temperatures (32-35ºC) make these animals the ideal host to *P. brasiliensis*. It is however important to stress that the isolation of *P. brasiliensis* from soil samples, an important aspect to be considered for the correct definition of the fungus natural habitat, is very hard to accomplish. This is probably due to the fungus slow growth and restricted ability to compete or survive in the presence of natural soil microbiota (Bagagli, Bosco et al. 2006).

The virulence, genetic and molecular profiles of *P. brasiliensis* isolates recovered from animals reveal to be highly variable (Sano, Defaveri et al. 1998; Sano, Tanaka et al. 1998; Hebeler-Barbosa, Montenegro et al. 2003), a trend also followed by clinical isolates collected from patients. This indicates that both humans and animals present a milieu required for the optimal growth of *P. brasiliensis* that is likely to be similar (Bagagli, Sano et al. 1998; Franco, Bagagli et al. 2000).

Although large advances have been made in determining *P. brasiliensis* ecological niche, it is of utmost importance to improve knowledge concerning the best environmental conditions for fungal development, such as temperature, salt tolerance and interaction with other soil microorganisms. This would help on defining the fungus ecological niche allowing to accurately determine the circumstances and conditions that allow the fungus to grow and disseminate in the host.

MORPHOLOGY

P. brasiliensis is a dimorphic fungus that, at temperatures around 37ºC, is present as yeast (the pathogenic form for humans), whereas at temperatures below 28ºC develops as mycelium (the non-pathogenic form) (Brummer, Castaneda et al. 1993). Mycelial phase of *P. brasiliensis* is characterized by the formation of white, small and irregular colonies (Fig. 2A). Usually it takes around 15 to 20 days (depending on the strain) to form these colonies, in temperatures ranging from 19 to 28ºC (Brummer, Castaneda et al. 1993). When in this morphological state, the cell wall is composed of two layers, being the outermost composed by β-1,3-glucan and the innermost having a reduced chitin composition when compared to the yeast phase (13% of the cell wall) (Kanetsuna, Carbonell et al. 1969). Microscopically, hyphae are multinucleated, septated and thin (Fig. 2B).

When cultured in media with reduced concentrations of carbohydrates and low temperatures, it is possible to observe the formation of diverse types of propagules such as arthroconidia or conidia (Samsonoff, Salazar et al. 1991). Unlike yeast or mycelia, these structures are uninucleated and small sized, whereas exhibiting thermal dimorphism that can give rise either to mycelia or yeast cells (McEwen, Restrepo et al. 1987).

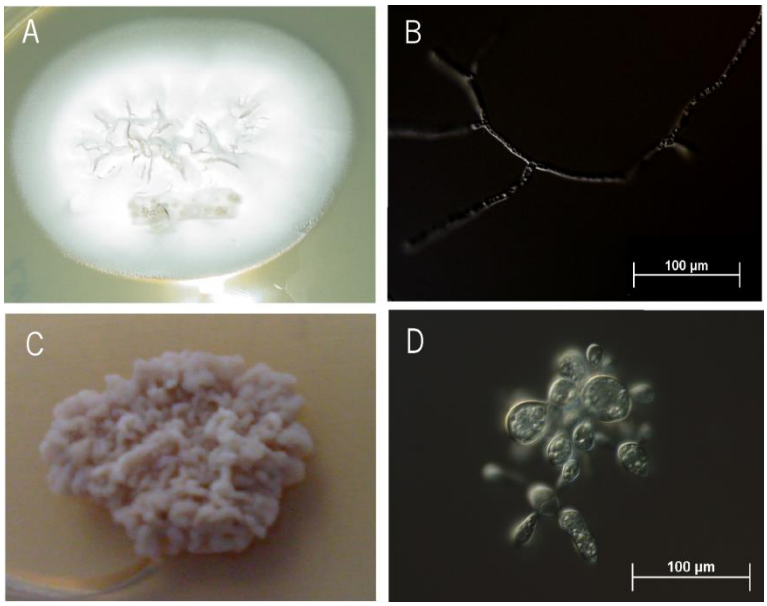

Figure 2 – Mycelial and yeast forms of *P. brasiliensis*. (A) Macroscopic features of the mycelial form; (B) Differential Interference Contrast (DIC) observation of mycelial cells (C); Macroscopic features of the yeast form; (D) Differential Interference Contrast (DIC) observation of yeast cells. (J. F. Menino *et al*, unpublished data).

The yeast phase of *P. brasiliensis* is characterized by cells with a soft, wrinkle and cream colored aspect, with sizes ranging from 4 to 30 µM (Brummer, Castaneda et al. 1993) (Fig. 2C). These cells have an extended duplication time, and as a consequence their growth becomes macroscopically visible only after 3 or 4 days of incubation. Most of the isolates have a long to elongated form, having a thick two-layered refractile cell wall (200 to 600 nm) and a cytoplasm that contains prominent lipid droplets. One of the hallmarks of the yeast phase of this fungus is the existence of multiple-budding mother cells, that can give rise to more than eight daughter cells (Fig. 2D). The appearance of such structures is usually denominated as “pilot’s wheel” (Brummer, Castaneda et al. 1993; San-Blas, Nino-Vega et al. 2002).

Yeast cells are also characterized for presenting variable areas with irregular shape, which can go from round to elongated or distorted cells, and by their multinucleated nature, with cells

presenting several nuclei with different sizes. The yeast cell wall is composed by α-1,3-glucan containing small amounts of α-1,3- or α-1,6-glycosidic linkages, and β-1,3-glucan (present in a much lesser extent) with a slime surface of complex mucopolysaccharides. Internally, it presents an elevated chitin composition (43% of the cell wall) (Kanetsuna, Carbonell et al. 1969).

The molecular basis underlying the pathogenic yeast-phase phenotypic characteristics of *P. brasiliensis* are still poorly understood. This is a relevant aspect, since it has been demonstrated that certain fungi modulate their morphology during host invasion to escape phagocytosis, therefore contributing to their pathogenicity (Chaffin 2008; Okagaki, Strain et al. 2010). Studies with *P. brasiliensis* demonstrate that yeast cells' shape and area can impact on the fungus virulence and disease progression (Svidzinski, Miranda Neto et al. 1999). Recent data on this matter highlighted the role of cell division cycle 42 (*CDC42*) in *P. brasiliensis* morphology (Almeida, Cunha et al. 2009). This gene encodes an essential GTPase that belongs to the Rho/Rac subfamily of Ras-like GTPases (Johnson 1999). Studies addressing the role of this molecule in various organisms (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, *Caenorhabditis elegans*) suggested that yeast budding frequency, temporal and spatial regulation of polarized growth, morphological switch control or even mating are attributed to its function (Fig. 3) (Miller and Johnson 1994; Drubin and Nelson 1996; Yaar, Mevarech et al. 1997; Steven, Kubiseski et al. 1998; Caviston, Tcheperegine et al. 2002; Howell, Savage et al. 2009).

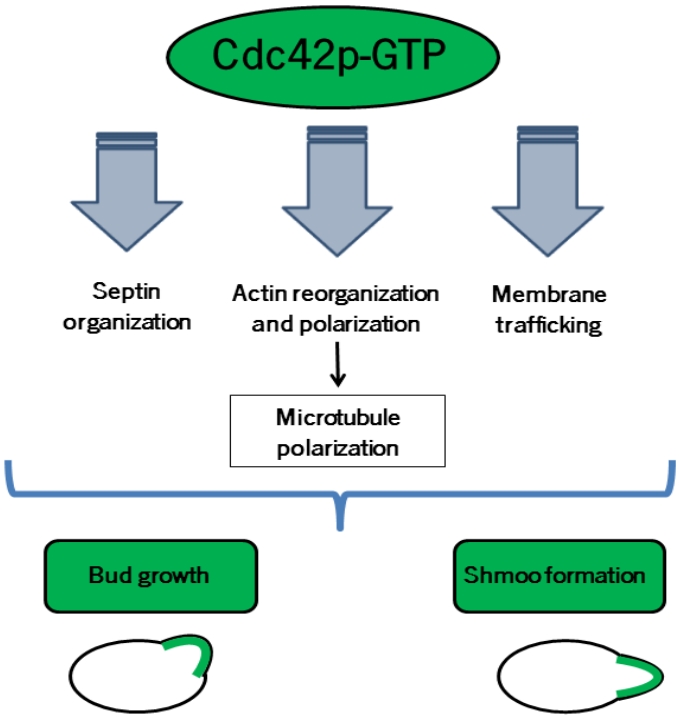

Figure 3 - Cdc42 during budding yeast polarization. Depending on the stimuli, budding yeast can polarize in different ways, whether leading to budding or formation of shmoos. The polarization of septins, actin, microtubule structures

and membrane trafficking allows a polarized growth, that leads during budding to the formation of a bud and during mating to the formation of a shmoo (adapted from Etienne-Manneville, 2004).

In 2009, Almeida and co-workers unravelled for the first time a role for PbCdc42p in *P. brasiliensis* morphology. Using antisense RNA (aRNA) technology, several mutants were obtained in unrelated *P. brasiliensis* isolates, with a large range of ranking of expression down-regulation of *PbCDC42*. The authors linked the expression of this gene to the area and shape of yeast cells, since down-regulation of *PbCDC42* resulted in cells with a more organized and controlled growth, with mutant cells being less elongated and polymorphic and more spherical when compared to the wild-type counterparts (Fig. 4). In addition to its crucial role in controlling yeast cell morphology, the authors also show that diminished levels of *PbCDC42* impair the virulence levels of the fungus, since increased phagocytosis by macrophages and abrogated virulence in a mouse model of infection was demonstrated (Almeida, Cunha et al. 2009).

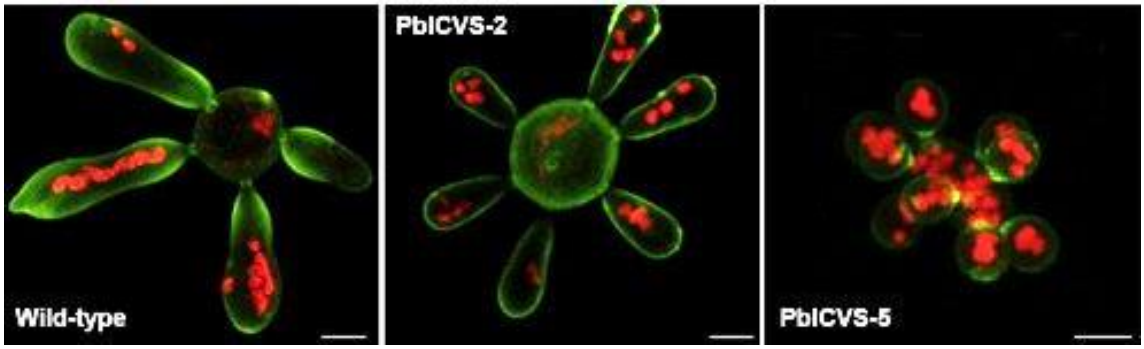

Figure 4 - Silencing of *PbCDC42* leads to a more homogenous cell growth. Confocal microscopy observation of PI and FITC-ConA double-stained wild-type and two PbCDC42 knock-down yeast strains, PbICVS-2 and -5. White bars correspond to 5 µm (adapted from Almeida *el at*, 2009).

Although these findings provide genetic support for the relevance of *PbCDC42* expression on the cell area, shape and virulence of *P. brasiliensis*, it becomes imperative to question if the high diversity of yeast cell shape and areas found for isolates from each cryptic group and among the different clades could be related with the expression levels of *PbCDC42* as a widespread event in *P. brasiliensis*. The clarification of these subjects could represent an important achievement for the discrimination of factors that can be directly or indirectly involved in the virulence of each isolate/cryptic group.

DIMORPHISM IN *P. BRASILIENSIS*

Temperature is a crucial factor to promote transition from the conidia/mycelial phase to the yeast phase in *P. brasiliensis*, a feature also shared with other dimorphic fungi such as *H.*

capsulatum and *B. dermatitidis* (Medoff, Kobayashi et al. 1987; Medoff, Painter et al. 1987). At environmental temperatures, *P. brasiliensis* occurs at the mycelial/conidia form. However, during the infective process, it changes to the yeast phase, in a process triggered in order to survive and disseminate throughout the host (Brummer, Castaneda et al. 1993; San-Blas, Travassos et al. 2000). The morphological changes undergone by *P. brasiliensis* throughout conidia/mycelium-to-yeast transition are clearly linked to its virulence, since alterations in the cell shape facilitates host tissue penetration, dissemination, intracellular colonization, and expression of virulence factors (San-Blas, Travassos et al. 2000). Some of the most significant alterations experienced by *P. brasiliensis* occur in the cell wall. During transition, extensive modifications occur, mainly due to a switch in glucan polymer linkage from β-1,3-glucan to α-1,3-glucan (Sorais, Barreto et al. 2010) and alterations in the lipid composition (Toledo, Suzuki et al. 1995). This is a reversible process, since the shift to temperatures below to those of the host allow transition to the conidia/mycelial phase (Medoff, Painter et al. 1987).

At the biochemical level, important changes occur, following a strictly regulated process. *Paracoccidioides* cells undergoing mycelium-to-yeast transition experience three main stages: stage 1, characterized by several degrees of uncoupling of oxidative phosphorylation, leading to a decrease on the cellular ATP levels, respiration rates and concentrations of electron transport components; stage 2, where cells decrease or cease respiration, demanding cysteine or other sulfhydryl-containing compounds to maintain viability; stage 3, characterized by the complete formation of yeast cells (Medoff, Painter et al. 1987). However, the molecular basis underlying this transition started to be uncovered only in the last decade. Several studies addressing these matters identified genes and overall pathways required for the thermodimorphic process and pathogenicity. The usage of techniques such as construction of expression sequence tags (ESTs) and library collections, studies based on DNA microarrays and subtractive suppression hybridization (SSH), *in silico* determination of over-expressed genes and cDNA representational difference analysis (RDA), have proven to be powerful methods of genetic analysis tools for the investigation of *P. brasiliensis* morphogenesis and virulence. In 2005, a large-scale gene expression analysis carried by Nunes and co-workers using high throughput microarray technology, enabled the identification of 2.583 genes displaying statistically significant modulation during transition (Nunes, Costa de Oliveira et al. 2005). Some of these genes encoded proteins involved in processes such as (i) signal transduction (ii) protein synthesis (iii) cell wall metabolism (iv) genome structure (v) oxidative stress response (vi) growth control (vii)

development. Among these, the gene encoding 4-hydroxyl-phenyl pyruvate dioxygenase (4-HPPD), an enzyme associated to the catabolism of aromatic amino acids, was highly overexpressed during the mycelium-to-yeast differentiation. This enzyme seems to be crucial during the mycelium-to-yeast transition, as its *in vitro* inhibition was able to stop growth and differentiation of the yeast phase of the fungus (Nunes, Costa de Oliveira et al. 2005). Studies using *in silico* electronic subtraction and cDNA microarrays also provided relevant data on genes being up or down-regulated in either one of the morphological states, such as genes related to cell cycle, stress response, drug resistance and signal transduction pathways (Felipe, Andrade et al. 2005). In a recent study, the inhibition of mitochondrial complexes III and IV or alternative oxidase (AOX) delayed mycelium-to-yeast transition of the fungus, whereas the association of AOX with complex III or IV inhibitors blocked the morphological switch. Expression of *AOX* was shown to be developmentally regulated throughout the differentiation process, being the highest expression levels achieved in the first 24 h and during the yeast exponential growth phase (Martins, Dinamarco et al. 2011).

To identify genes specifically expressed during infection, ESTs from infected mice were isolated. Costa and co-workers showed that, during liver infection, genes involved in the utilization of multiple carbon sources were activated, which included glucose and glyoxylate cycle substrates. In addition, genes for nitrogen metabolism and biosynthesis, as well as lipid biosynthesis, were highly expressed. These findings suggest that nitrogen and lipid compounds are probably not easily obtained from the host, while the availability of carbohydrates for energy maintenance is not limited (Costa, Borges et al. 2007). Bailao and co-workers found that several genes related to melanin biosynthesis, iron acquisition and cell defense were being highly expressed during infection in a mouse model of infection (Bailao, Schrank et al. 2006). However, when using blood as culture medium, only genes related to cell wall remodeling/synthesis were being up-regulated (Bailao, Schrank et al. 2006). Furthermore, microarray data revealed that genes involved in respiratory and metabolic processes of sugars, amino acids, proteins and lipids, synthesis of transcription factors and transporters (small peptides, sugars, ions and toxins), and methionine/cysteine metabolism were up-regulated in the yeast phase of the fungus, whereas in the mycelial phase they were differentially expressed (Monteiro, Clemons et al. 2009).

Significant differences in the expression levels of genes related to the sulfur metabolism were described when comparing both morphological phases of *P. brasiliensis*. In one study, genes coding enzymes involved in the "*de novo*" synthesis of cysteine were found to be up-regulated in

the yeast phase, suggesting that *P. brasiliensis* actively synthesize cysteine from inorganic sulfate. Relevantly, the authors claim that inorganic sulfate is not required during the transition process, since cells can undergo transition between both morphological phases in the absence of these compounds (Andrade, Paes et al. 2006). In a different study, a large-scale analysis of gene expression revealed that transcriptional factors responsible for activation or repression of the inorganic sulfur assimilatory pathway were up-regulated in the yeast phase, though in different extents. The authors suggest that, although *P. brasiliensis* cannot use inorganic sulfur as single sulfur source, it can use both organic and inorganic pathways during the dimorphic transition processes (Ferreira, Marques Edos et al. 2006).

Although important advances were achieved in comprehending how *P. brasiliensis* morphological growth is controlled, the dimorphic events underlying the infectious process are far from being genetically well characterized. This is mainly due to the impossibility to perform gene disruptions in *P. brasiliensis*, a fact that has been hampering functional testing on putative master regulators mediating mycelium-to-yeast transition.

SULFUR METABOLISM

As referred above, genes from the sulfur metabolic pathway are differentially expressed during mycelium-to-yeast transition. This is a relevant aspect since, in addition to temperature, the inability of *P. brasiliensis* yeast cells to assimilate inorganic sulfur (a characteristic shared by *H. capsulatum* and *B. dermatitidis*) is the only condition known to affect this dimorphic process (Boguslawski and Stetler 1979; Paris, Duran-Gonzalez et al. 1985; Medoff, Kobayashi et al. 1987; Maresca and Kobayashi 1989). These species are auxotrophic for organic sulfur compounds such as cysteine, cystine or methionine in the yeast phase, being unable to grow on their absence. On the other hand, the mycelial phase is prototrophic to organic sulfur compounds, as these fungus can use both organic and inorganic sulfur (Boguslawski and Stetler 1979; Paris, Duran-Gonzalez et al. 1985; Medoff, Painter et al. 1987; Maresca and Kobayashi 1989). Sulfur compounds are involved in amino acid synthesis and in other major synthetic pathways. While methionine is required for the synthesis of the great majority of proteins in all organisms, cysteine is crucial for their structure, stability and catalytic function (Marzluf 1997). The complex regulatory circuitry of sulfur metabolism must operate in order to insure the fulfillment of the cell requirements, either by controlling the level of entry of sulfur into the assimilatory pathway or via distinct steps within the main pathway itself (Marzluf 1997; Kopriva,

Suter et al. 2002). The assimilation of inorganic sulfur by fungi leads to "*de novo*" synthesis of cysteine, which is dependent on the organism needs for organic sulfur. The process begins with the sulfate uptake by the cell through sulfate permeases present in cell membranes, in an energy-dependent process. Once inside the cell, inorganic sulfur is phosphorylated, leading to the formation of adenosine-5-phosphosulfate (APS) which is on its turn phosphorylated, yielding a high-energy sulfate donor, 3'-phosphoadenosine-5' phosphosulfate (PAPS), known as "active sulfate" (Fig. 5).

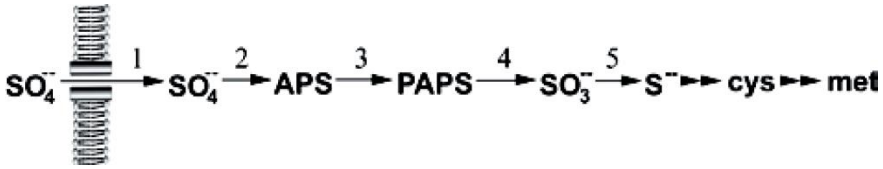

Figure 5 – Schematic representation of the inorganic sulfur assimilatory pathway. 1 – sulfate permease; 2 – ATP sulfurylase; 3 – APS kinase; 4 – PAPS reductase; 5 – sulfite reductase. (Adapted from Pilsyk and Paszewski, 2009).

In the final stage of this process, PAPS is reduced to sulfite and sulfide by the enzymes PAPS reductase and sulfite reductase, respectively, in order to generate cysteine (Fig. 5).

In organisms such as plants and fungi, the presence of a lateral branch whereby PAPS is converted to Choline-O-Sulfate results in the production of an internal pool off inorganic sulfate, that can re-enter the pathway, depending on the cell cysteine requirements (Marzluf 1997). Recent studies have addressed the genetics underlying the differential sulfur requirements of *P. brasiliensis* species. Transcriptomic analysis reported so far identified numerous genes involved in the sulfur assimilatory pathway that are differentially expressed, depending on the morphological state of the cell. Methionine permease was found up-regulated in the yeast phase (Marques, Ferreira et al. 2004). Similarly, ATP sulfurylase, APS kinase, Choline sulfatase and PAPS reductase have their expression levels highly up-regulated in the yeast phase compared to the mycelial phase (Felipe, Andrade et al. 2005; Andrade, Paes et al. 2006). More recently, *METR*, the homologue of *cys-3* and *metR* genes from *Neurospora crassa* and *Aspergillus nidulans*, respectively, were found with a 35-fold increase in the yeast phase compared to the mycelial phase (Ferreira, Marques Edos et al. 2006). *METR* is a member of the bZIP family of DNA-binding proteins. The bZIP domain consists of a leucine zipper, responsible for dimerization, and an immediate upstream basic region, essential for specific binding to DNA (Marzluf 1997). Under conditions of extracellular abundance of inorganic sulfur, *METR* acts as a transcriptional

factor that promotes the expression of genes encoding for permeases and enzymes involved in the acquisition of extracellular inorganic sulfur (Marzluf 1997). *SCONC*, the homologue of *scon-2*, *MET30* and *sconC* genes from *N. crassa*, *S. cerevisiae*, and *A. nidulans*, respectively, was also found with a 1,2-fold increase expression in the yeast phase compared to the mycelial phase (Ferreira, Marques Edos et al. 2006). This gene is a member of SCF, a multiprotein ubiquitin ligase complex (Marzluf 1997; Thomas and Surdin-Kerjan 1997; Patton, Willems et al. 1998), that in conditions of organic sulfur abundance leads to the proteolysis of MetR , "switching off" the inorganic sulfur assimilatory pathway (Fig. 6). The cross-talk of these two regulators has been suggested to be responsible for *P. brasiliensis* yeast-phase auxotrophy for organic sulfur compounds, by preventing pathogenic yeast cells to assimilate inorganic sulfur.

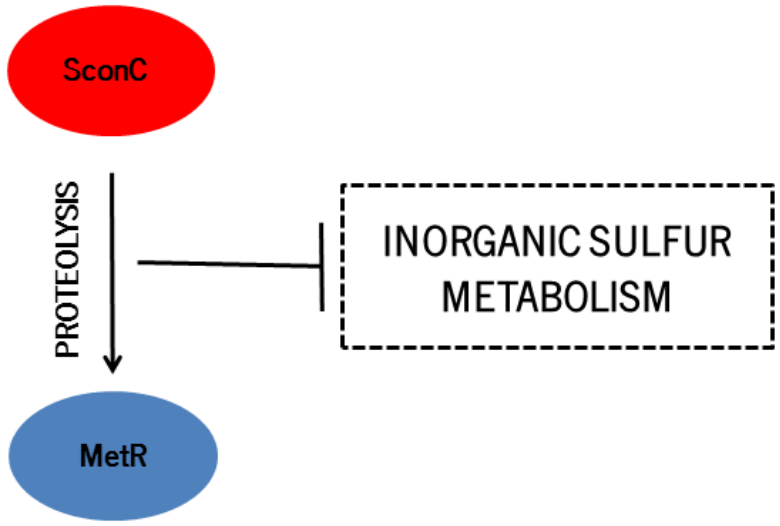

Figure 6 – Schematic representation of sulfur-mediated catabolic repression by proteolysis of MetR. In conditions of abundance of organic sulfur, SconC promotes ubiquitin-mediated proteolysis of MetR, inhibiting the transcription of enzymes required for inorganic sulfur assimilation (adapted from Goldman *et al*, 2012).

Although considerable information concerning a possible link between *P. brasiliensis* dimorphism and the sulfur metabolism has been provided in the last few years, this matter must be further dissected. Since conidia/mycelium-to-yeast transition is an essential step for the development of PCM, one cannot exclude the hypothesis that the fungus virulence can depend on the sulfur metabolism. Therefore, an approach at the molecular level on the sulfur metabolism could shed light on the unraveling of novel virulence determinants.

GENETICS AND MOLECULAR TOOLS

Despite the recent advances on the development of molecular tools to study *P. brasiliensis* genetic/genomic trends, there is still a long way to go towards a definitive elucidation of these matters. Importantly, the lack of an accurately described sexual phase in *P. brasiliensis* has also hindered the usage of classic genetic approaches to study this fungus. Over the past few years, the usage of techniques such as pulsed-field gel electrophoresis (PFGE), DNA hybridization and microfluorometry led to the estimation of *P. brasiliensis* genome size ranging from 23 to 31 mega base-pairs (Mb) for the yeast phase (Cano, Cisalpino et al. 1998; Feitosa, Cisalpino et al. 2003), similarly to what is described for other pathogenic fungi such as *H. capsulatum* and *C. immitis* (Pan and Cole 1992; Carr and Shearer 1998). Interestingly, in these studies some isolates were found to present twice the genome content previously referred, suggesting that *P. brasiliensis* yeast cells could have a diploid DNA content. In 2007, Almeida and co-workers developed an optimized Flow-cytometry (FCM) protocol, and by analyzing the *GP43* gene, *P. brasiliensis* was shown to have a haploid DNA content per nuclei, with a genome size ranging from 26.3 to 35.5 Mb for the yeast phase. A similar genome size was described for conidia (30.2 Mb), allowing one to exclude the hypothesis of ploidy shift during morphogenesis (Almeida, Matute et al. 2007). Moreover, the authors showed no association between genome size/ploidy and the clinical/epidemiological feature of the studied isolates.

More recently, the genome of two *P. brasiliensis* strains (Pb03 and Pb18) and one *P. lutzii* strain (Pb01) was completely sequenced (data available at http:// www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). The genome sizes ranged from 29.1 to 32.9 Mb, encoding 7.61 to 8.13 genes. Additionally, 94% of Pb18 assembly was successfully anchored to an optical map consisting of five linkage groups, possibly corresponding to five complete chromosomes. Interestingly, this study also suggest a genetic predisposition for Onygenales to degrade plant and animal substrates, enabling species such as *P. brasiliensis* to transfer from soil to animal hosts (Desjardins, Champion et al. 2011).

The development of manageable molecular techniques has recently allowed increasing knowledge on *P. brasiliensis* genomics, mainly regarding functional genomic studies by means of genetic random insertion mutagenesis via *Agrobacterium tumefaciens* mediated-transformation (ATMT) (Bundock, den Dulk-Ras et al. 1995). *A. tumefaciens* is a bacterial plant pathogen carrying a tumor-inducing (Ti) plasmid containing a DNA segment (T-DNA) that is randomly and separately inserted into the plant genome during infection (Hoekema, Roelvink et al. 1984).

Using this organism, Bundock and co-workers developed an efficient protocol to allow the integration of the T-DNA segment from *A. tumefaciens* into eukaryotic organisms other than plants, such as *S. cerevisiae* (Bundock, den Dulk-Ras et al. 1995). The authors also demonstrated that the route of integration of foreign DNA into the recipient organism is dependent on host factors, since integration in *S. cerevisiae* was accomplished by homologous recombination, instead of random integration in the genome.

Based on this technique, an efficient protocol was developed by Almeida and co-workers in order to insert exogenous DNA into *P. brasiliensis* genome (Almeida, Carmona et al. 2007), by co-cultivation of *A. tumefaciens* and *P. brasiliensis* cells. Several parameters were revealed to be essential during the process, such as the ratio of *A. tumefaciens*/ *P. brasiliensis* cells, co-cultivation conditions (time of air-drying, light exposure) and recovery time of host cells.

More recently, this methodology was employed to perform genetic manipulation in *P. brasiliensis* by means of gene expression down-regulation using antisense-RNA sequences targeting specific genes of the host. This technique employs the ATMT methodology, and through *in silico* analysis an *in vitro* manipulation, an artificial vector containing a resistance marker (e.g. hygromycin B phosphotransferase gene) and the aRNA sequence (Fig. 7) is inserted into *P. brasiliensis* genome, that depending on intrinsic factors, can lead to the reduction of the expression of the target gene (Menino, Almeida et al. 2012).

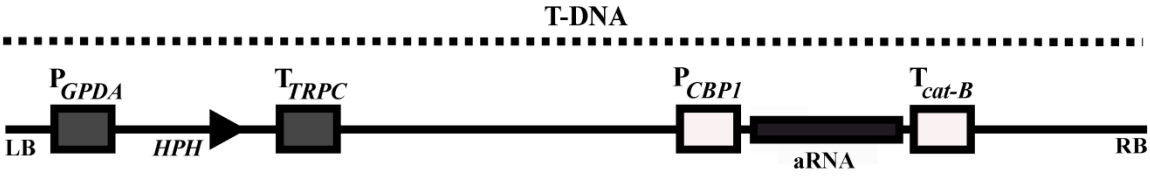

Figure 7 – Transfer-DNA (T-DNA) construct for aRNA silencing of a *P. brasiliensis* target gene via Agrobacterium tumefaciens-mediated transformation. T-DNA harboring the hygromycin B phosphotransferase (HPH) gene driven by the *A. nidulans* glyceraldehyde 3-phosphate (GPDA) promoter and transcriptional terminator (TRPC) with aRNA oligonucleotide under the control of the calcium-binding protein (CBP1) promoter from *H. capsulatum*. The construct is carried out in the pUR5750 vector (adapted from Menino *et al*, 2012).

Altogether, this methodologies are currently being applied by several research groups in the context of *P. brasiliensis* genetics, and it is now possible to study the structural organization and dynamic processes in *P. brasiliensis* by the observation of fluorescent-tagged proteins, and to

perform targeted down-regulation of gene expression (Almeida, Cunha et al. 2009; Hernandez, Almeida et al. 2010; Menino, Almeida et al. 2012). Using this approach, one can access the function of each gene in its pathway, and understand how *P. brasiliensis* control disease-related processes such as dimorphism, temperature-dependent behavior, and metabolic pathways vital for its survival inside the host.

PARACOCCIDIOIDOMYCOSIS

EPIDEMIOLOGY: DEMOGRAPHICS-GEOGRAPHICAL DISTRIBUTION

Paracoccidioidomycosis (PCM), a disease caused by *Paracoccidioides* species, is characterized by long latency periods (Brummer, Castaneda et al. 1993). As previously referred, this fact has hindered the precise determination of the site where the infection initiated and the exact ecological niche and route of infection followed by *P. brasiliensis*. However, over the years data has been collected supporting that the infectious process most likely starts by the inhalation of asexual propagules of the saprobic mycelial phase of the fungus existing in nature. Epidemiological data further support this via of infection, as determined by the appearance of PCM in farmers from agricultural regions (Brummer, Castaneda et al. 1993). Once the propagules reach the lower airway, a complex is formed leading to the generation of secondary systemic lesions arising through lymphatic and blood dissemination to various organs of the host (San-Blas, Nino-Vega et al. 2002; Wiwanitkit 2010; Marques 2012). It is estimated that the annual incidence of the disease in endemic areas is around 1 new case per 100.000 inhabitants, with a mortality rate of 1.65 cases per 1.000.000 inhabitants (Restrepo, McEwen et al. 2001). This disease is more common among farmers and rural workers between 30 and 40 years old, although 3 to 10% of the reported cases are from children and young adults (Brummer, Castaneda et al. 1993). Relevantly, this disease affects mainly males, in a proportion of 113 males to 1 woman. It is believed that the presence of estrogens, especially estradiol, is the main reason behind this differential gender-infection, since it seems to block conidia/mycelium-to-yeast transition (Restrepo, Salazar et al. 1984; Aristizabal, Clemons et al. 1998).

PCM is considered an autochthonous disease from northern Argentina to southern Mexico, with higher incidence in Brazil, accounting for 80% of the reported cases, followed by Columbia, Venezuela and Argentina (Fig. 8), (Brummer, Castaneda et al. 1993).

Figure 8 – Geographic distribution of PCM in Central and Latin America. Blue specifies areas with incidence of the disease (adapted from Duane *et al*, 2013).

In Brazil, PCM is the leading death cause of non-immunosuppressed patients due to systemic mycosis, and ranked as tenth as cause of death among infectious and parasitic diseases. Moreover, PCM is one of the responsible diseases for death among individuals with Human Immunodeficiency Virus (HIV) (Benard and Duarte 2000; Wanke and Aide 2009). Interestingly, reports of clinical PCM cases were shown in United States, Europe and Japan (Ajello and Polonelli 1985; Miyaji and Kamei 2003). The most reliable explanation for this fact is related with the population flow from Latin American to these countries, or short periods of immigration related with work or tourism in Latin America.

PATHOBIOLOGY AND CLINICAL FORMS

As previously referred, *P. brasiliensis* conidia produced by the saprobic mycelia act as infectious propagules (McEwen, Bedoya et al. 1987; Brummer, Castaneda et al. 1993). After inhalation of conidia by the host, a complex morphological shift to the pathogenic yeast phase occurs. The infectious process can give rise either to asymptomatic conditions or active disease, initially causing pulmonary lesions in the lungs of the host with the persistence of viable *P. brasiliensis* cells (called latent foci). Another possible outcome is the formation of extrapulmonary latent foci, by the dissemination of the fungus to other organs and tissues (Benard 2008).

PCM occurs mostly in immunocompetent individuals, with a gender-trend towards males (Brummer, Castaneda et al. 1993; Borges-Walmsley, Chen et al. 2002). Comorbidities such as alcoholism, malnutrition and acquired or iatrogenic immunosuppression disrupting host-agent equilibrium account as major reasons for the development of active disease (Benard 2008).

The standard diagnosis method of PCM is based on the microscopic identification of *P. brasiliensis* in clinical specimens or tissue. In fact this is an effective and inexpensive method, as it relies on the direct examination using 10% potassium hydroxide applied to a smear sample (Wanke and Aide 2009). In patients presenting oral or other skin lesions, the most common technique is based on the histological examination of tissue samples, using silver methenamine or periodic acid-Schiff stain. To better define the diagnosis and to evaluate treatment responses and disease recurrence, serological tests such as double-immunodiffusion, immunoenzymatic assays and counter-immunoelectrophoresis were proven to be valuable tools (Del Negro, Pereira et al. 2000; de Camargo 2008; Wanke and Aide 2009). Recently, the usage of molecular techniques such as PCR and real-time PCR revealed to increase the specificity of PCM diagnosis (Gomes, Cisalpino et al. 2000; Motoyama, Venancio et al. 2000; Buitrago, Merino et al. 2009). Moreover, some authors have also developed immunoenzymatic assays as an attempt to detect specific *P. brasiliensis* antigens like GP43 and GP70 in patients with PCM (Mendes-Giannini, Bueno et al. 1989; Gomez, Figueroa et al. 1998; Marques da Silva, Colombo et al. 2003; Marques da Silva, Queiroz-Telles et al. 2004).

Treatment of PCM is dependent on the severity of the disease and the clinical picture presented by the patient. The therapeutic strategies involve the elimination of the fungus, mainly based in sulphonamides, amphotericin B and azole derivatives (Yasuda 2005; Wanke and Aide 2009). In the absence of treatment, PCM is a fatal disease.

There are two different patterns which the disease may progress: the acute/subacute form and the chronic form.

Acute form: This is the most common form of infection in children, youth and adults under 30 years old, representing 10% of all PCM cases. The involvement of the monocytic phagocytic system results in manifestations such as fever, weight loss, lymph node enlargement, hepatosplenomegaly and bone marrow dysfunction (Fig. 9). In this clinical form lung involvement is very rare, and the clinical picture is characterized by the rapid progression of the disease, with

the patient's general condition being seriously impaired within weeks or few months. The rate of treatment success is approximately around 70% (Ramos and Saraiva Ldo 2008; Buitrago, Bernal-Martinez et al. 2011).

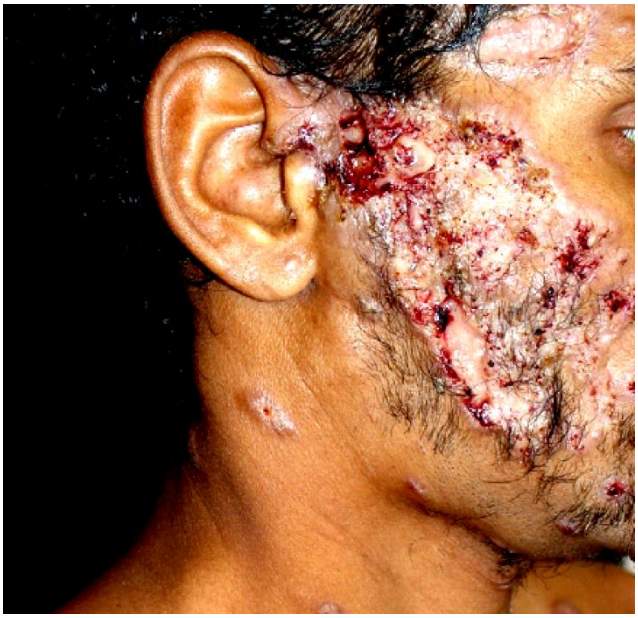

Figure 9 – Acute form of PCM. Large ulcerative lesion on face with infiltrated borders and hemorrhagic borders (adapted from Marques *et al*, 2012).

Chronic form: This form affects mostly adult males, and is characterized by a slow progression of the disease, contrarily to the acute/subacute form. Due to this fact, the need of health assistance is only necessary several months after the onset of the disease. The fungus dissemination throughout the oral mucosa (Fig. 10) prompts the patient to seek dental consultation. The most common symptoms are related to a single organ or system (unifocal) or to several organs (multifocal). This form of PCM usually involves oropharynx, skin, lymph nodes, adrenal glands and the lungs and mucosae of the upper respiratory tract, as opposed to the acute/subacute form. In some rare cases, the central nervous system, bones, joints, genital organs, eyes, thyroid and organs of the cardiovascular system may be compromised. The most common sequels can be both respiratory insufficiency and Addison's disease (Colombo, Faical et al. 1994; Ramos and Saraiva Ldo 2008).

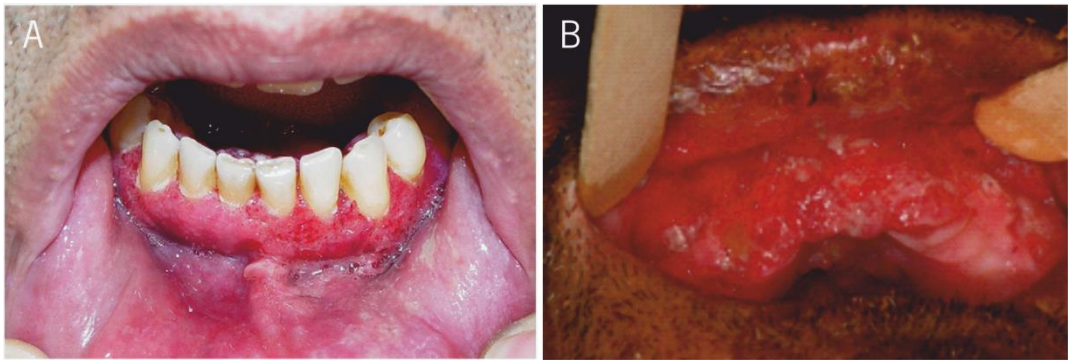

Figure 10 – Chronic form of PCM. (A) Superficial ulcerative lesion with hemorrhagic dots on gingiva. (B) Ulcerative and infiltrative lesion with multiple hemorrhagic dots and crusts on the upper gingiva and lip. (Adapted from Marques *et al*, 2012).

VIRULENCE FACTORS

Several aspects have to be considered for the determination of the specific attributes of *P. brasiliensis* to invade the host and disseminate, causing disease. Among others, the fungus dimorphism, the optimal temperature for fungal growth, adherence to host cells, cell wall components and enzyme production, are aspects that one has to explore for the identification of possible virulence factors of the fungus underlying the infectious process.

As referred previously, *P. brasiliensis* dimorphic conidia/mycelium-to-yeast transition can be considered a virulence factor, since this is a mandatory process that the fungus as to undergo to survive and disseminate in the host (Brummer, Castaneda et al. 1993; Silva-Vergara, Martinez et al. 2000; San-Blas, Nino-Vega et al. 2002; Klein and Tebbets 2007). This morphological switch leads to the remodeling of the cell wall constituents, mainly composed by lipids, proteins and polysaccharides (Kanetsuna and Carbonell 1970). During transition, the chitin content of the cell wall has a 3-fold increase, being α-1,3 glucan the glucoside bond in the yeast phase, whereas in the mycelial phase it is β-1,3 glucan (San-Blas and San-Blas 1977). San-Blas and co-workers showed that the virulence of different *P. brasiliensis* isolates is directly correlated with the levels of α-glucan. By reducing α-glucan levels by extended *in vitro P. brasiliensis* cultures, the fungus' virulence revealed to decrease (San-Blas 1985). Although these studies point α-glucan as a virulence factor, it is still not well understood. On the other hand, GP43, a 43-KDa glycoprotein that participates in the interaction with the host at different levels, is recognized as the key *P. brasiliensis* virulence factor. This protein was characterized as being an adhesin, as supported by experiments with a hamster model of infection. The adhesion of GP43 to extracellular laminin and fibronectin was demonstrated to enhance the fungus pathogenicity (Vicentini, Gesztesi et al. 1994). Popi and co-workers verified that GP43 impairs phagocytosis and fungicidal activity of

macrophages from resistant and susceptible mice, allowing *P. brasiliensis* to evade host defense mechanisms (Flavia Popi, Lopes et al. 2002). Recently, isolates with a specific GP43 genotype were found to be less virulent, whereas its expression was shown to be regulated not only at the transcriptional level but also at the protein/secretion level (Carvalho, Ganiko et al. 2005). Another element identified as a putative virulence factor was the pigment melanin, highly common in dimorphic fungi. Melanized *P. brasiliensis* cells are less phagocytized and more resistant to reactive oxygen species produced by host cells (da Silva, Marques et al. 2006; Silva, Thomaz et al. 2009).

As previously referred, *P. brasiliensis* CDC42 is a pivotal molecule for the definition of the fungus morphology (Almeida, Cunha et al. 2009). The authors also presented evidences linking this gene to virulence, since down-regulation of *CDC42* expression levels increased phagocytosis by murine macrophages. Depending on the level of *CDC42* down-regulation, virulence could be abrogated, as demonstrated using a mouse model of infection (Almeida, Cunha et al. 2009).

Extracellular phospholipase B (PLB) was equally identified as a putative virulence factor, as co-cultures with *P. brasiliensis* cells and alveolar macrophages resulted in higher expression levels of *PLB*. This up-regulation decreases the expression of pro-inflammatory cytokines, a circumstance that can impair host innate immunity (Soares, de Andrade et al. 2010). More recently, HAD32, the putative member of the haloacid dehalogenase (HAD) superfamily of hydrolases, was demonstrated to play an important role in *P. brasiliensis* virulence. By knocking-down *P. brasiliensis HAD32*, Hernandez and co-workers obtained mutants with lower adherence capacity to human epithelial cells and decreased virulence, as was demonstrated in a mouse model of infection (Hernandez, Almeida et al. 2010).

The usage of differential transcriptomic analysis has allowed researchers to identify genes that can putatively be considered virulence factors or, at least, be involved in the infectious process. However, the low number of molecular tools hampers the deeper exploration of the functionality of these genes. Given this, the exploration and modulation of morphology-related genes can represent a worthy focus of study, since *P. brasiliensis* dimorphic behavior is the central aspect underlying the infectious process.

HOST IMMUNE RESPONSES TO *P. BRASILIENSIS*

Upon *P. brasiliensis* infection, the host must activate immunological non-specific and specific mechanisms to sustain fungus proliferation and disease. While many individual exposed to the

fungus develop an asymptomatic infection, others are highly susceptible, developing conspicuous PCM (Brummer, Castaneda et al. 1993).

The initial contact of *P. brasiliensis* cells with host tissue triggers a congestive-exudative inflammatory reaction, characterized by a predominant afflux of neutrophils. Eventually, these cells are replaced by macrophages, which are arranged in loose nodules and multinucleated giant cells. During the inflammatory process, epithelioid histiocytes are found and a lympho-plasmacytic halo is formed. At this stage, the formation of a PCM granuloma consists of a nodular arrangement of epithelioid histiocytes and multinucleated giant cells of the Langhans and foreign body types, many of these cells containing fungi. Usually, a central area of suppuration and inflammatory exudate rich in lymphocytes is formed, with plasma cells and eosinophils permeating or surrounding the granulomatous reaction being found. Fibrosis is generally seen surrounding the granulomas or necrotic areas, that are gradually replaced by fibrous scar tissue (Moreno and Guzman de Espinosa 1976; de Camargo and de Franco 2000).

It is thought that the formation of the granuloma is related to host immune responses to components of the cell wall being expressed by *P. brasiliensis* (Calich, da Costa et al. 2008; Fortes, Miot et al. 2011). The analysis of epithelioid granulomas from skin or mucosal biopsies of PCM patients reveal the presence of macrophages and epithelioid cells surrounded by a peripheral mantle of T-cells, with the predominance of a CD4+ population (Moscardi-Bacchi, Soares et al. 1989). This seems to indicate that T-lymphocytes and macrophages are actively involved in the process of granuloma formation, therefore playing an important role against *P. brasiliensis*.

Although the available literature is clear on the clarification of how the host develops an immune response against the fungus at later stages of infection, most of the studies focusing on the comprehension of the initial mechanisms triggered in the host by *P. brasiliensis* are performed using *in vitro* and *in vivo* approaches, using as model of infection either resistant or susceptible mice. This is mainly due to the fact that PCM infection and disease in humans is usually diagnosed long periods after infection (Brummer, Castaneda et al. 1993; Borges-Walmsley, Chen et al. 2002).

The main host defence immune mechanisms against *P. brasiliensis* are those cell-mediated, namely linked to T-helper 1 (Th1) type of immune response, rather than humoral (Taborda, Juliano et al. 1998; Kashino, Fazioli et al. 2000; Souza, Correa et al. 2001).

The first steps of resistance against *P. brasiliensis* infection triggered by the host rely on macrophages, natural killer (NK) cells, neutrophils and monocytes, and their antifungal activity is induced either by the fungus or cytokines produced by phagocytes (Calich, Vaz et al. 1998; Pagliari, Pereira et al. 2010).

Macrophages, as host and effector cells for these intracellular fungi, are of major importance, being alveolar macrophages thought to be crucial for the initial steps of fungal contention. Studies with susceptible mice reveal that alveolar macrophages recovered after *P. brasiliensis* infection are unable to produce hydrogen peroxide, whereas those from resistant mice produce high levels of this compound (Cano, Singer-Vermes et al. 1995). Several studies on nitric oxide (NO) production by macrophages refer opposite effects of this compound on the fungicidal ability of activated macrophages, depending on the stage of infection. Some authors support the idea that high production levels of NO in initial steps of infection increase fungicidal ability of macrophages, while others claim that this higher production can interfere with acquired-immune responses, leading to the suppression of T-cell mediated immunity (Bocca, Hayashi et al. 1998; Gonzalez, de Gregori et al. 2000; Nascimento, Calich et al. 2002; Gonzalez, Aristizabal et al. 2004).

On the other hand, studies on the role of Natural Killer (NK) cells reveal that their function in PCM varies according to the host or the site where these are driven. In the peripheral blood of PCM patients, NK cells were found in great numbers, but with low cytotoxic activity (Peracoli, Soares et al. 1991), whereas splenic-NK cells from mice were shown to be immunoprotective (Jimenez and Murphy 1984).

Moreover, the production of cytokines by host cells is crucial for the correct modulation and development of an appropriate response against *P. brasiliensis*. Interferon-gamma (IFN-g) was found to be the most important protective cytokine in models using susceptible, intermediate and resistant mice (Cano, Kashino et al. 1998; Souto, Aliberti et al. 2003). While TNF-α and IL-12 also play a protective role for containing the disease (Deepe, Romani et al. 2000; Arruda, Franco et al. 2002; Souto, Aliberti et al. 2003), IL-4 can be either protective or disease promoting, depending on hosts' genetic background (Pina, Valente-Ferreira et al. 2004).

Additionally, the secretion of antimicrobial proteins such as cell-wall-degrading enzyme lysozyme, the iron-chelating protein lactoferrin, the membrane-permeabilizing members of the defensin, cathelicidin, and pentraxin families (Calich, da Costa et al. 2008) complement the action NK and phagocytes (Soares, Calvi et al. 2001; Calvi, Peracoli et al. 2003; Pagliari, Pereira et al. 2010).

The development of the immune response is a controlled mechanism, since an exacerbated pro-inflammatory profile can be detrimental for the host. CD4+ CD25+ Regulatory T cells (Treg) were shown to play a major role in controlling immune responses to *P. brasiliensis*. On their absence during the infectious process, an exacerbated inflammatory response can lead to the development of autoimmune diseases (Moraes-Vasconcelos, Grumach et al. 2005), while their excessive activation may be associated with susceptibility (Ferreira, de Oliveira et al. 2010).

INNATE-IMMUNE MECHANISMS: *P. BRASILIENSIS* RECOGNITION BY TOLL-LIKE RECEPTORS

Cells from the innate-immune system sense the presence of strange/invading microorganisms using conserved, transmembrane or intracytoplasmatic receptors, named Pattern Recognition Receptors (PRRs). These receptors have the ability to sense conserved molecular structures shared by microorganisms, known as Pathogen-Associated Molecular Patterns (PAMPs) (Medzhitov 2001; Janeway and Medzhitov 2002). Among these PRRs, the Toll-Like Receptors (TLRs) are the most widely known, along with non-TLRs such as nucleotide-binding oligomerization domain (NOD)-like proteins and the C-type lectin receptors (CLRs) (Akira, Uematsu et al. 2006). Upon recognition of the invading organism, the activation of PRRs leads to the subsequent activation of cells from the innate-immune system and production of mediators, that are used to eliminate the invading microorganism and control the adaptive immune response (Medzhitov 2001; Schnare, Barton et al. 2001).

Since the discovery of TLRs, several studies have focused on the elucidation of their roles on the recognition of a large variety of fungal pathogens. To the best of our knowledge, 13 TLRs have been described so far, and either individually or forming dimers, these receptors have the ability to recognize a wide variety of pathogen-derived products (Fig. 11). The importance of these receptors relies on their ability to activate cells expressing these receptors and signal for the recruitment of phagocytes to the site of infection, thus leading to microbial killing (Akira and Takeda 2004; Reis e Sousa 2004).

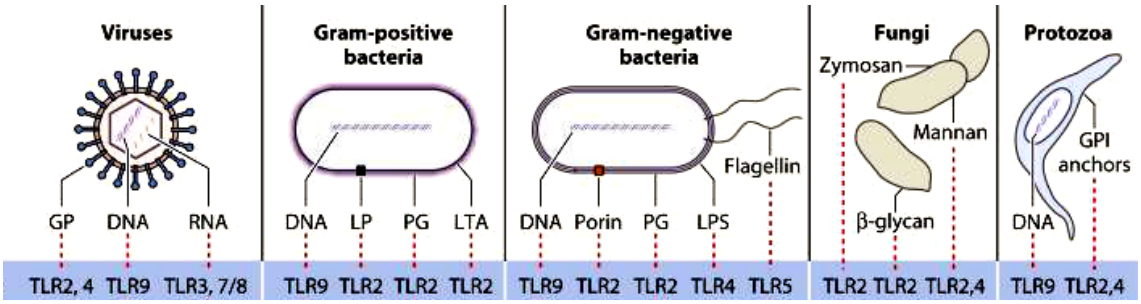

Figure 11 - Ligands of toll-like receptors (TLRs). TLRs are able to recognize a variety of pathogen-derived products: lipopolysaccharide (LPS) is the ligand for TLR4; bacterial lipoproteins are recognized by a TLR2/6 dimer; triacylated lipopeptides by a TLR2/1 dimer; CpG oligonucleotides by TLR9; flagellin by TLR5. TLR11 in mice senses uropathogenic bacteria. A TLR2/6 dimer recognizes zymosan for anti-fungal responses. Anti-viral responses are mediated by TLR4 which senses F protein from RSV, TLR3 which senses double-stranded RNA (poly I:C), TLR7 and TLR8 which sense single-stranded RNA (ss RNA). Protozoal glycosyl-phosphatidyl-inositol (GPI-) anchor proteins are recognized by TLR2. Products of inflamed tissue are sensed by TLR4 (adapted from Mogensen, 2009).

In 2002, Netea and co-workers were the first to describe the role of TLRs on the recognition of a fungal pathogen (Netea, Van Der Graaf et al. 2002). As described by the author, TLR4 plays a protective role against *C. albicans*, as mice lacking the receptor were more susceptible to the disease, due to an impairment of chemokine expression and neutrophil recruitment (Netea, Van Der Graaf et al. 2002). TLR2 was also accessed in the context of *Candida* infections. While some authors show a protective role of this TLR for this pathogen, others claim it is dispensable (Netea, Sutmuller et al. 2004; Villamon, Gozalbo et al. 2004). MyD88, an important adaptor protein recruited by several TLRs for the signaling pathway, was also described to play a protective role during *C. albicans* infections. This molecule was shown signal for increased phagocytosis, killing and synthesis of cytokines by *Candida*-infected cells (Marr, Balajee et al. 2003; Villamon, Gozalbo et al. 2004). Studies with *Cryptococcus neoformans* also addressing these matters suggest that TLR4 and TLR2 marginally contribute to host response against this fungus, while others support a protective role for TLR2 and MyD88 (Yauch, Mansour et al. 2004; Biondo, Midiri et al. 2005; Nakamura, Miyagi et al. 2006). Several studies focusing on another pathogenic fungus, *Aspergillus fumigatus*, reveal a protective role for TLR2 and 4 on hosts' activation of an appropriate immune response, due to their contribution for the activation of polymorphonuclear neutrophils (PMN) and cytokine secretion (Wang, Warris et al. 2001; Meier, Kirschning et al. 2003; Netea, Warris et al. 2003; Bellocchio, Moretti et al. 2004).

In the past few years, *P. brasiliensis* infections have been target of immunological-related studies, specifically those aiming to understand how the innate-immune system is triggered via TLRs. In 1985, Calich and co-workers provided data on the role played by TLR4 on *P. brasiliensis*

recognition. According to the author, TLR4-deficient mice intraperitonially infected with the fungus were more resistant to infection (Calich, Singer-Vermes et al. 1985). Recently, *in vitro* and *in vivo* experiments presented evidences showing that TLR4 recognition of *P. brasiliensis* lead to severe fungal infection, related to a pro-inflammatory pattern (Loures, Pina et al. 2010). In these experiments, TLR4 knock-out macrophages infected *in vitro* presented decreased fungal loads, associated with impaired synthesis of nitric oxide, IL-12, and macrophage chemotactic protein 1 (MCP-1). Accordingly, *in vivo* infection of TLR4-defective mice resulted in reduced fungal burdens and decreased levels of pulmonary nitric oxide, pro-inflammatory cytokines, and antibodies. On the other hand, *in vivo* infection of wild-type mice had as outcome the production of high levels of IL-12 and tumor necrosis factor alpha (TNF-α), besides cytokines of the Th17 type of CD4 T cells. This pattern suggests a pro-inflammatory role for TLR4 signaling upon *P. brasiliensis* recognition, which can be deleterious to the host. TLR2 was also investigated in the scope of *P. brasiliensis* infections. This receptor was shown to be important in regulating the development of Th17-driven immune responses, since its absence results in increased Th17 immunity, ultimately causing a diminished expansion of regulatory T cells and increased lung pathology (Loures, Pina et al. 2009).

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Although some major advances were accomplished for a better comprehension on host responses to *P. brasiliensis*, the peculiarities of this fungus such as its dimorphic behavior, morphological characteristics, multinucleated nature and budding pattern, are some of the aspects that still have to be addressed in a host-fungus approach. In particular, a more profound investigation on the role that immunological elements such as TLRs may play during the infectious process can be of utmost importance for the risk stratification of individuals to PCM and for the development of therapeutic strategies.

CHAPTER 2

MORPHOLOGICAL HETEROGENEITY OF *PARACOCCIDIOIDES BRASILIENSIS*: RELEVANCE OF THE RHO-LIKE GTPASE *PBCDC42*

The results presented over this chapter were published:

(i) in an international peer reviewed journal:

Menino JF, Osório NS, Sturme MH, Barros D, Gomes-Alves AG, Almeida AJ, Ludovico P, Costa P, Goldman GH, Rodrigues F. (2012) Morphological heterogeneity of *Paracoccidioides brasiliensis:* relevance of the Rho-like GTPase *PbCDC42*. *Medical Mycology*, 50(7):768-74.

(ii) in conference proceedings:

Menino JF, Barros D, Gomes-Alves AG, Hernández O, Almeida AJ, Rodrigues F. Morphological heterogeneity of *Paracoccidioides brasiliensis*: characterization and relevance of the Rho-like GTPase Pbcdc42. 26th Fungal Genetics Conference. Pacific Grove, USA. 2011. *(oral communication).*

Menino JF, Osorio N, Sturme M, Barros D, Gomes-Alves AG, Almeida AJ, Costa P, Goldman G, Rodrigues F. Morphological heterogeneity of *Paracoccidioides brasiliensis*: relevance of the Rho-like GTPase *PbCDC42*. Microbiotec, 01-03 December, Braga, Portugal, 2011 *(poster presentation).*

ABSTRACT

Paracoccidioides brasiliensis budding pattern and polymorphic growth were previously shown to be closely linked to the expression of *PbCDC42* and to influence the pathogenesis of this fungus. In this work, a detailed morphogenetic evaluation was carried out for the yeast-form of 11 different clinical and environmental *P. brasiliensis* isolates from the 3 phylogenetic lineages (S1, PS2 and PS3) and one isolate from *Paracoccidioides lutzii* species (Pb01), as well as a *PbCDC42* knock-down strain. High variations in the shape and size of mother and bud cells for each isolate were observed and revealed no characteristic morphologic profile for each phylogenetic group. In all isolates studied, the bud size and shape were demonstrated to be highly dependent on the mother cell. Importantly, we found strong correlations between *PbCDC42* expression and both the shape of mother and bud cells and the size of the buds in the evaluated isolates and knock-down strain. Our results revealed that *PbCDC42* expression can explain approximately 80 % of both mother and bud cell shape and 19 % of bud cell size, thereby supporting that the *PbCDC42* expression level is a relevant predictor of *P. brasiliensis* morphology. Altogether, these findings quantitatively describe the polymorphic nature of the *P. brasiliensis* yeast form and provide additional support for the key role of *PbCDC42* expression on yeast cell morphology.

INTRODUCTION

The fungus *Paracoccidioides brasiliensis* is a etiological agent of paracoccidioidomycosis (PCM), an endemic disease restricted to Latin America with high incidence in Brazil, Colombia and Venezuela (Restrepo, McEwen et al. 2001). *P. brasiliensis* is a thermo dimorphic fungus growing as mycelium at environmental temperatures and shifting to the pathogenic yeast-form at host temperatures (Brummer, Castaneda et al. 1993). The yeast-form of *P. brasiliensis* shows a multiple-budding pattern and presents a variable size with irregular shape (Kashino, Calich et al. 1987). To date, several studies have focused on the characterization of genes that are involved in the regulation of *P. brasiliensis* yeast morphology or other phenotypical traits (Kurokawa, Lopes et al. 2005; Almeida, Cunha et al. 2009). Our previous work has highlighted the role of the cell division cycle 42 (*CDC42*) gene on mother and bud cell size, as knocking-down *PbCDC42* resulted in more spherical cells with reduced virulence when compared to wild-type cells (Almeida, Cunha et al. 2009). Also, specific inhibition of the interactions of *C. albicans* CDC42

with its downstream effectors was demonstrated to inactivate and even reverse the pathogenicity-related morphologic transition of *C. albicans* (Su, Li et al. 2007). In this study, we performed a quantitative characterization of *P. brasiliensis* yeast cell morphology and the number of buds of several clinical and environmental isolates belonging to the four phylogenetic lineages S1, PS2, PS3 and "Pb01-like" (Teixeira, Theodoro et al. 2009). *PbCDC42* expression levels of the *P. brasiliensis* isolates were quantified and their correlation with the evaluated morphological parameters was tested.

MATERIALS AND METHODS

Microorganisms and culture media. *Paracoccidioides* wild-type and mutant strains used in this study are listed in Table 1. For maintenance, *Paracoccidioides* isolates were sub-cultured every seven days on brain heart infusion (BHI) (Duchefa, The Netherlands) solid medium supplemented with 1 % glucose. For the morphological and expression studies, *Paracoccidioides* yeast cells were grown in BHI liquid medium supplemented with 1 % glucose and gentamicin (50µg/mL) at 37 °C with aeration on a mechanical shaker (220 rpm). Cell growth was monitored at different time points over a period of 148 h and cells were collected during the exponential growth phase (72h of growth, $1.65 \pm 0.8 \times 10^7$ cells/mL) for all experiments.

Table 1. *Paracoccidioides* isolates used in this study.

Phylogenetic group	Isolate	Location	Source	Reference
P. lutzii	Pb01	Goiás, Brazil	Chronic PCM	(Teixeira, Theodoro et al. 2009)
S1	Pb18	Sao Paulo,Brasil	Chronic PCM	(Teixeira, Calich et al. 1987)
S1	PbT8B1	Botucatu,Brasil	Armadillo	(Hebeler-Barbosa, Montenegro et al. 2003)
PS2	Pb03	São Paulo,Brasil	Chronic PCM	(Morais, Barros et al. 2000)
PS2	Pb04	São Paulo,Brasil	Chronic PCM	(Morais, Barros et al. 2000)
PS2	Pb02	Caracas,Venezuela	Chronic PCM	(Morais, Barros et al. 2000)
PS3	PbGarcia	Antioquia, Colombia	Chronic PCM	(Hoyos, McEwen et al. 1984)
PS3	PbGarcia /PbICVS-6	N.A.*	Genetically modified	(Almeida, Cunha et al. 2009)
PS3	Pb60855	Antioquia, Colombia	Chronic PCM	(Gomez, Nosanchuk et al. 2001)
N.S.**	Pb54180	N.S.**	Chronic PCM	N.S.**
N.S.**	PbMeredia	N.S.**	Chronic PCM	N.S.**
N.S.**	PbM. Benitez	N.S.**	Chronic PCM	N.S.**

*N.A. – Not applicable; **N.S. – Not specified

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated using TRIzol methodology (Invitrogen, USA) and RNA samples were subsequently treated with 2U of DNaseI (Ambion, USA) by incubation for 1h at 37 ◦C. Absence of DNA contamination was assessed by PCR amplification for the *GP43* gene. Total RNA (1µg) was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad, France) following manufacturer's instructions and 1µL of cDNA used as a template for real-time quantification using the SsoFast EvaGreen SuperMix (Bio-Rad, France). Real-time quantification of *PbCDC42* transcripts and the endogenous reference β-tubulin (*TUB2)* were carried out on a CFX96 Real-Time System (Bio-Rad, France) using previously described primers (Almeida, Cunha et al. 2009). All measurements were done in triplicate, and melting curve analysis performed to exclude non-specific amplification.

Microscopy. To accurately determine morphometric properties of cell populations, cell samples were taken from exponentially growing cultures and fixed as described (Almeida, Cunha et al. 2009), and microscopic images recorded with a DXC390 digital camera (Sony, Japan) using a 40 X objective on a motorized Axioplan 2 microscope (Zeiss, Germany). In these images cell size and shape were analyzed using StereoInvestigator software (MicroBrightField, Williston, USA). Cell size was evaluated as projected area in μm^2, and cell shape was rated in values ranging from 0 (perfect ellipse) to 1 (perfect circle). A minimum of 150 mother cells and respective buds were analyzed for each morphological parameter. Representative images of isolates were acquired by Differential Interference Contrast Microscopy on an Olympus BX61 microscope equipped with a high-resolution DP70 digital camera.

Statistical analysis. Data are reported as the mean ± standard error of the mean (SEM) and all assays were repeated at least three times. All statistical analysis was performed using SPSS statistics 19 package. The One-way ANOVA and Tukey's Post Test was applied for all multiple comparisons. Linear regression analysis was performed applying Pearson coefficient, taking into account the correlation (R^2), degrees of freedom (df_1 and df_2) and slope of the curve (β). For all data analysis statistical significance was considered at the level of 0.001 (2-tailed, 95 % confidence interval).

RESULTS

HETEROGENEOUS MORPHOLOGY AND BUD NUMBER OF *P. BRASILIENSIS* YEAST-FORM

For morphometric analysis of the *P. brasiliensis* yeast-form, we selected 11 clinical and environmental isolates (Table 1) and performed a quantitative analysis of size and shape of at least 150 mother cells and respective buds. Microscopic observations confirmed the highly polymorphic nature of *P. brasiliensis* and revealed, for each isolate, a great heterogeneity in terms of mother and bud cell size and shape (Fig. 1.1). The results indicated high variations between isolates, especially regarding mother and bud cell size, ranging from those displaying large mother and bud cells (Pb02 and Pb54180, Fig. 1.1A, B and C) to those isolates showing smaller cells (PbT8B1, PbMeredia and Pb03, Fig. 1.1A, B and C). Cell shape among isolates showed less variability, with Pb02 being the strain with more elongated cells (Fig 1.1A, D and E). Analyzing the morphological data in Fig. 1.1, Pb02 stands out from the rest of the isolates, with statistically significant differences ($p<0.001$) concerning all the parameters analyzed.

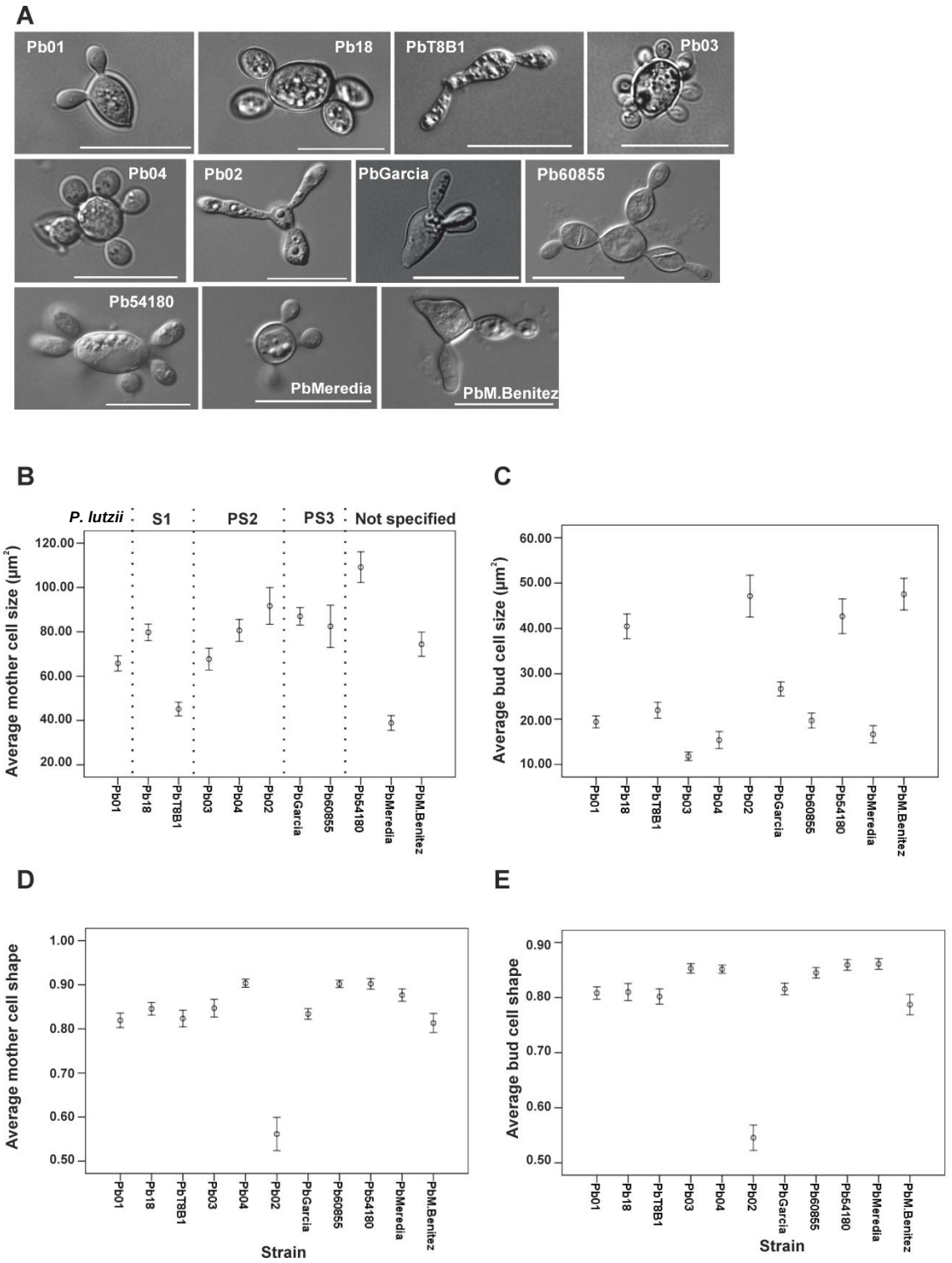

Figure1.1 - *P. brasiliensis* yeast cells reveal high morphological heterogeneity. (A) Differential Interference Contrast microscopy observation of wild-type yeast cells. White bars correspond to 20 µm (100x magnification). Morphometric analysis using StereoInvestigator software of 150 mother cells and respective buds of eleven distinct wild-type *P. brasiliensis* isolates for average yeast cells size (µm²) of (B) mother cell and (C) bud cell and average yeast cells shape [given in values ranging from 0 (perfect ellipse) to 1 (perfect circle)] of (D) mother cell and (E) bud cell. All the differences were statistically significant among isolates ($p < 0.001$).

In general, a high variation in the average bud number per mother cell was observed between and within each phylogenetic group (Fig. 1.1A and Fig. 1.2). Isolate Pb03 in particular presented a high number of buds (>5 buds) compared to the other isolates with on average only two or three buds.

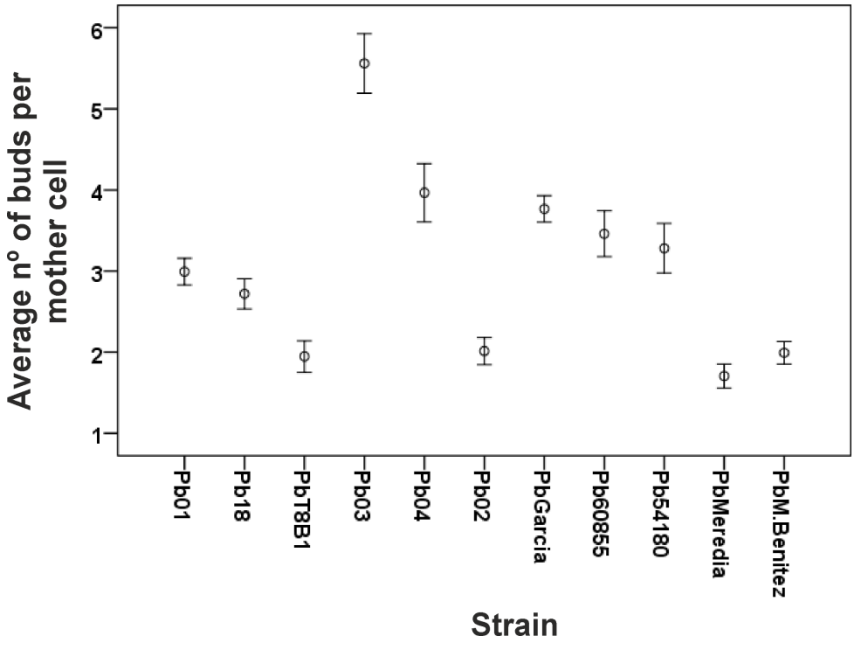

Figure 1.2 - *P. brasiliensis* yeast cells reveal distinct number of buds in the same isolate and among isolates. Morphometric analysis using StereoInvestigator software of 150 mother cells and respective buds of eleven distinct wild-type *P. brasiliensis* isolates for average number of buds per mother cell. All the differences were statistically significant among isolates ($p < 0.001$).

BUD SHAPE IS HIGHLY DEPENDENT ON THE MOTHER CELL SHAPE

To model the relationship between mother cell size and the corresponding bud size, as well as the shape and correlation with the number of buds per cell, a linear regression analysis was performed. This allowed the identification of significant predictors (independent variable; considered here as mother cell size and shape) of a dependent variable (bud size and shape), allowing to infer bud cell morphological features by analyzing those of the mother cell. Our data show that mother cell shape is a strong predictor of the bud cell shape. The results indicated that around 96 % of the bud shape can be explained by the shape of the mother cell (Fig. 2B), suggesting an inherited phenotypic feature. Elongated and polymorphic mother cells gave rise to elongated and highly polymorphic buds (Fig. 1.1A, D and E). For the mother size versus bud size,

we could observe that 24 % of the bud cell size could be explained by the size of the mother cell (Fig. 2A).

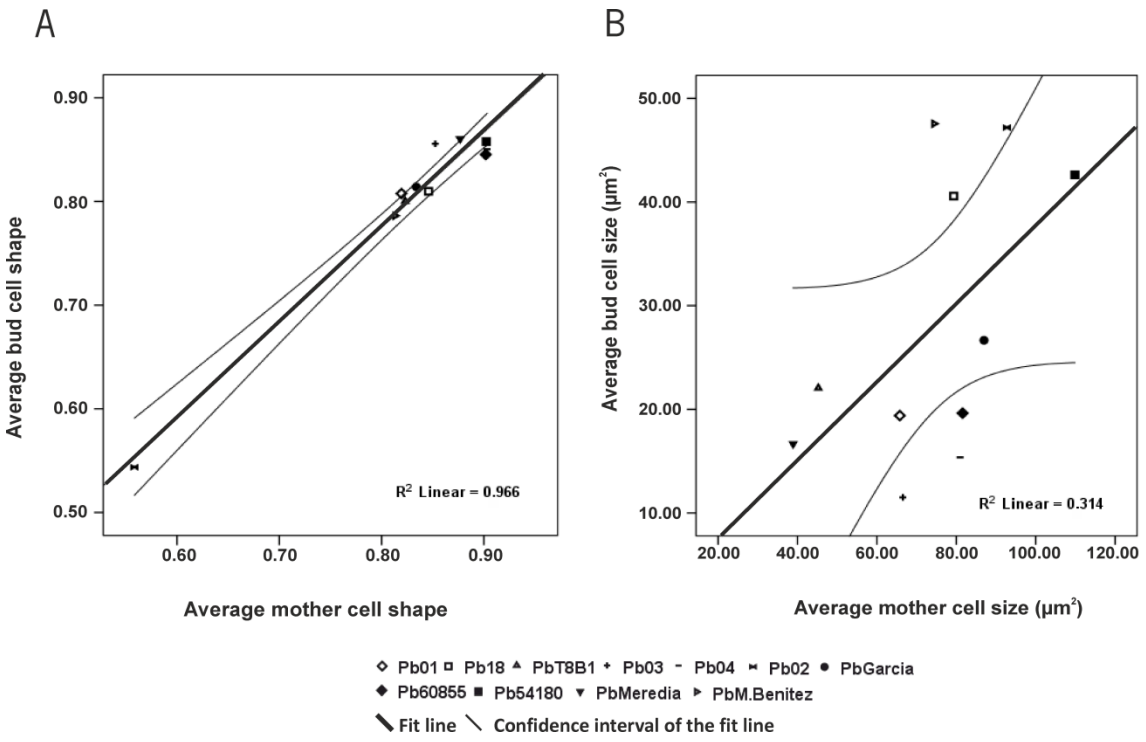

Figure 2 - *P. brasiliensis* bud cells shape and size (µm²) are positively correlated with mother cells shape and size (µm²), respectively. (A) Linear regression analysis applied to average mother cell shape and average bud cell shape per strain. Approximately 96 % of bud cell shape can be explained by mother cell shape (adjusted R^2 = 0.962, df_1=1, df_2=9, β=0.983). (B) Linear regression analysis applied to average mother cell size and average bud cell size per strain. Approximately 24 % (adjusted R^2 = 0.238, df_1=1, df_2=9, β=0.560) of bud cell size can be explained by mother cell size.

Although generally bigger mother cells generated larger bud cells (Fig. 1.1A. B and C) the weaker correlation found for size when compared with shape might indicate that other factors are also involved (Almeida, Cunha et al. 2009). Altogether, these data indicate that regarding cell morphology, mother cell shape is a stronger predictor of bud cell shape, and that to a lesser extent bud cell size can be deduced from the mother size, indicating that *P. brasiliensis* morphology is in part conserved throughout the progeny.

PBCDC42 EXPRESSION IS A STRONG PREDICTOR OF *P. BRASILIENSIS* YEAST CELLS MORPHOLOGICAL PARAMETERS

Taking into consideration our previous work that suggested a role for *PbCDC42* in *P. brasiliensis* morphology and virulence (Almeida, Cunha et al. 2009), we questioned whether the expression levels of this gene were predictive of the previously analyzed morphological parameters. We observed a clear trend for higher *PbCDC42* expression levels in isolates with higher mother and bud cell size and more elliptic cell shape (e.g. Pb02, Fig. 1.1A and Fig. 3). Consistently, lower *PbCDC42* expression levels were associated with less polymorphic, rounder and smaller isolates (e.g. Pb03, Fig. 1.1A and Fig. 3).

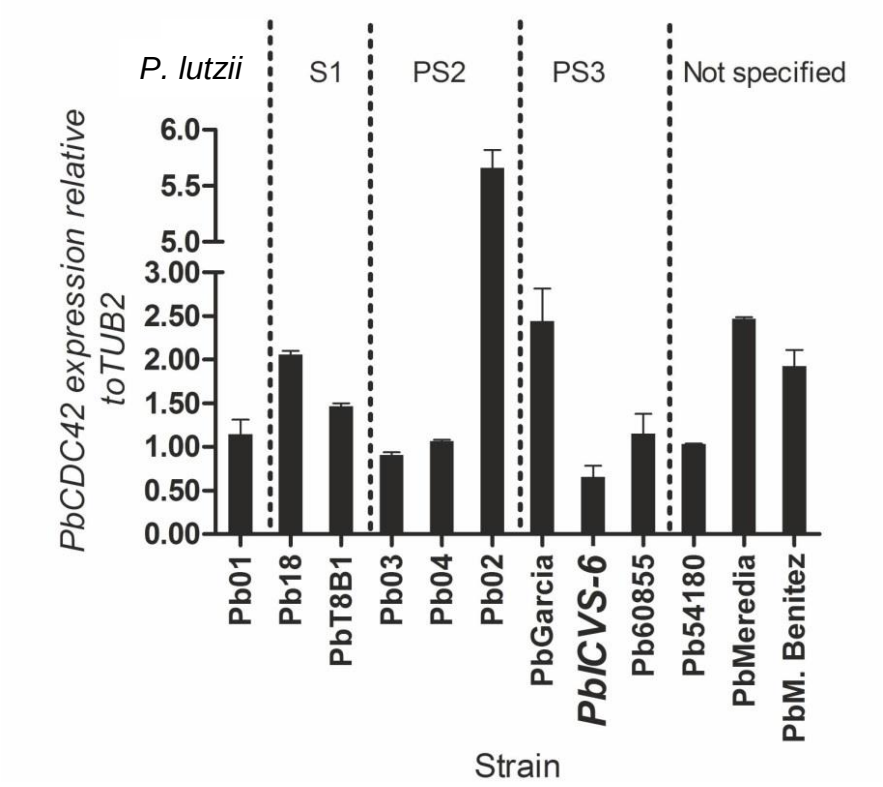

Figure 3 - *PbCDC42* is heterogeneously expressed among the different analyzed isolates and demonstrates no correlation to the cryptic speciation. *PbCDC42* expression levels obtained by qRT-PCR were normalized against the internal reference *TUB2*. All the differences were statistically significant among isolates ($p<0.001$).

To assess the predictor effect of *PbCDC42* expression on the analyzed morphological parameters, we performed a linear regression analysis. Per strain each morphological parameter (considered the dependent variables) was plotted against their corresponding *PbCDC42* expression levels (considered as the independent variable). Our data showed that there was no significant correlation between mother cell size and *PbCDC42* expression levels (data not shown).

Conversely, a positive correlation indicating that *PbCDC42* expression explained 19 % of bud cell size was detected (Fig. 4C). Importantly, both mother and bud cell shape is strongly correlated with *PbCDC42* expression. Approximately 79 % of mother cell shape and 80 % of bud cell shape can be explained by *PbCDC42* expression (Fig. 4A and B). The correlations observed between the shape parameter and the *PbCDC42* expression levels were negative, meaning that higher *PbCDC42* expression levels result in more elongated and elliptic cells. To confirm these trends, we applied the correlation analysis to the morphological parameters of strain PbGarcia and those of its genetically modified counterpart PbICVS-6 (Almeida, Cunha et al. 2009) (with 25±5 % *PbCDC42* expression level relative to wild-type strain PbGarcia (Table 1 and Fig. 3) (Fig. 4D, E and F). The data show that reducing *PbCDC42* expression in the PbICVS-6 strain resulted in quantitative morphological alterations that fitted to the confidence intervals of the fit line for the values obtained for the wild-type isolates. Overall, the correlations obtained were conserved for the *PbCDC42* knockdown strain PbICVS-6, indicating that the expression of this gene can be considered a good predictor for *P. brasiliensis* cell morphology.

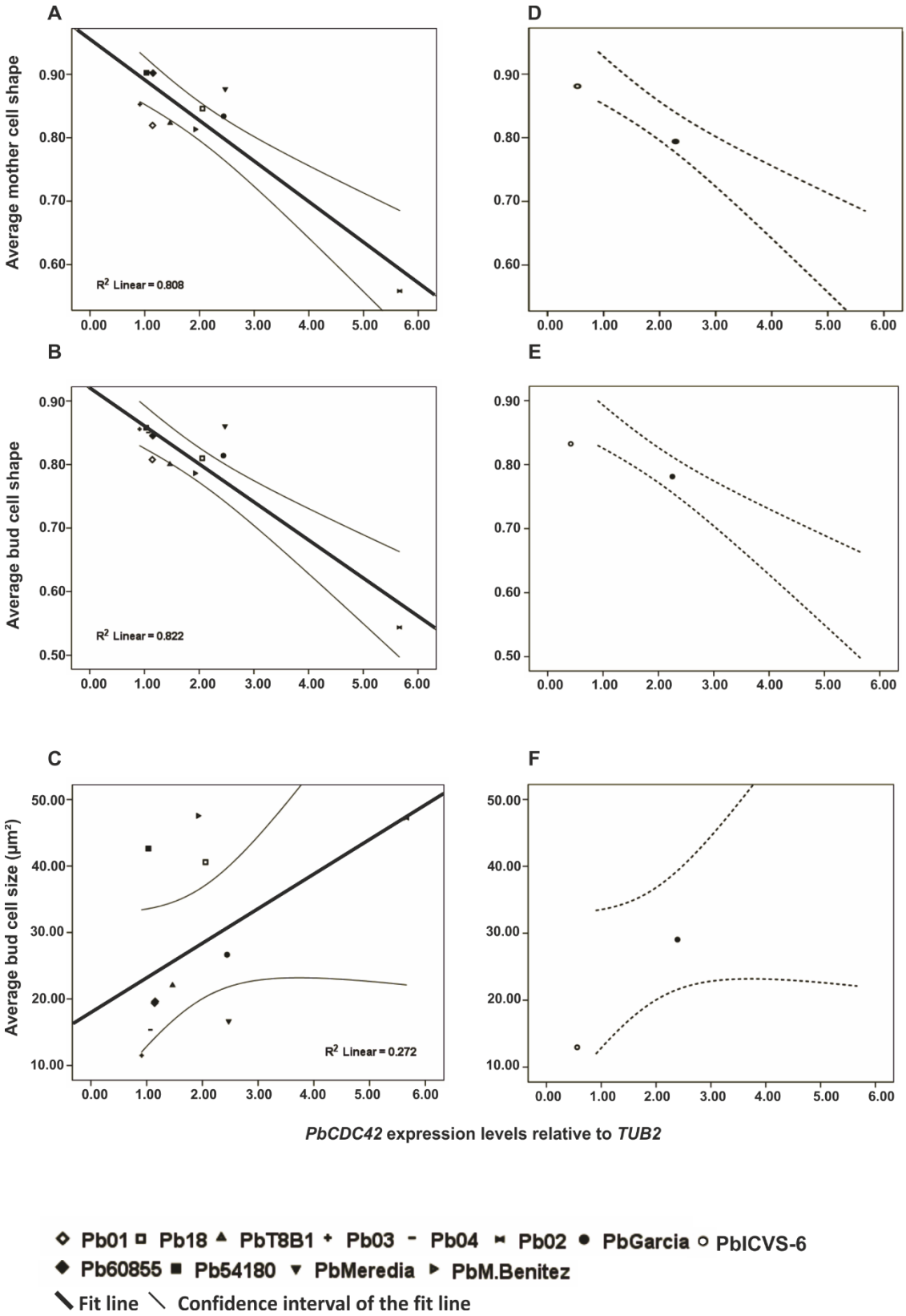

Figure 4 - Morphology of *P. brasiliensis* isolates is strongly correlated with *PbCDC42* expression. (A) Linear regression analysis applied to average *PbCDC42* expression per strain versus average mother cell shape. Approximately 79 % (adjusted R^2 = 0.787, df_1=1, df_2=9, β=-0.899) of mother cell shape can be explained by

PbCDC42 expression (increasing expression levels of *PbCDC42* lead to more elliptical and longer mother cells). (B) Linear regression analysis applied to average *PbCDC42* expression per strain versus average bud cell shape. Approximately 80 % (adjusted R^2 = 0.802, df_1=1, df_2=9, β=-0.906) of bud cell shape can be explained by *PbCDC42* expression (increasing expression levels of *PbCDC42* lead to more elliptical and longer bud cells). (C) Linear regression analysis applied to average *PbCDC42* expression per strain versus average bud cell size (μm^2). Approximately 19 % (adjusted R^2 = 0.191, df_1=1, df_2=9, β=-0.521) of the bud cell size can be explained by *PbCDC42* expression (increasing expression levels of *PbCDC42* lead to larger bud cells). (D, E and F) Linear trends observed for wild-type isolates are followed by the *PbCDC42* knock-down strain PbICVS-6. Morphological parameters from PbGarcia wild-type strain and one *PbCDC42* knock-down strain (PbICVS-6) applied to the confidence intervals of the fit line for the values in wild-type isolates for (D) average *PbCDC42* expression per strain versus average mother cell shape, (E) average *PbCDC42* expression per strain versus average bud cell shape and (F) average *PbCDC42* expression per strain versus average bud cell size (μm^2). *PbCDC42* expression levels obtained by RT-PCR were normalized with the internal reference *TUB2*.

DISCUSSION

Morphological transition during host invasion is a relevant factor for fungal pathogenesis (Karkowska-Kuleta, Rapala-Kozik et al. 2009). Moreover, recent data showed that the variability of cell size within a fungal cell population affects pathogenicity via alteration of phagocytosis (Almeida, Cunha et al. 2009; Okagaki, Strain et al. 2010). *P. brasiliensis* is known to have a highly polymorphic yeast-form, even within the same isolate (Kashino, Calich et al. 1987; Brummer, Castaneda et al. 1993; Svidzinski, Miranda Neto et al. 1999). This aspect is still poorly characterized, especially at the level of how gene expression can modulate the cell size and shape. We have previously shown that *PbCDC42* expression levels are associated with the size and shape of *P. brasiliensis* within a single isolate (Almeida, Cunha et al. 2009), raising the question whether *PbCDC42* expression levels are correlated to the morphological features of wild-type *P. brasiliensis* isolates in general.

Our findings revealed that *P. brasiliensis* has, besides its multiple-budding and highly polymorphic nature, no clearly defined cell size, shape and bud number for each isolate or even each phylogenetic group. A high variability in yeast cell morphology was uncovered between isolates within and between the phylogenetic groups, demonstrating that the morphologic characteristics of this fungus cannot be considered a predictor for phylogenetic classification. However, correlations between mother and bud morphological parameters, in particular shape and size, where found, demonstrating that bud cell morphology is highly dependent on mother

cell morphological traits. The microscopic analysis herein presented gives new insights on the morphological characterization of different isolates from each phylogenetic group as well as of each isolate. Moreover, the role of *PbCDC42* expression on the yeast morphology was further established using both wild-type isolates and a *PbCDC42* knockdown strain. We found strong statistical correlations between *PbCDC42* expression and mother and bud cell shape as well as bud cell size, indicating that this variable may explain the existing heterogeneity among *P. brasiliensis* strains. The data herein presented represent novel quantitative insights on the complex polymorphic nature of *P. brasiliensis*, and give indications for morphological factors that can influence the virulence of each isolate, as suggested before (Svidzinski, Miranda Neto et al. 1999; Su, Li et al. 2007). The results also reinforce that PbCdc42p could be a good drug target for therapy against paracoccidioidomycosis, as suggested for *Candida albicans* infections [6].

CHAPTER 3

P. BRASILIENSIS VIRULENCE IS AFFECTED BY SCONC, THE NEGATIVE REGULATOR OF INORGANIC SULFUR ASSIMILATION

The results presented over this chapter were published:

(i) in an international peer reviewed journal:

Menino JF, Saraiva M, Gomes-Rezende J, Sturme M, Pedrosa J, Castro AG, Ludovico P, Goldman GH, Rodrigues F, *P. brasiliensis* virulence is affected by SconC, the negative regulator of inorganic sulfur assimilation. *PLOS ONE (Accepted for publication).*

(ii) in conference proceedings:

Menino JF, Saraiva M, Goldman G, Castro AG, Pedrosa J, P Ludovico, F Rodrigues. *P. brasiliensis* virulence is affected by the negative regulator of inorganic sulfur assimilation pathway SconC. XXIX Jornadas de Biologia de Leveduras "Prof Nicolau Van Uden", 15-16 june, Lisbon, Portugal, 2012. *(oral communication).*

Menino JF, Saraiva M, Goldman G, Castro AG, Pedrosa J, P Ludovico, F Rodrigues. Gene silencing of the negative regulator of inorganic sulfur assimilation SconC affects virulence of the fungal pathogen *Paracoccidioides brasiliensis*. XXXVII Jornadas Portuguesas de Genética, 29-31 May, Lisbon, Portugal, 2012. *(oral communication).*

ABSTRACT

Conidia/mycelium-to-yeast transition of *Paracoccidioides brasiliensis* is a critical step for the establishment of paracoccidioidomycosis, a systemic mycosis endemic in Latin America. Thus, knowledge of the factors that mediate this transition is of major importance for the design of intervention strategies. So far, the only known pre-requisites for the accomplishment of the morphological transition are the temperature shift to 37°C and the availability of organic sulfur compounds. In this study, we investigated the auxotrophic nature to organic sulfur of the yeast phase of *Paracoccidioides*, with special attention to *P. brasiliensis* species. For this, we addressed the role of SconCp, the negative regulator of the inorganic sulfur assimilation pathway, in the dimorphism and virulence of this pathogen. We show that down-regulation of *SCONC* allows initial steps of mycelium-to-yeast transition in the absence of organic sulfur compounds, contrarily to the wild-type fungus that cannot undergo mycelium-to-yeast transition under such conditions. However, *SCONC* down-regulated transformants were unable to sustain yeast growth using inorganic sulfur compounds only. Moreover, pulses with inorganic sulfur in *SCONC* down-regulated transformants triggered an increase of the inorganic sulfur metabolism, which culminated in a drastic reduction of the ATP and NADPH cellular levels and in higher oxidative stress. Importantly, the down-regulation of *SCONC* resulted in a decreased virulence of *P. brasiliensis*, as validated in an *in vivo* model of infection. Overall, our findings shed light on the inability of *P. brasiliensis* yeast to rely on inorganic sulfur compounds, correlating its metabolism with cellular energy and redox imbalances. Furthermore, the data herein presented reveal SconCp as a novel virulence determinant of *P. brasiliensis*.

INTRODUCTION

Paracoccidioides brasiliensis is a dimorphic fungus and a causative agent of paracoccidioidomycosis, an endemic mycosis affecting the population from Latin America countries such as Brazil, Colombia and Venezuela (Brummer, Castaneda et al. 1993). The infective process comprises a temperature-dependent morphological switch of the fungus from the conidia/mycelium phase at environmental temperatures (around 26°C) to the pathogenic yeast phase at the mammalian host temperature (around 37°C) (Brummer, Castaneda et al. 1993; Restrepo, McEwen et al. 2001). In addition to the well-studied temperature requisite, the

knowledge of other regulators mediating both morphogenesis and virulence in *P. brasiliensis* is scarce. Thus, a better understanding of *P. brasiliensis* metabolic processes is essential to unravel its virulence determinants, offering novel targets for prophylaxis and/or therapeutics intervention.

Several studies show that sulfur metabolism is directly correlated with virulence of bacteria and dimorphic fungi like *Histoplasma capsulatum* (Maresca, Lambowitz et al. 1981; Fu, Paietta et al. 1989; Senaratne, De Silva et al. 2006; Bhave, Muse et al. 2007). Sulfur compounds play a role in the formation of functional thiol (-SH) groups, crucial for innumerous cellular components and signaling processes (Ziegler 1985; Dickinson and Forman 2002). Despite the relevance of sulfur as constituent of essential organic molecules such as coenzyme-A and glutathione (Brosnan and Brosnan 2006), the preferential pathway of sulfur assimilation, from either organic or inorganic compounds, in pathogenic fungi is not completely understood. Sulfur requirements of *P. brasiliensis* differ between the mycelium and the yeast phase of the fungus. Similarly to other dimorphic fungi, the growth of *P. brasiliensis* in the yeast phase depends on organic sulfur sources (Boguslawski and Stetler 1979; Paris, Duran-Gonzalez et al. 1985; Medoff, Painter et al. 1987). In contrast, and despite being auxotrophic for organic sulfur compounds such as cysteine or methionine, *P. brasiliensis* is prototrophic in the mycelial phase (Boguslawski and Stetler 1979; Paris, Duran-Gonzalez et al. 1985; Medoff, Painter et al. 1987).

Recent transcriptomic approaches revealed that genes related to sulfur metabolism are differentially expressed in the mycelium and yeast phase of *P. brasiliensis* (Medoff, Painter et al. 1987; Ferreira, Marques Edos et al. 2006). In particular, expression of inorganic sulfur metabolism-related genes was markedly up-regulated in the yeast phase of this fungus (Marques, Ferreira et al. 2004; Felipe, Andrade et al. 2005; Andrade, Paes et al. 2006; Ferreira, Marques Edos et al. 2006; Tavares, Silva et al. 2007). For example, a 35-fold increase in the expression of *METR*, the positive regulator of the inorganic sulfur assimilation pathway, was observed (Ferreira, Marques Edos et al. 2006). Another differentially expressed gene is *SCONC*, which as *METR* is highly expressed in the yeast phase and down-regulated in the mycelial phase of *P. brasiliensis* (Ferreira, Marques Edos et al. 2006). *SCONC* gene encodes a negative regulator of the inorganic sulfur assimilatory pathway, orchestrating the repression of genes from the sulfur assimilatory pathway by promoting MetR proteolysis (Marzluf 1997; Nunes, Costa de Oliveira et al. 2005). However, the low levels of *SCONC* in the mycelial phase allow *P. brasiliensis* to use inorganic sulfur. Therefore, the cross-talk of these two regulators has been suggested to be responsible for the yeast-phase auxotrophy for organic sulfur compounds, by preventing the assimilation of

inorganic sulfur by the pathogenic yeast cells. Although the molecular bases underlying the distinct sulfur requirements of *P. brasiliensis* are presently more elucidated, the physiological aspects behind the sulfur-dependent dimorphic behavior are far from being understood. A recent transcriptome study showed that *SCONC* was being highly expressed in *P. brasiliensis* yeast cells recovered from infected mice than in those cultured *in vitro* (Costa, Borges et al. 2007). Therefore, a stronger repression of inorganic sulfur assimilation seems to be in place during infection, indicating that yeast cells alter their metabolism *in vivo*, most probably due to higher access to organic sulfur. It is likely that this change plays a role in the fungus pathogenesis. For these reasons, a better understanding of the impact played by SconCp on the sulfur-dependent dimorphic processes in *P. brasiliensis* is crucial, as it may highlight a new *P. brasiliensis* virulence factor.

To address these questions, we down-regulated the expression of *SCONC* in isolates from different *Paracoccidioides* species and investigated its impact both on the inorganic sulfur assimilatory pathway and on the dimorphic transition. In addition, we evaluated the role of SconCp as a possible *P. brasiliensis* virulence factor, using an *in vivo* mouse model of infection. We herein present evidence that *P. brasiliensis* SconCp acts as a regulator of dimorphism by modulating the inorganic sulfur metabolism, thereby influencing the virulence of this pathogenic fungus.

MATERIAL AND METHODS

Microorganisms and culture media: *Paracoccidioides* wild-type species and *SCONC* down-regulated strains are listed in Table 1. Yeast cells were maintained at 37°C by subculturing in brain heart infusion (BHI) (Duchefa) solid media supplemented with 1% glucose and gentamicin (50 µg/mL). For the expression studies, *Paracoccidioides* yeast cells were grown in synthetic McVeigh Morton (MMvM) liquid medium (Restrepo and Jimenez 1980) at 37°C with aeration on a mechanical shaker (220 rpm). For the *in vivo* assays, yeast cells were grown in BHI liquid medium supplemented with 1% glucose and gentamicin (50 µg/mL) at 37°C with aeration on a mechanical shaker (220 rpm). Cell growth was monitored for 148 h by microscopic counting using a Neubauer counting chamber and cells were collected during the exponential growth phase (72 h of growth, $1.65 \pm 0.8 \times 10^7$ cells/mL) for the infection.

Table 1. *Paracoccidioides* isolates used in this study.

Phylogenetic group	Isolate	Location	Source	Reference
P. lutzii	Pb01	Brazil	Chronic PCM	(Teixeira, Theodoro et al. 2009)
P. lutzii	Pb01 As*SCONC* D	N.A.*	This study	
PS3	Pb60855	Colombia	Chronic PCM	(Gomez, Nosanchuk et al. 2001)
PS3	Pb60855 As*SCONC* A	N.A.*	This study	
PS3	Pb60855 As*SCONC* B	N.A.*	This study	

*N.A. – Not applicable

Agrobacterium tumefaciens strain LBA1100 (Beijersbergen, Dulk-Ras et al. 1992) was used as the recipient for the binary vector constructed in this study. Bacterial cells were maintained at 28°C in Luria Bertani (LB) medium containing kanamycin (100 µg/ml). *Escherichia coli* JM109 competent cells (Promega) were grown at 37°C in LB medium supplemented with appropriate antibiotics and were used as the host for plasmid amplification and cloning.

For the morphological transition, complete MMvM (MMvM +Cys/+SO_4^{2-}) [supplemented with L-cysteine (1.7 mM) as organic sulfur source and $MgSO_4.7H_2O$ (2 mM) and $(NH_4)_2SO_4$ (15 mM) as inorganic sulfur sources], MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) and MMvM without organic sulfur compounds supplementation (MMvM -Cys/+SO_4^{2-}) were used. Briefly, for the yeast-to-mycelium transition yeast cells were cultured at 37°C to the exponential growth phase in complete MMvM, washed 3 times with sterile phosphate-buffered saline (PBS), and inoculated at a final concentration of $1x10^6$ cells/mL in the appropriated medium. The cultures were then transferred to a mechanical shaker (220 rpm) at 26°C and cultured until complete transition was accomplished. For the mycelium-to-yeast transition, mycelium was cultured at 26°C in complete MMvM medium, and washed 3 times with sterile PBS. The cultures were then transferred to a mechanical shaker (220 rpm) at 37°C and cultured till no more morphological changes were being observed.

Construction of Paracoccidioides SCONC antisense-RNA (AsSCONC) isolates: Plasmid DNA extraction, recombinant DNA manipulations, and *E. coli* transformation procedures were performed as described elsewhere (Almeida, Carmona et al. 2007). *P. brasiliensis* wild-type

strain ATCC 60855 DNA was extracted from yeast cultures during exponential growth and a high-fidelity proof-reading DNA polymerase (NZYTech) was employed to amplify an aRNA oligonucleotide sequence targeting the coding sequence of *SCONC*. The As*SCONC* sequence was inserted into the plasmid pCR35 under the control of the calcium-binding protein (CBP1) promoter region from *H. capsulatum* as previously described (Rappleye, Engle et al. 2004). The aRNA cassette was subsequently cloned into the transfer DNA (T-DNA) region of the binary vector pUR5750 (Figure 1A) and mobilized to *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.

Insertion of recombinant T-DNA harboring the As*SCONC* cassette and a hygromycin B resistance marker into the genome of *Paracoccidioides* yeast cells was accomplished by *A. tumefaciens*-mediated transformation (ATMT) (Almeida, Carmona et al. 2007). Briefly, *A. tumefaciens* LBA1100 carrying binary vector pUR5750 harboring the As*SCONC* sequence was grown overnight in LB liquid medium with antibiotics at 28°C with agitation. Bacterial cells were spun down, washed, set to an OD_{660nm} of 0.30 in induction medium (IM) (de Groot, Bundock et al. 1998) with acetosyringone (Sigma, USA) (200µM), and re-incubated at 28°C until an OD_{660nm} of approximately 0.80. *Paracoccidioides* yeast cells were grown in BHI batch cultures to the exponential growth phase and cells were washed with IM and adjusted to a final concentration of $1x10^8$ cells/ml using direct microscopic counts (Neubauer counting chamber procedures). A 1:10 *A. tumefaciens*/ *Paracoccidioides* ratio was inoculated onto sterile Hybond N membrane (Amersham Biosciences, USA) on solid IM for co-cultivation at 25°C for 3 days. Prior to incubation, co-cultivation plates with cellular mixtures were air dried in a safety cabinet for 30 min. Following co-cultivation, membranes were transferred to BHI liquid medium containing cefotaxime (200 µg/ml), cells dislodged by aid of a spatula, and the cell suspension incubated for 48 h at 36°C, 200 rpm, before plating on selective BHI media (HygB 75 µg/ml). Selection plates were monitored for colony forming ability at 36°C for 15 days. Randomly selected HygB resistant transformants confirmed by PCR were tested for mitotic stability and selected for further assays. An identical procedure was followed to obtain the *Paracoccidioides lutzii* Pb01 As*SCONC* transformant.

Quantitative real-time polymerase chain reaction (qRT-PCR): *Paracoccidioides* yeast cultures were inoculated from a single colony and grown to exponential phase in complete MMvM at 37°C (200

rpm). Medium was refreshed once after 4 days. Total RNA (1 µg) from wild-type and As*SCONC* transformants was isolated according to TRIzol protocol (Invitrogen, USA) and RNA samples were subsequently treated with 2U of DNaseI (Ambion, USA) by incubation for 1 h at 37°C. The absence of DNA contamination in the samples was confirmed by the lack of PCR amplification of the *GP43* gene in the isolated RNA. Total RNA (1 µg) was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad, France) following manufacturer's instructions and 1 µL of cDNA used as a template for real-time quantification using the SsoFast EvaGreen SuperMix (Bio-Rad, France) following manufacturer's instructions. Real-time quantification was carried out on a CFX96 Real-Time System (Bio-Rad, France) using threshold cycle (Ct) values for β-tubulin (*TUB2)* transcripts as the endogenous reference. The primer sequences were designed and synthesized by NZYTech and are described in Table 2. All measurements were performed in triplicate. A single melting peak was obtained for each gene analyzed in all samples.

Table 2. List of primers used in this study.

Gene name	Sequence (5' to 3')
β-tubulin (*TUB2*)	**Forward** *aga aca tga tgg ctg ctt cc*
	Reverse *gcg cat ctg atc ttc gac ttc*
Sulfur controler (*SCONC*)	**Forward** *gaa tgg tgc gaa cat cac ag*
	Reverse *cca gga tta tct caa aaa gc*
bZIP transcription factor (*METR*)	**Forward** *ttc ttg agc cac cga ttc tcc*
	Reverse *gga gcg cac cgt taa gga g*
Sulfate permease (*SP*)	**Forward** *tgg tca gtt ggc ttg tga ac*
	Reverse *tta gca tca acc tgg gga ac*
Choline-*O* sulfatase (*CHS*)	**Forward** *tga aca acg ctt gac cag tg*
	Reverse *cgg aag aca tat cat ggt acc*
ATP sulfurylase (*MET3*)	**Forward** *cgt tgg agg aaa ggt tga ag*
	Reverse *ctc gat gca tgg gat tta tc*
PAPS reductase (*MET16*)	**Forward** *cca att cct aga acc gca ag*
	Reverse *gag ttt gga gag cat gtc gag*
Sulfite reductase (*MET10*)	**Forward** *ccc acc gat atc cat acc ac*
	Reverse *tcc ata ggc ctc ctt gaa ga*

Microscopy: To evaluate cell morphology during the dimorphic processes, four morphotypes were determined according to Nunes and co-workers (2005) (Nunes, Costa de Oliveira et al. 2005). *Paracoccidioides* cells were collected during the transition process (yeast-to-mycelium or mycelium-to-yeast) in complete MMvM, MMvM +Cys/-SO_4^{2-} and MMvM -Cys/+SO_4^{2-} and fixed as previously described (Almeida, Cunha et al. 2009). A total of 300 morphological units were counted for each culture (in triplicate), and the estimation of each morphological state (from yeast to mycelium and vice-versa) was performed in terms of percentage. Morphological units were counted using a Zeiss Axioskop equipped with a Carl Zeiss AxioCam (Carl Zeiss, Jena).

Determination of P. brasiliensis growth curve in yeast cells: Yeast cells of *P. brasiliensis* wild-type Pb60855 and As*SCONC* transformants were grown in MMvM, MMvM +Cys/-SO_4^{2-} and MMvM -Cys/+SO_4^{2-} at 37°C and samples were collected to determine cell number, using a standard Neubauer system, at different times until stationary growing phase was reached. All cultures were started from an initial concentration of $1x10^5$ cells/mL.

ATP/NADPH measurements: ATP measurements were performed according to (Ashe, De Long et al. 2000). Briefly, cells were collected by centrifugation and the pellet was frozen with liquid nitrogen and stored at -80°C. For the measurements, the pellet was mixed with 200 µL of 5% trichloroacetic acid (TCA) and vortexed twice for one minute, with one minute interval on ice. The mix was centrifuged for 1 min at 4°C and 10 µL of the supernatant were added to 990 µL of reaction buffer (25 mM HEPES, 2 mM EDTA, pH 7.75). Of this mixture, 100 µL were added to 100 µL of Enliten Luciferin/Luciferase Reagent (Promega) and luminescence was measured on a ThermoScientific Fluoroskan Ascent FL.

NADPH measurements were performed according to manufacturer's instructions (NADP/NADPH Assay Kit, Abcam). Briefly, cells were collected by centrifugation and washed with ice-cold PBS. After pelleting $2x10^5$ cells for each sample, 400 µL of NADP/NADPH extraction buffer was added. Two freeze/thaw cycles (20 min on dry-ice followed by 10 min at room-temperature) were applied, and after vortexing for 10 sec the supernatant was collected. Of each supernatant, 50 µL were incubated with 100 µL of NADP cycling mix for 5 min at room temperature. Next step, 10 µL of NADPH developer were added to each sample and the mixture was incubated at room temperature for 1 hour. Total NADP ($NADP^+$ + NADPH) and NADPH only were measured at OD_{450nm} on a Bio-Rad 680 Micro-plate Reader.

Pulse with inorganic sulfur sources: *P. brasiliensis* wild-type Pb60855 and As*SCONC* yeast strains were cultured to the exponential phase of growth in complete MMvM (MMvM +Cys/+SO_4^{2-}) at 37°C (200 rpm). Cells were collected, washed three times in sterile PBS, and transferred to MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) to a final concentration of 1x10^6 cells/mL. A pulse with inorganic sulfur sources [$MgSO_4 \cdot 7H_2O$ (final concentration 2 mM) and $(NH_4)_2SO_4$ (final concentration 15 mM)] was given to all the cultures, and samples were collected 15 and 30 min after the pulse for posterior analysis.

Measurement of reactive oxygen species: Intracellular reactive oxygen species (ROS) were detected by dihydrorhodamine (DHR-123) or dihydroethidium (DHE) (Molecular Probes) staining. For evaluation of H_2O_2 levels, cells were incubated with 15 mg/mL of DHR-123 for 90 min at 30°C in the dark, washed with PBS and measured by flow cytometry. For evaluation of O_2^- levels, cells were incubated with 5 mM DHE for 10 min at 30°C in the dark, washed with PBS and evaluated by flow cytometry. All measurements were performed in a BD™ LSR II flow cytometer. A minimum of 100,000 cells per sample was acquired at low/medium flow rate. Offline data was analyzed with the flow cytometry analysis software package FlowJo 7.6.1.

In vivo infection: Eight-week-old C57BL/6 male mice were obtained from Charles River (Barcelona,Spain). Mice were housed under specific-pathogen-free conditions with food and water *ad libitum*. C57BL/6 WT mice were infected intravenously (i.v.) with 1x10^6 *P. brasiliensis* yeast cells grown to the exponential phase in BHI liquid medium (either wild-type Pb60855 or each of the corresponding As*SCONC* transformants). Prior to infection, cells were washed 3 times with lipopolysaccharide (LPS)-free PBS (Gibco), passed through a syringe to eliminate cell clumps, and submitted to Neubauer counting procedures (each mother and bud cells were considered as individual counts). Mice survival was monitored for 80 days.

Ethics statement: This study was approved by the Portuguese national authority for animal experimentation Direção Geral de Veterinária (ID:DGV 594 from 1st June 2010). Animals were kept and handled in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and of the Council.

Statistics: Data are reported as the mean ± standard error of the mean (SEM) and all assays were repeated at least three times. All statistical analyses were performed using the GraphPad Prism Software version 5.01. The survival curves, representative of two independent experimental infections (Fig. 7, n=20 mice), were compared using the Chi square Logrank Test. For the experiments comparing two groups (see Figure 1B, 2B-F, 5, 6), a two-tailed unpaired Student *t* test was performed. Welch's correction was applied when making multiple comparisons. For all data analysis statistical significance was considered at the level of 0.05 (2-tailed, 95% confidence interval).

RESULTS

SILENCING OF THE *PARACOCCIDIOIDES SCONC* GENE LEADS TO THE UP-REGULATION OF GENES INVOLVED IN THE INORGANIC SULFUR METABOLISM

To evaluate the impact of *SCONC* down-regulation in the yeast phase of *P. brasiliensis,* we constructed an antisense RNA sequence targeting the coding sequence of the *SCONC* gene (Figure 1A) for genome integration using ATMT methodology (Almeida, Carmona et al. 2007; Almeida, Cunha et al. 2009). Two independent *SCONC* down-regulated transformants for *P. brasiliensis* wild-type 60855 (Pb60855 As*SCONC* A and Pb60855 As*SCONC* B) were selected. Due to the yet impossible gene disruption and complementation in *P. brasiliensis* (Almeida, Carmona et al. 2007; Almeida, Cunha et al. 2009), we replicated our findings by down-regulating *SCONC* in the strain Pb01 of *Paracoccidoides lutzii*. For this strain one clone (Pb01 As*SCONC* D) was also selected for analysis. The percentage of reduction in the expression levels of *SCONC* in the As*SCONC* transformants was calculated by comparison to expression levels in the respective wild-type strains (Figure 1B). For Pb60855 As*SCONC* A and Pb60855 As*SCONC* B the percentages of *SCONC* down-regulation were around 73% and 30%, respectively, whereas for Pb01 As*SCONC* D it was around 50%.

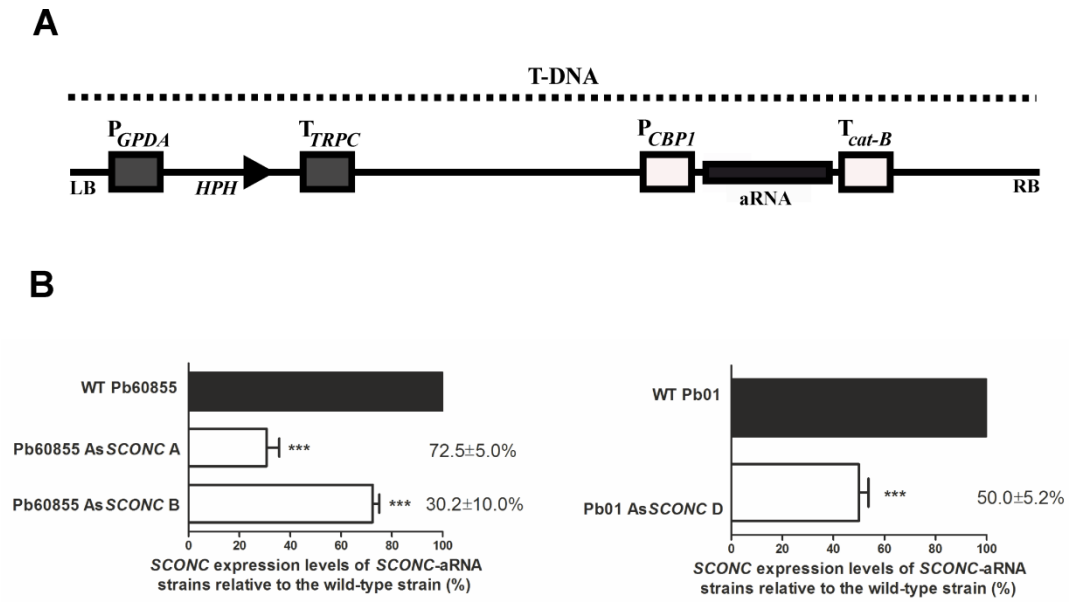

Figure 1. Silencing of *SCONC* expression by targeted antisense RNA in *Paracoccidioides* species cells decreases mRNA levels. (A) Structure of aRNA Transfer DNA (T-DNA) cassette inserted into *P. brasiliensis* yeast cells via ATMT in order to silence *SCONC*. The antisense RNA (As*SCONC*) sequence was placed under control of the calcium binding protein promoter from *H. capsulatum* (CBP1) with hygromycin B phosphotransferase (*HPH*) gene under control of the glyceraldehyde 3-phosphate promoter from *A. nidulans* (PGPDA). (B) Gene expression levels of *SCONC* in *Paracoccidioides* clones harboring silencing oligonucleotides targeting the *SCONC* coding sequence compared to the respective wild-type strain. Cells were grown to the exponential phase in complete MMvM (MMvM +Cys/+SO_4^{2-}) at 37°C and collected for gene expression evaluation. Asterisks represent statistical differences between wild-type and down-regulated strains (***$p<0.001$). Percentage of gene expression levels reduction was obtained comparing *SCONC* levels of each clone to those of the wild-type strains. *SCONC* expression levels determined by qRT-PCR were normalized to expression of the internal reference gene β-tubulin (*TUB2*). Bars represent means and standard deviations.

To evaluate the impact of *SCONC* silencing in the inorganic sulfur assimilatory pathway (Figure 2A), we analyzed the expression levels of several genes described to be involved in this pathway (Marzluf 1997) using exponential yeast cells cultured in complete MMvM. Although similar differences in the expression of the targeted genes were found for both transformants As*SCONC* A and B, differences for As*SCONC* A were consistently more pronounced, probably due to the better efficiency of *SCONC* silencing in this transformant (Figure 1B). The expression of the gene encoding sulfate permease (*SP*), the membrane transporter responsible for inorganic sulfate uptake in *P. brasiliensis* (Ferreira, Marques Edos et al. 2006), was found to be significantly up-regulated in Pb60855 As*SCONC* A when compared to the wild-type strain (Figure 2B). Also up-regulated in the As*SCONC* transformants was the expression of several downstream genes of the

inorganic sulfur assimilatory pathway (Figure 2C-E) and that of choline sulfatase (*CHS*) (Figure 2F), an enzyme of the lateral branch of the inorganic sulfur assimilation pathway, that uses as substrate choline-*O*-sulfate, a osmoprotectant and an additional intracellular source of inorganic sulfur (Marzluf 1997). Overall, our data is in line with previous reports (Burton and Metzenberg 1972; Piotrowska, Natorff et al. 2000; Uthman, Dockal et al. 2005; Ferreira, Marques Edos et al. 2006) showing a key role for *SCONC* as a negative transcriptional regulator of the inorganic sulfur metabolism.

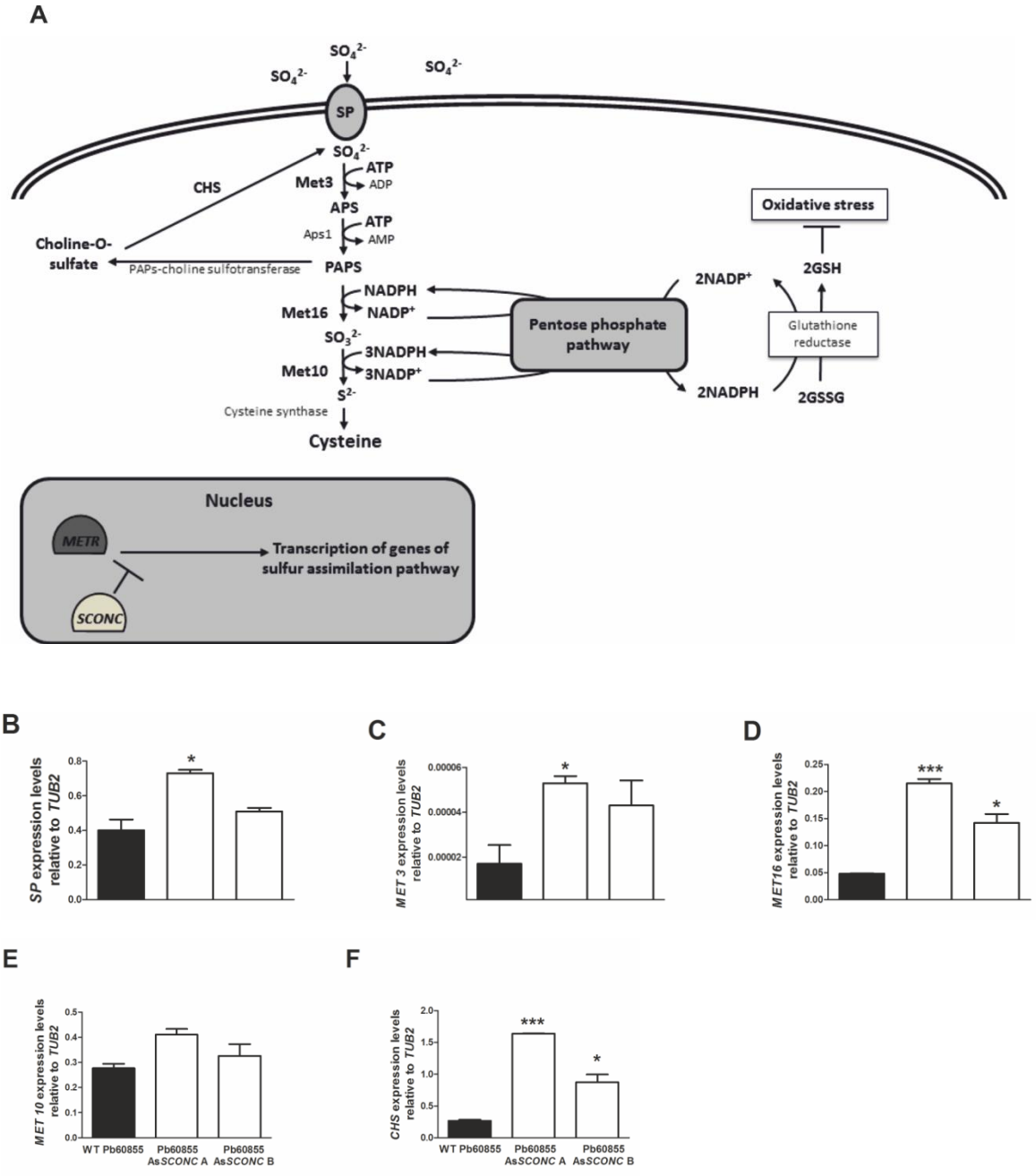

Figure 2. Sulfur assimilatory pathway and interplay with the glutathione system and pentose phosphate pathway.

(A) SconCp orchestrates MetRp proteolysis, therefore resulting in the transcriptional repression of several genes from this pathway. The interplay between sulfur assimilatory pathway and the pentose phosphate pathway for the inter-conversion of $NADP^+$ and NADPH will impact on the levels of reduced glutathione (GSH), therefore on cells redox balance. (B) *SCONC* down-regulation results in increased expression levels of inorganic sulfur assimilation pathway related genes. Expression profiles of genes coding sulfate permease (*SP*), ATP sulfurylase (*MET 3*), PAPS reductase (*MET 16*), sulfite reductase (*MET 10*) and choline-*O* sulfatase (*CHS*) in wild-type strain Pb60855 and two down-regulated clones, Pb60855 As*SCONC* A and Pb60855 As*SCONC* B grown to the exponential phase in complete MMvM (MMvM +Cys/+SO_4^{2-}) at 37°C. Asterisks represent statistical differences between wild-type strain and aRNA clones (*p<0.05; ***p<0.001). *SCONC* expression levels determined by qRT-PCR were normalized to expression of the internal reference gene β-tubulin (*TUB2*). Bars represent means and standard deviations.

SCONC DOWN-REGULATION PROMOTES MYCELIUM-TO-YEAST TRANSITION IN THE ABSENCE OF ORGANIC SULFUR SOURCES, BUT DOES NOT SUPPORT YEAST GROWTH

Considering that *Paracoccidoides* conidia/mycelium-to-yeast morphological switch is a critical step in the infective process (Brummer, Castaneda et al. 1993) and that the high expression of *SCONC* may be responsible for the organic-sulfur auxotrophy of the yeast phase (Ferreira, Marques Edos et al. 2006), we next investigated the effects of *SCONC* down-regulation on both transitions yeast-to-mycelium and mycelium-to-yeast. To address the yeast-to-mycelium transition, we cultured yeast cells of *Paracoccidioides* wild-type and As*SCONC* transformants at 26°C in three different media: complete MMvM, MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) and MMvM without organic sulfur compounds supplementation (MMvM -Cys/+SO_4^{2-}). The presence of each morphotype (Fig. 3.1A) during the transition process was evaluated over time. As expected, the yeast-to-mycelium transition was successfully accomplished in both wild-type strains (Pb60855 and Pb01) and in the respective As*SCONC* transformants, in complete medium or in the absence of either inorganic or organic sulfur compounds (Fig. 3.1B, 3.2A and 3.3A). To investigate the mycelium-to-yeast transition, we cultured mycelium of wild-type strains and As*SCONC* transformants in the above mentioned media at 37°C. Both Pb60855 and Pb01 wild-type strains were able to completely convert to the yeast phase in complete MMvM and MMvM without inorganic sulfur supplementation (Fig. 3.1C and 3.3B). The same was observed for all the As*SCONC* transformants (Fig. 3.1C 3.2B and 3.3B). However, wild-type strains were unable to switch from the mycelial to the yeast phase or other intermediate phases in MMvM without organic sulfur (Fig. 3.1C and 3.3B). These results confirmed the auxotrophy of the yeast phase of *Paracoccidioides* species for organic sulfur compounds, as previously reported (Medoff, Painter et al. 1987; Ferreira, Marques Edos et al.

2006). Conversely, the morphological switch of the As*SCONC* transformants to the yeast phase in MMvM without organic sulfur was compromised, occurring at a lower efficiency (Figure 3C and Supp. Figures 1B and 2B).

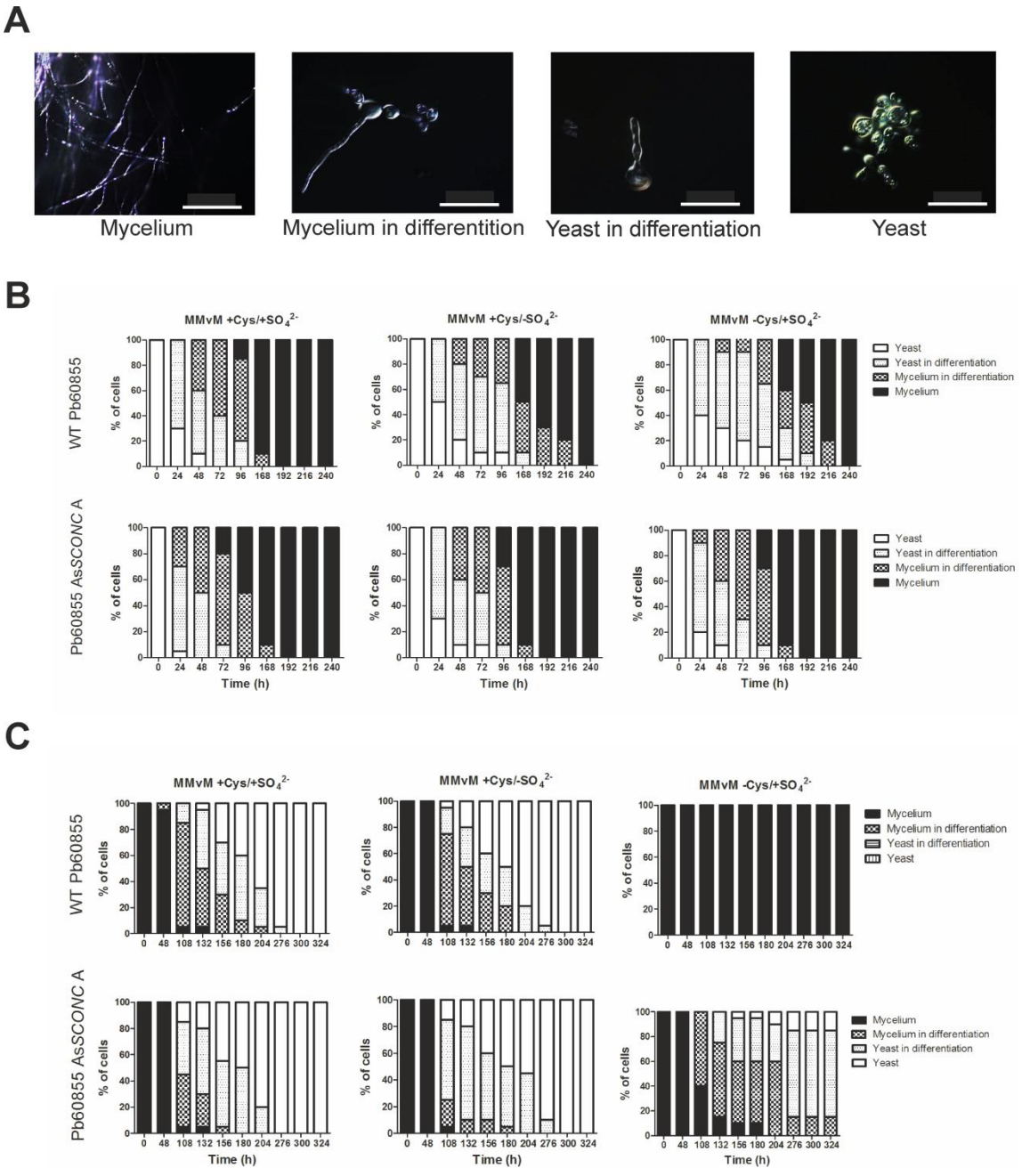

Figure 3.1. Down-regulation of *SCONC* in *P. brasiliensis* allows mycelium-to-yeast transition in the absence of organic sulfur compounds. (A) Representative DIC pictures of each morphotype considered during the morphological transition. Magnification: x40 (White bars represent 200 µm). (B) Evaluation of Pb60855 and Pb60855 As*SCONC* A morphotypes during yeast-to-mycelium transition at 26°C in complete MMvM (MMvM +Cys/+SO_4^{2-}), MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) and MMvM without organic sulfur compounds supplementation (MMvM -Cys/+SO_4^{2-}); (C) Evaluation of Pb60855 and Pb60855 As*SCONC* A morphotypes during

mycelium-to-yeast transition at 37°C in complete MMvM (MMvM +Cys/+SO_4^{2-}), MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) and MMvM without organic sulfur compounds supplementation (MMvM -Cys/+SO_4^{2-}).

For all As*SCONC* transformants, the cultures under transition contained a considerable percentage of yeast cells (approximately 10% for Pb60855 As*SCONC* A, approximately 5% for Pb60855 As*SCONC* B and approximately 30% for Pb01 As*SCONC* D). Nonetheless, the majority of cells were in intermediate phases and no mycelial cells were present in cultures with Pb60855 As*SCONC* A and Pb01 As*SCONC* D. Although our transcriptional data indicates that *SCONC* down-regulation would allow the yeast cells to consume inorganic sulfur compounds by the de-repression of the corresponding metabolic pathway (Fig. 2A), a complete transition was not observed in the absence of organic sulfur compounds.

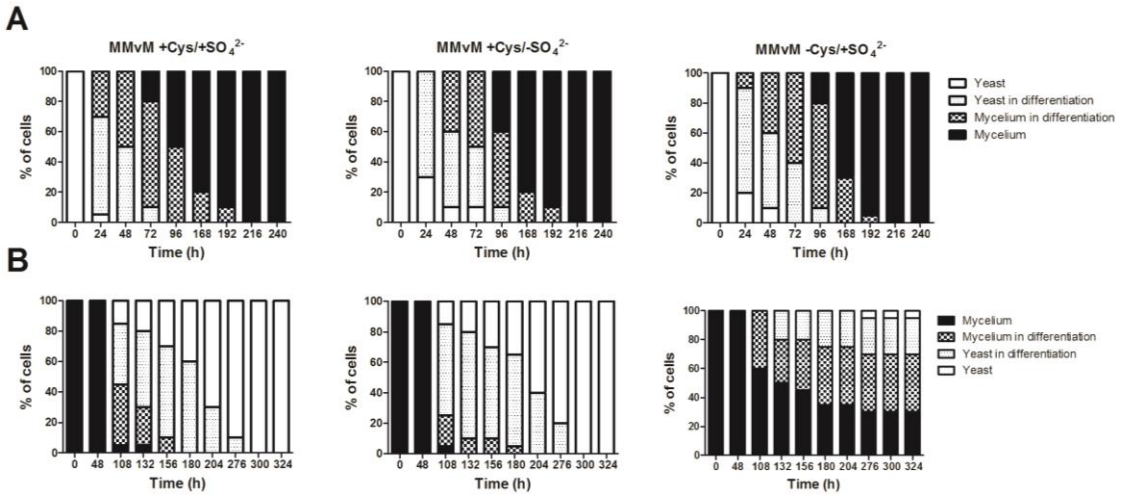

Figure 3.2. . Down-regulation of *SCONC* in *P. brasiliensis* allows mycelium-to-yeast transition in the absence of organic sulfur compounds. Evaluation of Pb60855 As*SCONC* B morphotypes during: (A) Yeast-to-mycelium transition at 26°C in complete MMvM (MMvM +Cys/+SO_4^{2-}), MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) and MMvM without organic sulfur compounds supplementation (MMvM -Cys/+SO_4^{2-}); (B) Mycelium-to-yeast transition at 37°C in complete MMvM (MMvM +Cys/+SO_4^{2-}), MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) and MMvM without organic sulfur compounds supplementation (MMvM -Cys/+SO_4^{2-}).

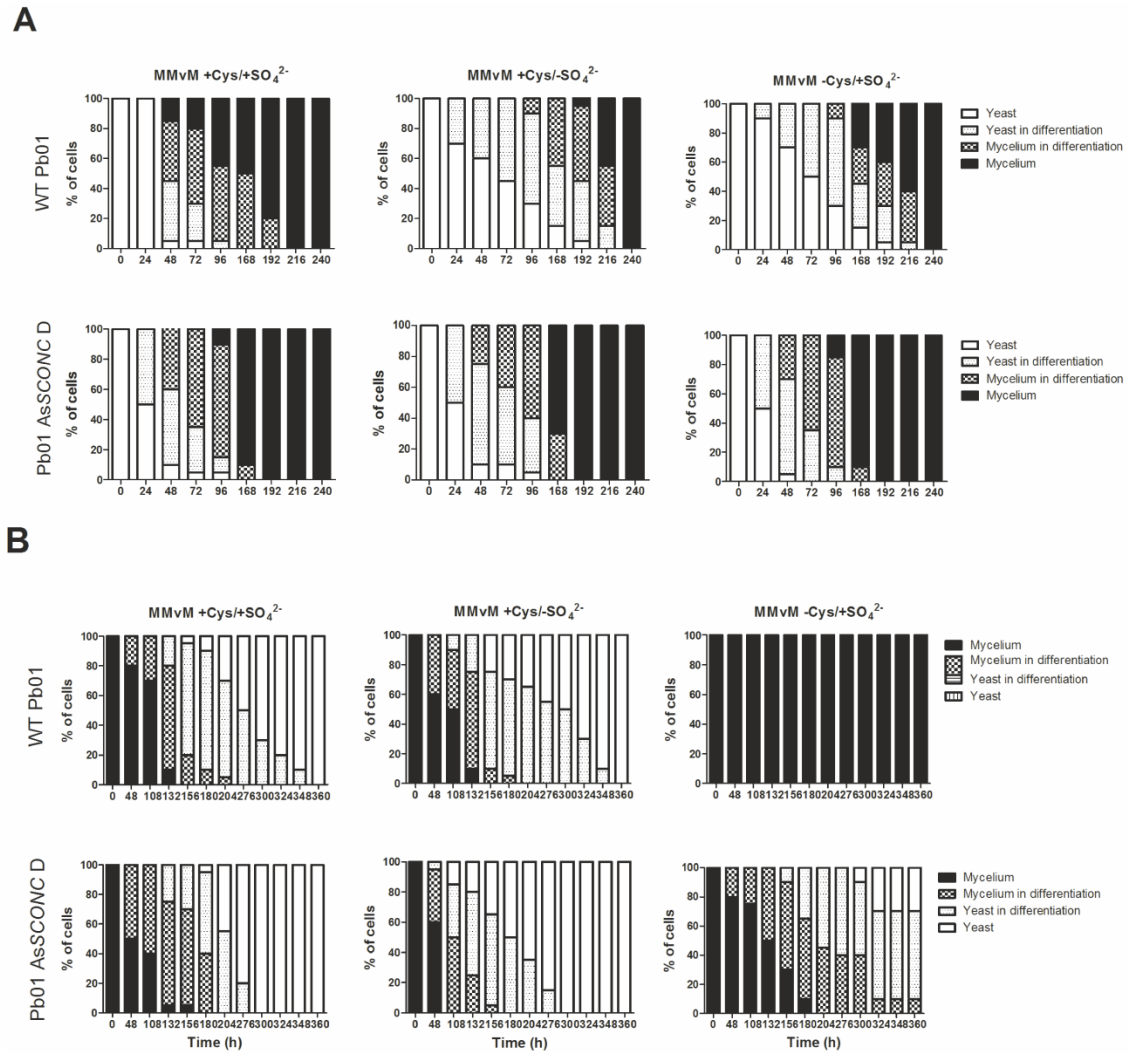

Figure 3.3. . Down-regulation of *SCONC* in *P. lutzii SCONC* allows mycelium-to-yeast transition in the absence of organic sulfur compounds. Evaluation of Pb01 and Pb01 As*SCONC* D morphotypes during: (A) Yeast-to-mycelium transition at 26°C in complete MMvM (MMvM +Cys/+SO_4^{2-}), MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) and MMvM without organic sulfur compounds supplementation (MMvM -Cys/+SO_4^{2-}); (B) Mycelium-to-yeast transition at 37°C in complete MMvM (MMvM +Cys/+SO_4^{2-}), MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) and MMvM without organic sulfur compounds supplementation (MMvM -Cys/+SO_4^{2-}).

To investigate if *SCONC* silencing was affecting the growth of *Paracoccidioides* yeast cells, we next analyzed the growth profiles of Pb60855 and Pb60855 As*SCONC* A in the three media previously mentioned. We have chosen to perform these assays with Pb60855 As*SCONC* A as it was the transformant with the lowest *SCONC* expression. As shown in Figure 4A, *P. brasiliensis* was not able to surpass its auxotrophy to organic sulfur sources, even when *SCONC* was down-regulated. Concomitantly, when in the presence of organic sulfur compounds, both wild-type Pb60855 and As*SCONC* A showed similar growth patterns, indicating that inorganic sulfur

compounds are not required for the growth of the yeast phase (Figure 4B). However, when both organic and inorganic sulfur sources were present in the medium, Pb60855 As*SCONC* A revealed a significantly lower growth rate and final optical density compared to the wild-type strain (Figure 4C), indicating that inorganic sulfur was having a negative effect on the biomass yield.

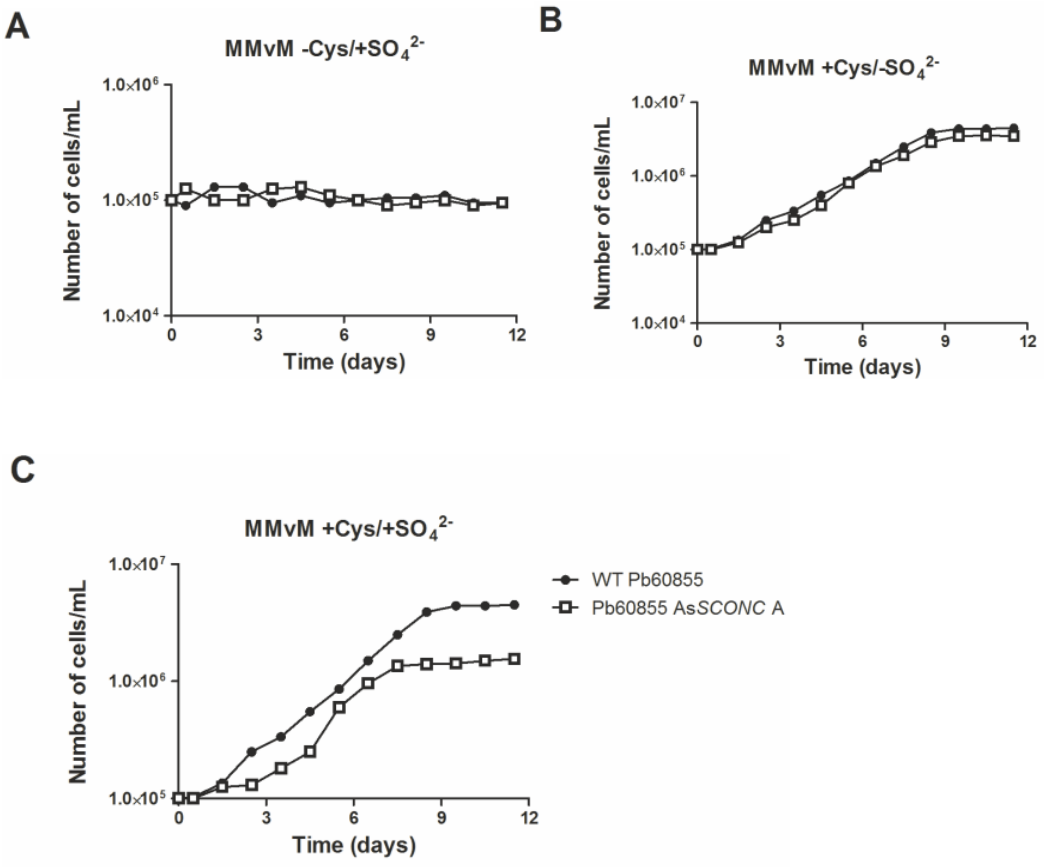

Figure 4. *P. brasiliensis* As*SCONC* transformants do not grow in medium supplemented with inorganic sulfur sources only. *P. brasiliensis* As*SCONC* transformants are unable to grow in medium supplemented with inorganic sulfur sources only (MMvM -Cys/+SO_4^{2-}) and do not sustain yeast growth together with low biomass yield in medium with both organic and inorganic sulfur sources (MMvM +Cys/+SO_4^{2-}). Pb60855 and Pb60855 As*SCONC* A yeast cells were grown in: (A) complete MMvM (MMvM +Cys/+SO_4^{2-}); (B) MMvM without inorganic sulfur supplementation (MMvM +Cys/-SO_4^{2-}); (C) MMvM without organic sulfur compounds supplementation (MMvM -Cys/+SO_4^{2-}); Cells were cultured at 37°C and samples were collected at specific time points to determine growth curves.

SILENCING OF *SCONC* REDUCES CELLULAR ATP LEVELS AND NADPH POOL THUS INCREASING ROS ACCUMULATION

As the growth rate and final optical density in complete MMvM were lower in cultures of the As*SCONC* transformants compared to wild-type cultures (Figure 4C), we hypothesized that inorganic sulfur metabolism was leading to a reduction on the ATP and NADPH cellular pools (Figure 2A). To confirm this hypothesis, both wild-type Pb60855 and Pb60855 As*SCONC* A and B strains were cultured in MMvM without inorganic sulfur compounds and pulsed with inorganic sulfur [$MgSO_4.7H_2O$ (2 mM) and $(NH_4)_2SO_4$ (15 mM)]. Intracellular levels of ATP and NADPH were subsequently measured after 0, 15 and 30 min. The ATP and NADPH pool was lower in the As*SCONC* transformants at basal levels (0 min) than the observed for the wild-type strain (Figures 5A and B), except for the NADPH levels in Pb60855 As*SCONC* B transformant. The pulse with inorganic sulfur compounds resulted in a significant decrease of the ATP and NADPH levels in the As*SCONC* transformants, while those of the wild-type strain remained constant (Figures 5A and B). Taking into consideration the ATP and NADPH requirements for the inorganic sulfur metabolism (Figure 2A), these results are consistent with the metabolic ability of the As*SCONC* transformants to use inorganic sulfur compounds.

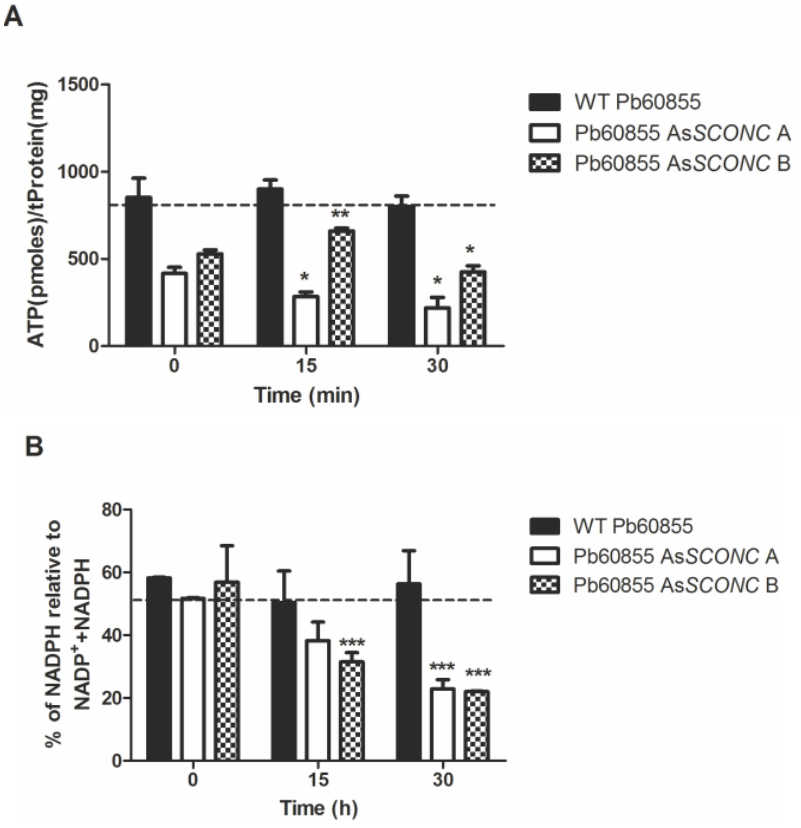

Figure 5. Down-regulation of *SCONC* impairs the ATP/NADPH pool in the presence of inorganic sulfur compounds. (A) ATP content in wild-type strain Pb60855, Pb60855 As*SCONC* A and Pb60855 As*SCONC* B, cultured at 37°C to

the exponential growth phase in MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) at time 0, and 15 and 30 min after a pulse with inorganic sulfur [$MgSO_4.7H_2O$ (2 mM) and $(NH_4)_2SO_4$ (15 mM)]. Asterisks represent significant differences between 0 min and either 15 min or 30 min for each strain (*p<0.05; ***p<0.01); (B) % of NADPH relative to $NADP^+$+NADPH in wild-type strain Pb60855, Pb60855 As*SCONC* A and Pb60855 As*SCONC* B cultured at 37°C to the exponential growth phase in MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) at time 0, and 15 and 30 min after a pulse with inorganic sulfur [$MgSO_4.7H_2O$ (2 mM) and $(NH_4)_2SO_4$ (15 mM)]. Asterisks represent significant differences between 0 min and either 15 min or 30 min for each strain (***p<0.001). Bar graphs indicate mean and standard deviation in three independent experiments. Statistical analysis was performed comparing 15 and 30 min to time 0 for each clone.

The cellular NADPH pool is critical for the maintenance of the reduced glutathione pool and thus for the maintenance of the cellular redox balance. Therefore, we next evaluated the accumulation of ROS such as superoxide anions and H_2O_2 in the wild-type strain and As*SCONC* transformants after a pulse with inorganic sulfur compounds [$MgSO_4.7H_2O$ (2 mM) and $(NH_4)_2SO_4$ (15 mM)]. This was performed by FACS analysis using dihydroethidium (DHE) and dihydrorhodamine 123 (DHR), respectively, as fluorescence markers (Mesquita, Weinberger et al. 2010). We found that both As*SCONC* transformants presented higher levels of superoxide anions and H_2O_2 at time 0 than those of the wild-type strain (Figure 6A and B). Moreover, there was a statistically significant increase of both ROS species overtime in the As*SCONC* transformants upon the inorganic sulfur pulse, while in the wild-type strain ROS levels were maintained (Figure 6A and B). These results reveal that by diverting NADPH to sulfur metabolism (Figure 2A) As*SCONC* cells accumulate more ROS such as H_2O_2 (Figure 6B). The alteration of the cellular redox balance in these cells most probably leads to an amplification loop of ROS generation that, in addition to the uncoupling of oxidative phosphorylation during *P. brasiliensis* mycelium-to-yeast transition, accounts for the observed increased levels of superoxide anions (Figure 6A).

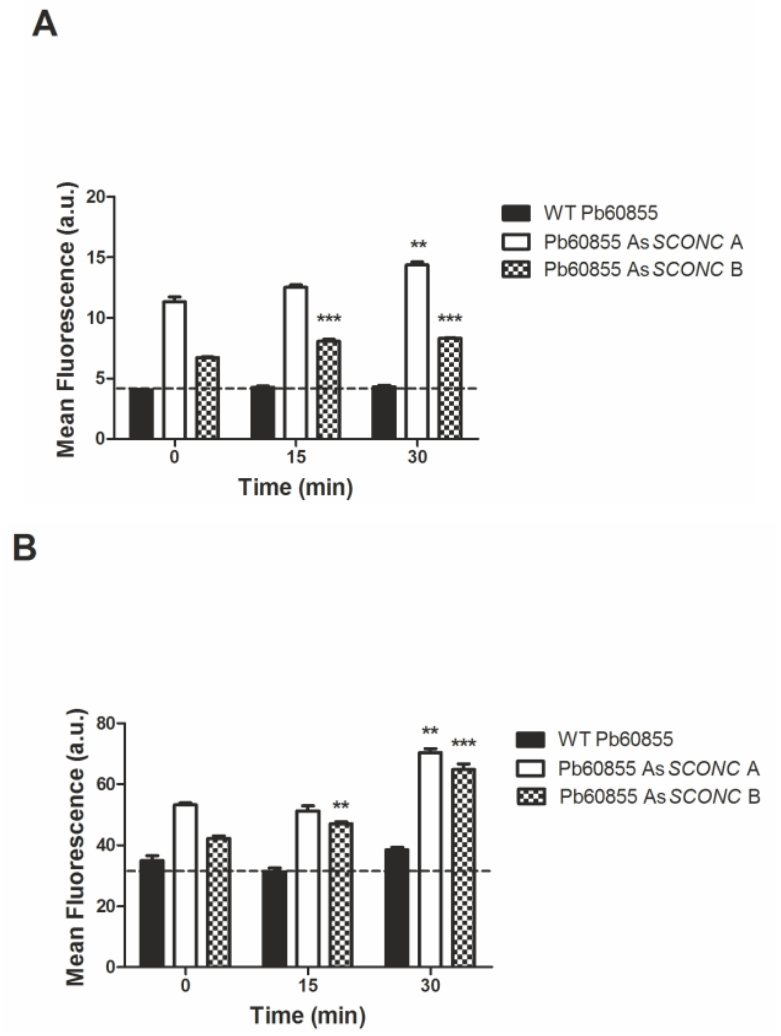

Figure 6. As*SCONC* transformants present high intracellular levels of reactive oxygen species.

(A) FACS measurements of superoxide anions using the probe dihydroethidium (DHE) in wild-type strain Pb60855, Pb60855 As*SCONC* A and Pb60855 As*SCONC* B, cultured at 37°C in MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) at time 0 and 15 and 30 min after a punch with inorganic sulfur compounds. Asterisks represent significant differences between 0 min and either 15 min or 30 min for each strain (**p<0.01; ***p<0.001); (B) FACS measurements of H_2O_2 using dihydrorhodamine 123 (DHR) in wild-type strain Pb60855, Pb60855 As*SCONC* A and Pb60855 As*SCONC* B, cultured at 37°C in MMvM without inorganic sulfur compounds supplementation (MMvM +Cys/-SO_4^{2-}) at time 0 and 15 and 30 min after a pulse with inorganic sulfur compounds [$MgSO_4.7H_2O$ (2 mM) and $(NH_4)_2SO_4$ (15 mM)]. Asterisks represent significant differences between 0 min and either 15 min or 30 min for each strain (**p<0.01; ***p<0.001). Bar graphs indicate mean and standard deviation of fluorescence/cell (arbitrary units) measured in $1x10^5$ cells per sample in three independent experiments. Statistical analysis was performed comparing 15 and 30 min to time 0 for each clone.

SCONCp CONTRIBUTES TO THE *IN VIVO* VIRULENCE OF *P. BRASILIENSIS*

So far our data clearly implicate SconCp in *Paracoccidioides* mycelium-to-yeast morphological switch, thus making SconCp a good candidate for a virulence factor of this fungus. To address

this question, C57BL/6 male mice were intravenously infected with 1X10^6 exponential yeast cells of wild-type Pb60855 strain and its respective As*SCONC* transformants. We found that 67 days post-infection all mice infected with the wild-type strain had succumbed (Figure 7), which is in accordance with the virulence pattern normally observed for this strain. In contrast, Pb60855 As*SCONC* B transformant revealed an intermediate virulence level in what regards the survival of the infected mice (Figure 7). Mice infected with Pb60855 As*SCONC* A transformant survived up to 80 days post-infection (Figure 7), suggesting a less virulent phenotype for this transformant. As observed before, the phenotype of each transformant when compared to the wild-type strain is correlated with the level achieved for *SCONC* down-regulation (which was highest for transformant A). Our results strongly suggest SconCp as an essential player on *P. brasiliensis* species virulence.

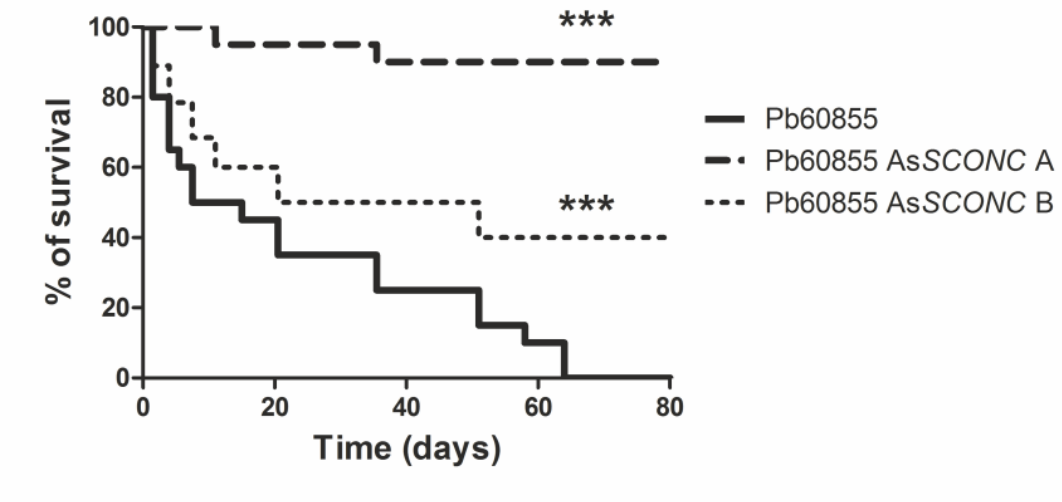

Figure 7. Silencing of *SCONC* decreases virulence of *P. brasiliensis* yeast cells. Representative survival curves of an experimental i.v. infection carried out in C57BL/6 mice (n=20) with 1x10^6 wild-type Pb60855, Pb60855 As*SCONC* A and Pb60855 As*SCONC* B yeast cells grown to the exponential phase in BHI. Asterisks represent significant differences between mice infected with Pb60855 and mice infected with either Pb60855 As*SCONC* A or Pb60855 As*SCONC* B.

DISCUSSION

Recent studies uncovered several genes involved in the pathogenicity of *P. brasiliensis* and its degree of virulence, including *CDC42* (Almeida, Cunha et al. 2009; Menino, Osorio et al. 2012), *GP43* (Stambuk, Puccia et al. 1988; Almeida, Unterkircher et al. 1998; de Almeida, de Moraes et al. 1998), *HAD32* (Hernandez, Almeida et al. 2012) and *AOX* (Hernandez, Garcia et al. 2011). However, the research on *P. brasiliensis* genetic determinants that govern conidia/mycelium-to-

yeast transition and subsequently fungal virulence has been highly neglected. The understanding of the mechanisms underlying the morphological transition of *P. brasiliensis* from the conidia/mycelial phase to the pathogenic yeast phase is essential, as this is critical for the establishment and development of paracoccidioidomycosis. In the last decades, the only known factor attributed to this morphological switch was the temperature shift that occurs once *P. brasiliensis* conidia/mycelium reach the lungs of the host (San-Blas, Restrepo et al. 1992; Brummer, Castaneda et al. 1993; Tercarioli, Bagagli et al. 2007).

In this study we evaluated the effect of SconCp, the negative regulator of the inorganic sulfur assimilatory pathway, in *P. brasiliensis* dimorphism and virulence. Knowing from previous studies that *SCONC* is highly expressed in the yeast phase *of P. brasiliensis*, and that it blocks the assimilation of inorganic sulfur sources (Marques, Ferreira et al. 2004; Andrade, Paes et al. 2006; Ferreira, Marques Edos et al. 2006; Tavares, Silva et al. 2007), we down-regulated *Paracoccidioides SCONC* using a gene silencing approach (Almeida, Cunha et al. 2009). The reduction of *SCONC* expression levels led to the up-regulation of several genes belonging to the inorganic sulfur pathway. These results are in line with data obtained for other fungi. Mutations in *sconC1* and *sconC2* genes from *Aspergillus nidulans* were shown to impair methionine-mediated sulfur metabolite repression, allowing the fungus to the produce sulfur-metabolism related enzymes (Natorff, Balinska et al. 1993; Piotrowska, Natorff et al. 2000). Characterization of a *Neurospora crassa* mutant in *SCON-2* gene also revealed a de-repression of the sulfur metabolism, contrarily to the wild-type strain (Burton and Metzenberg 1972). In *Saccharomyces cerevisiae*, mutations in *MET30* (the homolog of *SCONC* from *P. brasiliensis*) revealed to impair the repression of the sulfur network (Thomas, Kuras et al. 1995).

Knowing that the conidia/mycelium-to-yeast transition is a requisite for the development of paracoccidioidomycosis (Brummer, Castaneda et al. 1993), we tested whether down-regulation of *SCONC* and consequent up-regulation of inorganic sulfur pathway-related genes impacted on the dimorphic process. We found that upon de-repression of the inorganic sulfur pathway by down-regulation of *SCONC*, the yeasts' auxotrophy for organic sulfur sources was surpassed. Consequently, the mycelium-to-yeast transition in media supplemented only with inorganic sulfur sources was possible. These data further support a regulatory role for SconCp during the dimorphic process, by repressing inorganic sulfur metabolism related genes.

Concomitantly, a similar occurrence was found upon down-regulation of *SCONC* in *Paracoccidioides* species other than *P. brasiliensis*, such as in *P. lutzii* species. This is a good

indicator that, although great sequence and morphological divergences separate these species, *P. brasiliensis* and *P. lutzii* share the same regulatory sulfur mechanisms and dimorphic traits. Although *P. brasiliensis* cells were able to assimilate inorganic sulfur sources, they could not accomplish a complete transition in the absence of organic sulfur compounds. Together with this, the fact that down-regulated transformants presented lower biomass yields in medium supplemented with both inorganic and organic sulfur sources, led us to question if the assimilation of inorganic sulfur was causing a metabolic imbalance, thereby affecting growth of *P. brasiliensis* yeast cells. The high demand of ATP and NADPH for inorganic sulfur metabolism (Figure 2A) could be hampering both the energy available for cellular processes and the ability of *P. brasiliensis* to scavenge ROS. Our results show that the usage of inorganic sulfur sources in As*SCONC* transformants indeed decreased the availability of total NADPH overtime. This fact, together with the natural low activity of glucose-6-phosphate dehydrogenase in the yeast phase of *P. brasiliensis*, the main cellular source of NADPH (Kanetsuna and Carbonell 1966), could explain the high accumulation of H_2O_2 and superoxide anions in As*SCONC* transformants. This is possibly resulting in a decrease of the reduced glutathione pool, thus impairing the cellular redox balance. In fact, an interplay between the sulfur assimilatory pathway and the formation of the reduced form of glutathione, a powerful anti-oxidant (Nuttall, Martin et al. 1998), is well described in some fungi (Townsend, Tew et al. 2004; Sato, Shimatani et al. 2011). A similar observation was reported upon selenium uptake by *S. cerevisiae* cells. Selenium metabolites share a highly similar chemical and physical nature with sulfur metabolites, and are both thought to follow the same metabolic routes. In conditions of sulfur deficiency, selenium uptake by *S. cerevisiae* cells led to an intracellular redox imbalance, linked to a disproportionate ration between the reduced form of glutathione and the oxidized one, a circumstance shown to be detrimental for cell viability (Mapelli, Hillestrom et al. 2012).

As for an implication of the inorganic sulfur metabolism in the energy available for cellular processes, we were able to detect a decrease on the intracellular pool of ATP in the As*SCONC* transformants. Since the uncoupling of oxidative phosphorylation during *P. brasiliensis* mycelium-to-yeast transition is known to reduce the levels of ATP (Medoff, Painter et al. 1987), the remaining ATP pool in the As*SCONC* transformants is likely not fulfilling the cellular requirements upon inorganic sulfur metabolism. Taken together, our findings on the alterations of the cellular pools of both NADPH and ATP can be accountable for the low biomass yields of the As*SCONC* transformants, and their inability to grow on the yeast phase using only inorganic sulfur sources.

Finally we also show that the down-regulation of *SCONC* profoundly alters the outcome of the infection in an *in vivo* mouse model of infection. In fact, the As*SCONC* transformants were less virulent to mice, being the degree of virulence correlated with the efficiency of *SCONC* silencing. Therefore, our data suggest a novel role for SconCp as a virulence factor for *P. brasiliensis*. Lack of virulence of *SCONC* down-regulated transformants is likely due to an impairment of the cells' antioxidant properties and energy in the form of ATP, as discussed herein. Since the silencing of this molecule can in fact abrogate the *in vivo* virulence of *P. brasiliensis,* it will be essential to explore the modulation of SconCp in *P. brasiliensis* as a tool to obtain an attenuated vaccine, and also possible ways to abrogate the expression or the activity of SconCp in a therapeutic perspective.

CHAPTER 4

TLR9 ACTIVATION DAMPENS THE EARLY INFLAMMATORY RESPONSE TO *PARACOCCIDIOIDES BRASILIENSIS*, IMPACTING HOST SURVIVAL

The results presented over this chapter were published:

(i) in an international peer reviewed journal:

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(ii) in conference proceedings:

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Menino JF, Saraiva M, Silva D, Guedes J, Ludovico P, Pedrosa J, Castro A, Rodrigues F. TLR9 activation plays an early-protective role against *Paracoccidioides brasiliensis* infection. XXXVII Jornadas Portuguesas de Genética, 29-31 May, Lisbon, Portugal, 2012. *(poster presentation).*

ABSTRACT

Paracoccidioides brasiliensis causes paracoccidioidomycosis, one of the most prevalent systemic mycosis in Latin America. Thus, understanding the characteristics of the protective immune response to *P. brasiliensis* is of interest as it may reveal targets for disease control. The initiation of the immune response relies on the activation of pattern recognition receptors, among which are TLRs. Both TLR2 and TLR4 have been implicated in the recognition of *P. brasiliensis* and regulation of the immune response. However, the role of TLR9 during the infection by this fungus remains unclear. We used *in vitro* and *in vivo* models of infection by *P. brasiliensis*, comparing wild type and TLR9 deficient ($^{-/-}$) mice, to assess the contribution of TLR9 on cytokine induction, phagocytosis and outcome of infection. We show that TLR9 recognizes either the yeast form or DNA from *P. brasiliensis* by stimulating the expression/production of pro-inflammatory cytokines by bone marrow derived macrophages, also increasing their phagocytic ability. We further show that TLR9 plays a protective role early after intravenous infection with *P. brasiliensis*, as infected TLR9$^{-/-}$ mice died at higher rate during the first 48 hours post infection than wild type mice. Moreover, TLR9$^{-/-}$ mice presented tissue damage and increased expression of several cytokines, such as TNF-α and IL-6. The increased pattern of cytokine expression was also observed during intraperitoneal infection of TLR9$^{-/-}$ mice, with enhanced recruitment of neutrophils. The phenotype of TLR9$^{-/-}$ hosts observed during the early stages of *P. brasiliensis* infection was reverted upon a transient, 48 hours post-infection, neutrophil depletion. Our results suggest that TLR9 activation plays an early protective role against *P. brasiliensis*, by avoiding a deregulated type of inflammatory response associated to neutrophils that may lead to tissue damage. Thus modulation of TLR9 may be of interest to potentiate the host response against this pathogen.

INTRODUCTION

Paracoccidioides brasiliensis is a causative agent of paracoccidioidomycosis (PCM), one of the most prevalent systemic mycosis in Latin America (Brummer, Castaneda et al. 1993). One of *P. brasiliensis* biological hallmarks is its particular temperature-dependent morphological dimorphism. This fungus switches from the environmental non-pathogenic mycelial/conidial form at ambient temperatures to the pathogenic multiple budding yeast form, with an high variability of cell sizes, when exposed to temperatures similar to those of the mammalian host (Almeida,

Cunha et al. 2009; Garcia, Hernandez et al. 2010; Menino, Osorio et al. 2012). The mechanism of infection by *P. brasiliensis* entails the inhalation of airborne conidia that, when in the lung and exposed to host temperatures, undergo a complex morphological switch to the pathogenic yeast form (McEwen, Bedoya et al. 1987; Brummer, Castaneda et al. 1993). PCM is divided in two different forms: the acute or sub-acute form and the chronic form, depending on the natural course of infection and clinical manifestations of the patient (Franco 1987; Brummer, Castaneda et al. 1993). The clinical manifestations rely on the virulence of *P. brasiliensis* infecting strain, the degree and type of immune response triggered, the tissues infected, and importantly, on intrinsic characteristics of the host (Shikanai-Yasuda, Telles Filho Fde et al. 2006; Benard 2008).

Despite the fact that a large number of individuals are exposed to the fungus, only a minority develops the disease, suggesting that, for the majority of the population, a protective immune response is developed (Musatti, Rezkallah et al. 1976; Franco 1987). Therefore, the understanding of the protective characteristics of the immune response to *P. brasiliensis* is of interest as it may reveal targets for disease control. The initiation of the immune response relies on the activation of the innate immune system upon recognition of pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov 2002). This recognition is mediated by the family of pattern recognition receptors (PRRs) that is composed by a large number of receptors in immune cells (Akira 2001; O'Neill 2006). Activation of PRRs culminates with the expression of several immune mediators, including pro- and anti-inflammatory cytokines and also with the activation of a series of microbicidal mechanisms that aim at eliminating the pathogen (Medzhitov 2001). The most widely recognized type of PRRs are the toll-like receptors (TLRs) (Kawai and Akira 2007). Over the past few years, several studies have demonstrated a relevant role for TLRs in the recognition of fungal pathogens, such as *P. brasiliensis*, *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* (Shoham, Huang et al. 2001; Netea, Van Der Graaf et al. 2002; Braedel, Radsak et al. 2004; Netea, Van der Graaf et al. 2004; Villamon, Gozalbo et al. 2004; Calich, da Costa et al. 2008; Carvalho, Pasqualotto et al. 2008). The role of MyD88, an adaptor protein used by all TLRs (with the exception of TLR3), during *P. brasiliensis* infection remains controversial, with some authors reporting that this protein is not essential for an effective response against *P. brasiliensis* (Gonzalez, Yanez et al. 2008), and others claiming that MyD88 is important for the activation of innate fungicidal mechanisms and for the induction of the effector and regulatory cells of the adaptive immune response (Loures, Pina et al. 2011). A role for both TLR2 and TLR4 in the recognition and internalization of *P. brasiliensis* has been

reported in human monocytes and neutrophils (Bonfim, Mamoni et al. 2009). In a model of experimental PCM, TLR2 deficiency leads to increased Th17 immunity associated with diminished expansion of regulatory T cells and increased lung pathology due to unrestrained inflammatory reactions (Loures, Pina et al. 2009). In contrast, *P. brasiliensis* recognition by TLR4 leads to an increased production of Th17 cytokines, enhanced pro-inflammatory immunity, and impaired expansion of regulatory T cells, resulting in a more severe form of infection (Loures, Pina et al. 2010). However, the involvement of TLR9 in *P. brasiliensis* infection has not yet been addressed. Several lines of evidence suggest that TLR9 may play a role in infection by *P. brasiliensis*, similarly to what is already described for other pathogenic fungi, such as *A. fumigatus*, *C. albicans* and *C. neoformans* (Bellocchio, Montagnoli et al. 2004; Nakamura, Miyazato et al. 2008; Ramirez-Ortiz, Specht et al. 2008; van de Veerdonk, Netea et al. 2008; Ramaprakash, Ito et al. 2009; Mansour, Tam et al. 2012). Firstly, *P. brasiliensis* DNA is known to have large numbers of CpG motifs (Souza, Correa et al. 2001), the natural ligand to TLR9 (Rutz, Metzger et al. 2004). Secondly, due to the fungus multinucleated nature (McEwen, Restrepo et al. 1987; Almeida, Matute et al. 2007), a high amount of DNA is expected to be released upon cell death during infection. Thirdly, previous studies show that, in *in vitro* models, *P. brasiliensis* DNA increases the phagocytic index of macrophages, whereas in *in vivo* models of *P. brasiliensis* infection, TLR9 activation may act as a Th1-promoting adjuvant in a time/concentration dependent-manner (Souza, Correa et al. 2001; Amaral, Garcia et al. 2005).

In this study, we investigated the role of TLR9 in the recognition of *P. brasiliensis*, and its influence on the infective process and evolution of the disease. Our results show that TLR9 recognizes *P. brasiliensis*, playing a major regulatory role during early times of *in vivo* infection with its absence making the host more prone to increased liver pathology and premature death, mainly mediated by neutrophils.

MATERIAL AND METHODS

Microorganisms and culture media: The strain ATCC 60855 of *P. brasiliensis* registered at the American Type Culture Collection (Rockville, MD) was used throughout this experiment. Yeast cells were maintained at 37°C by subculturing in brain heart infusion (BHI) (Duchefa) solid media supplemented with 1 % glucose and gentamicin (50 µg/mL). For both *in vitro* and *in vivo* assays,

yeast cells were grown in BHI liquid medium supplemented with 1 % glucose and gentamicin (50 µg/mL) at 37ºC with aeration on a mechanical shaker (220 rpm). Cell growth was monitored for 148 h by microscopic counting using a Neubauer's Chamber and cells were collected during the exponential growth phase (72 h of growth, 1.65 ± 0.8 x 10^7 cells/mL) for all the experimental assays.

Ethics statement: This study was approved by the Portuguese national authority for animal experimentation Direção Geral de Veterinária (ID: DGV 594 from 1st June 2010). Animals were kept and handled in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and of the Council.

Mice: Eight-week-old C57BL/6 mice were obtained from Charles River (Barcelona, Spain) and eight-week-old TLR9$^{-/-}$ (generated in a C57BL/6 background) were kindly provided by P. Vieira (Pasteur Institute of Paris, France). Mice were housed under specific-pathogen-free conditions with food and water *ad libitum*.

In vivo infection: C57BL/6 WT or TLR9$^{-/-}$ mice were infected intravenously (i.v.) with 1x10^6 *P. brasiliensis* yeast cells grown to the exponential phase in BHI liquid medium. Prior to infection, cells were washed 3 times with lipopolysaccharide (LPS)-free phosphate-buffered saline (PBS) (Gibco), passed through a syringe to eliminate cell clumps, and submitted to Neubauer counting procedures (each mother and bud cells were considered as individual counts). Mice were monitored daily for 32 days. Mice showing severely impaired mobility in the first 48 hours of infection were sacrificed and liver and blood were collected for later analysis. For the peritoneal cavity model of infection, mice were injected intraperitoneally (i.p.) with 1x10^6 *P. brasiliensis* yeast cells grown and treated as described above. Mice were sacrificed 6 hours post-infection and, after peritoneal lavage with 4 ml of PBS, total leukocyte number was determined.

Histological studies: Liver from dying mice were harvested in the time-period comprehending 48 h post-infection, fixed in 3.8% phosphate-buffered formalin and embedded in paraffin. Light-microscopy studies were performed on tissue sections stained with hematoxylin and eosin (HE) as previously described (Oliveira, Fraga et al. 2005). The histological analysis was performed by

the presence of necrotic areas and the type of inflammatory infiltrate (when present) in each field of 10× objective.

Neutrophil depletion: C57BL/6 WT and TLR9$^{-/-}$ mice were made neutropenic by treatment with the monoclonal antibody (MAb) RB6-8C5, as previously described (Appelberg, Castro et al. 1994; Appelberg, Castro et al. 1995). Briefly, mice were injected i.v. in the lateral tail vein with 200 mg of MAb RB6-8C5, and 6 h later infected i.v. with 1x10^{6} *P. brasiliensis* yeast cells grown to the exponential phase in BHI liquid medium and treated as indicated before. Mice were monitored as indicated before during the first 2 days of infection.

Preparation of fungal DNA: *P. brasiliensis* DNA was extracted from exponentially growing cells. Briefly, cells were disrupted using a mix of lysis buffer (1 mM EDTA, 10 mM Tris–HCl, 1% SDS, 100 mM NaCl) and phenol/chloroform/isoamylalcohol (25:24:1). Heat-shock treatment (45 min at 65°C) was performed and the aqueous phase was treated using a column-based method (in-column proteinase K and RNAse treatments were performed). To elute DNA, LPS-free water was used.

In vitro infection: Bone marrow-derived macrophages (BMDMs) from C57BL/6 WT and TLR9$^{-/-}$ mice were prepared as described previously (Oliveira, Fraga et al. 2005; Zhang, Majlessi et al. 2009; Neves, Silvestre et al. 2010). BMDMs were seeded in 24-well plates at 5 ×10^{5} cells/well and kept at 37°C and 5% CO_2 atmosphere. Cells were challenged with 5 μg of *P. brasiliensis* DNA extracted from exponentially growing cells. In other experiments, BMDMs from C57BL/6 WT and TLR9$^{-/-}$ mice seeded as described above were challenged with *P. brasiliensis* yeast cells grown to the exponential phase, with late-stationary growing cultures or with *P. brasiliensis* lysed cells, using a 2:1 multiplicity of infection (MOI; yeast/ BMDMs ratio) for 24 h. Prior to infection, fungal cells were washed 3 times with LPS-free PBS, passed through a syringe to eliminate cell clumps, and submitted to Neubauer counting procedures (mother and bud cells were considered as individual counts). Supernatants from stimulated BMDMs were collected 24 h post-infection and stored at -80°C for later cytokine analysis.

P. brasiliensis phagocytosis assays: BMDMs from C57BL/6 WT and TLR9$^{-/-}$ prepared as previously described (Oliveira, Fraga et al. 2005) were seeded in 24-well plates at 5 ×10^{5}

cells/well, kept at 37°C and 5% CO_2 atmosphere and infected with *P. brasiliensis* yeast cells grown to the exponential phase using a 2:1 multiplicity of infection (MOI; yeast/macrophage ratio) for 3 h. The wells were then washed three times with PBS to eliminate non-phagocytized cells, BMDMs were lysed with sterile H_2O and the remaining phagocytized yeast cells were collected to determine total phagocytosis, using a Neubauer's Chamber. For the experiments, *P. brasiliensis* cells treated with DNase were also used. Briefly, after collecting and washing, *P. brasiliensis* yeast cells grown to the exponential phase were incubated at 37°C for 1 h with 20 μg of DNaseI (Ambion). As control, heat-inactivated DNase (70°C for 1 h) was used.

ELISA: Cytokine levels were measured in serum collected from infected animals or in supernatants of infected cell cultures by capture enzyme-linked immunosorbent assay (ELISA) (eBioscience). The ELISA procedure was performed according to the manufacturer's protocol, and absorbances were measured with a Bio-Rad 680 Micro-plate Reader.

Flow cytometry: Quantification of neutrophils and mononuclear cells influx to the peritoneal cavity during the i.p. infection with *P. brasiliensis* was performed by flow cytometry (FCM) on a BD LSR II flow cytometer. Cells collected from the peritoneal cavity were stained, using specific antibodies for CD11b, CD11c, GR-1 and Ly6G to distinguish neutrophils and mononuclear cells populations, and a minimum of 100,000 cells per sample was acquired at low/medium flow rate. Offline data was analyzed with the flow cytometry analysis software package FlowJo 7.6.1.

Real-time polymerase chain reaction (RT-PCR): Total RNA (1 μg) was isolated according to TRIzol methodology (Invitrogen). For liver samples, small portions were homogenized in between microscopy slides, suspended in 1 mL of TRIzol reagent and kept at -80°C for later analysis. For the i.p. experiment around $5x10^5$ cells from the peritoneal exudates were used, suspended in 250 uL of TRIzol reagent and stored at -80°C for later analysis. RNA integrity was checked by the presence of clear 18S and 28S rRNA bands in agarose gel electrophoresis. The absence of DNA contamination in the samples was confirmed by the absence of PCR amplification of the ubiquitin gene in the isolated RNA. Total RNA (1 μg) was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad) following manufacturer's instructions and 1 μL of cDNA used as a template for real-time quantification using the SsoFast EvaGreen SuperMix (Bio-Rad) following manufacturer's instructions. Real-time quantification was carried out on a CFX96 Real-Time

System (Bio-Rad) using threshold cycle (Ct) values for ubiquitin transcripts as the endogenous reference. The primer sequences were designed and synthesized by TIB Mol. Biol. and were as follows: UBQ forward, *TGG CTA TTA ATT ATT CGG TCT GCA T*; UBQ reverse, *GCA AGT GGC TAG AGT GCA GAG TAA*; IL-10 forward, *TTT GAA TTC CCT GGG TGA GAA*; IL-10 reverse, *GCT CCA CTG CCT TGC TCT TAT T*; IL-17 forward, *CTC AGA CTA CCT CAA CCG TTC CA*; IL-17 reverse, *TTC CCT CCG CAT TGA CAC A*; TNF forward, *GCC ACC ACG CTC TTC TGT CT*; TNF reverse, *TGA GGG TCT GGG CCA TAG AAC*; MIP2 forward, *CTC AGT GCT GCA CTG GT*; MIP2 reverse, *AGA GTG GCT ATG ACT TCT GTC T*; IL-6 forward, *TCG TGG AAA TGA GAA AAG AGT TG*; IL-6 reverse, *TAT GCT TAG GCA TAA CGC AC TAG*. All measurements were performed in triplicate. A single melting peak was obtained for each gene analyzed in all samples.

Statistics: Data is reported as the mean ± standard error of the mean (SEM) and all assays were repeated at least three times. All statistical analysis was performed using the GraphPad Prism Software version 5.01. For the experiments comparing two groups (see Fig. 1A and B, 4 and 5), a two-tailed unpaired Student *t* test was performed. Welch's correction was applied when making multiple comparisons. The One Way ANOVA test was performed in data presented in Fig. 1C using Turkey's multiple comparison post-test. The survival curves, representative of three independent experimental infections (Fig. 2, n=21 mice), are represented using the Kaplan-Meier estimator, and Gehan-Breslow-Wilcoxon test was applied. For all data analysis statistical significance was considered at the level of 0.05 (2-tailed, 95% confidence interval).

RESULTS

TRIGGERING OF TLR9 BY *P. BRASILIENSIS* MODULATES MACROPHAGE CYTOKINE PRODUCTION AND PHAGOCYTOSIS

Since previous studies have reported the recognition of fungal DNA by TLR9 (van de Veerdonk, Kullberg et al. 2008), we questioned if *P. brasiliensis* triggers TLR9-mediated responses in macrophages. For that, we stimulated BMDMs generated from wild-type (WT) and TLR9$^{-/-}$ mice with purified *P. brasiliensis* DNA. Our results showed that purified *P. brasiliensis* DNA induced the secretion of TNF-α and IL-6 by BMDMs in a TLR9 dependent way (Fig. 1A). Next, we tested if TLR9 could also recognize *P. brasiliensis* DNA in the yeast cellular context and whether this depended on the cellular physiological stage and integrity. We stimulated WT and TLR9$^{-/-}$ BMDM

with either *P. brasiliensis* cells from exponential or late-stationary growing cultures or with lysed cells. We found that BMDMs stimulated with *P. brasiliensis* yeast-form produced TNF-α in a TLR9 dependent manner (Fig. 1B), although the response was higher for late-stationary growing and lysed cells.

In addition to cytokine expression, TLR triggering also associates with the onset of phagocytosis (Sanjuan, Dillon et al. 2007; Blander 2008). We next investigated if TLR9 activation by the yeast-form of *P. brasiliensis* impacted phagocytosis. The percentage of phagocytosis of *P. brasiliensis* by BMDMs was significantly reduced when *P. brasiliensis* yeast cells were treated with DNase or when TLR9$^{-/-}$ BMDMs were used (Fig. 1C). When heat inactivated DNase was used, the percentage of phagocytosis by WT macrophages was similar to that observed when no treatment was performed, whereas TLR9$^{-/-}$ macrophages maintained the phagocytic profile, indicating that DNase is not interfering with phagocytosis. Thus, our data indicate that TLR9 activation is required for maximal *P. brasilisensis* phagocytosis.

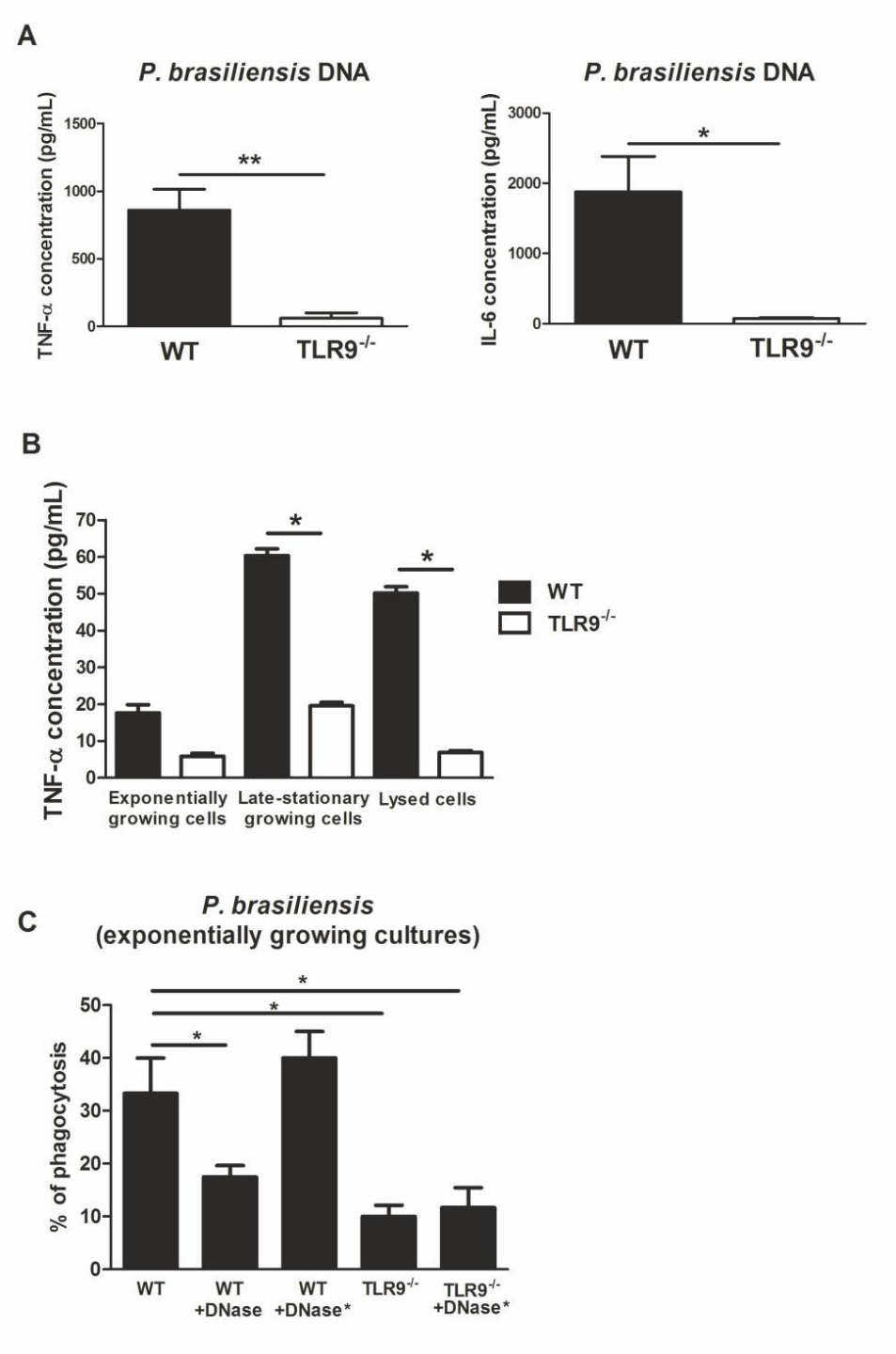

Figure 1 – Outcome of *in vitro* stimulation of macrophages from WT and TLR9$^{-/-}$ mice. (A) - Protein levels after stimulation with 5 µg of *P. brasiliensis* DNA for 24 h. Asterisks represent significant differences between WT and TLR9 TLR9$^{-/-}$ mice (**P<0.01, *P<0.05). IL-6 was also found with higher expression levels in dendritic cells from TLR9$^{-/-}$ mice when compared to dendritic cells from WT mice (data not shown) (B) – Protein levels after stimulation with wild-type *P. brasiliensis* ATCC 60855 yeast cells grown either to the exponential or late-stationary phase for 24 h using a MOI of 2:1, and lysed cells. Asterisks represent significant differences between WT and TLR9$^{-/-}$ mice. IL-6 levels were below detection limits; (C) - Percentage of total phagocytosis of wild-type *P. brasiliensis* ATCC 60855 yeast cells grown to the exponential phase using a MOI of 2:1. Asterisks represent significant differences between

macrophages from WT mice stimulated with *P. brasiliensis* yeast cells and either macrophages from WT mice stimulated with *P. brasiliensis* yeast cells pre-treated with DNase (WT+DNase) (*P<0.05) or macrophages from TLR9$^{-/-}$ mice stimulated with *P. brasiliensis* yeast cells (TLR9$^{-/-}$) (*P<0.05). Pre-treatment of *P. brasiliensis* yeast cells with DNase does not affect phagocytosis by TLR9$^{-/-}$ macrophages (data not shown). When cells were treated with heat inactivated DNase, the phagocytic profile of WT macrophages is restored (WT$^{-/-}$+DNase*), while for TLR9$^{-/-}$ macrophages it is not altered (TLR9$^{-/-}$+DNase*).

TLR9 HAS A PROTECTIVE ROLE IN THE EARLY PHASE OF *P. BRASILIENSIS* INFECTION

Given the *in vitro* impact of *P. brasiliensis* recognition by TLR9, we next sought to investigate a role for this receptor during the course of an *in vivo* experimental infection. WT and TLR9$^{-/-}$ mice were intravenously infected with *P. brasiliensis* and the survival rates followed over time. We found that 24 days post-infection 100% of TLR9$^{-/-}$ mice had succumbed, whereas for WT mice this was only observed 31 days post-infection (Fig. 2).

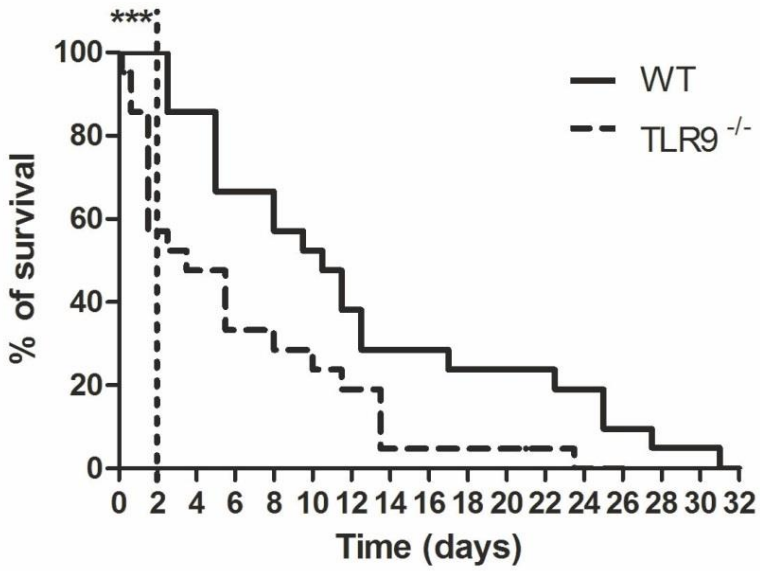

Figure 2 – Outcome of *in vivo* intravenous infection of WT and TLR9$^{-/-}$ mice with *P. brasiliensis*. Representative survival curves using the Kaplan-Meier estimator of an experimental intravenous infection carried out in WT and TLR9$^{-/-}$ C57BL/6 mice (n= 21) with 1x10^{6} wild-type *P. brasiliensis* ATCC 60855 yeast cells grown to the exponential phase. The estimated mean survival for WT mice is 13.6±2.0, while for TLR9$^{-/-}$ mice is 6.8±1.4 (Gehan-Breslow-Wilcoxon test was applied). Data is expressed as percentage of live animals. The observed differences during the first 48 h post- infection were statistically significant (***P<0.001).

Furthermore, the estimated mean survival for WT mice was of 13.6±2.0 days, while for TLR9$^{-/-}$ mice it was reduced to 6.8±1.4 days (p<0.001; Fig. 2). Even more striking was the observation that during the first 48 h post-infection, TLR9$^{-/-}$ infected mice showed severe impaired mobility,

with a significant number of animals being humanely sacrificed as a result. As shown in Fig. 2, during this early period the mortality of the TLR9$^{-/-}$ mice was approximately 43%, while WT mice showed no signs of disease. Thus, our data highlight an unexpected protective role of TLR9 during the early phases of *P. brasiliensis* infection.

ABSENCE OF TLR9 ASSOCIATED WITH INCREASED IMMUNOPATHOLOGY EARLY DURING *P. BRASILIENSIS* INFECTION

In view of the key protective role observed for TLR9 during the early stages of *P. brasiliensis* infection, we next investigated several parameters that could be associated with the premature death of TLR9$^{-/-}$ infected animals. Since during the initial period of infection (the first 48h) differences in the lungs of infected animals are difficult to assess, we performed a histological analysis of the liver of the infected animals. We found that whereas the livers of infected WT animals showed a normal structure, those of TLR9$^{-/-}$ hosts presented small areas of granulocytes/neutrophil infiltrates and of necrosis (Fig. 3).

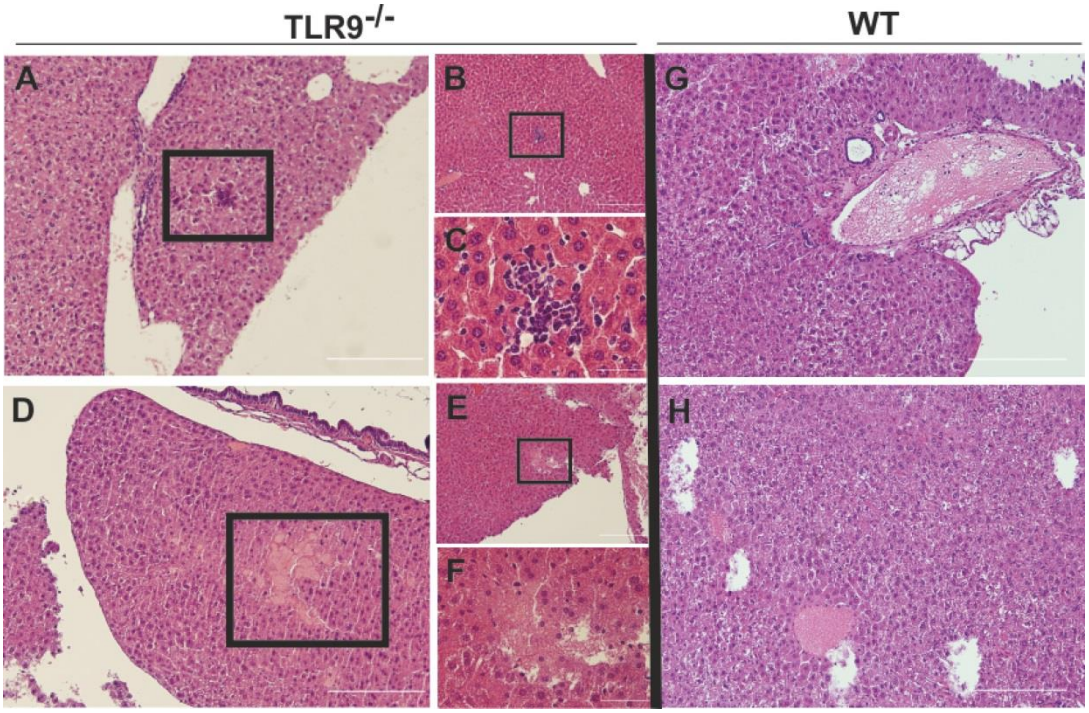

Figure 3 –Histological sections of liver stained with HE from TLR9$^{-/-}$ C57BL/6 mice (A to F) and WT C57BL/6 mice (G and H) intravenously infected with 1x10^6 wild-type *P. brasiliensis* ATCC 60855 yeast cells grown to the exponential phase. Magnification: x10 (A, B, D, E, G and H); x40 (F) and x60 (C). Squares represent either tissue with inflammatory infiltrates (A and B) or with necrotic areas (D and E). Figures C and F represent magnifications of B and E, respectively. Results are from one representative experiment of two independent experiments. White bars represent 200 μm.

To dissect further if the histological differences observed were associated to the intensity of the immune response between WT and TLR9$^{-/-}$ animals, we assessed cytokine expression in the liver and blood of infected animals. In the liver, the expression of both pro- and anti-inflammatory cytokines, namely TNF-α, IL-6 and IL-10, was increased in the absence of TLR9 (Fig. 4A-E). Likewise, a significant increase of circulating levels of IL-6 was observed in TLR9$^{-/-}$-infected mice (Fig. 4G). Moreover, a trend towards higher levels of circulating TNF-α was detected (Fig. 4F) whereas those of IL-10 were below detection limit (data not shown).

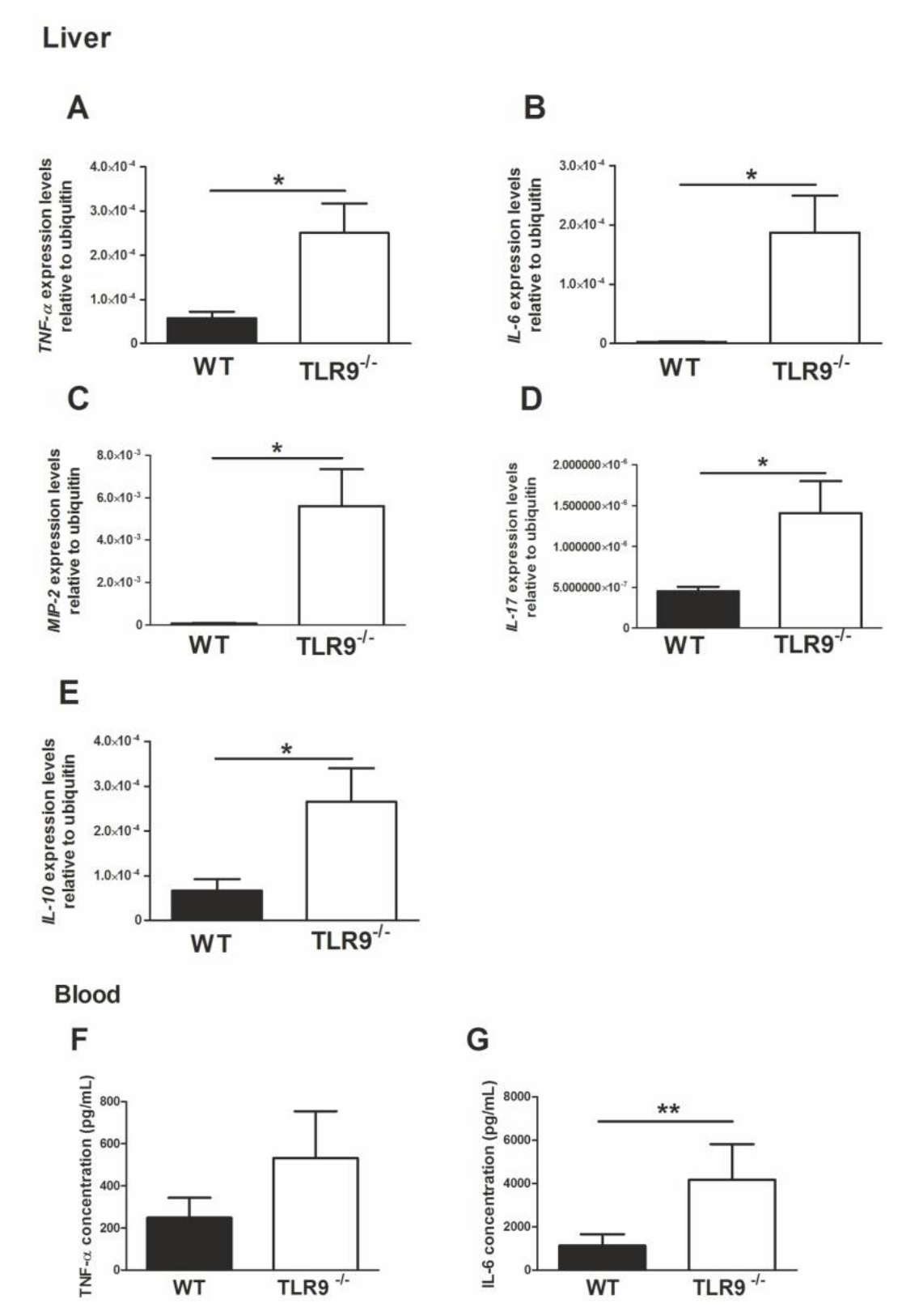

Figure 4 – Cytokine levels detected upon intravenous infection of WT and TLR9$^{-/-}$ C57BL/6 mice (n=21) with 1x10^{6} wild-type *P. brasiliensis* ATCC 60855 yeast cells grown to the exponential phase. Samples were taken from dying mice during the first 48 h post-infection. As control, samples from the same number of healthy WT mice (n=9) were

collected. (A-E) – Expression profile of TNF-α, IL-6, MIP-2, IL-17, and IL-10 at mRNA level in liver from the mice. Asterisks represent significant differences between WT and TLR9$^{-/-}$ mice (*P<0.05); (F and G) - Protein levels of TNF-α and IL-6 detected via ELISA in the blood serum of challenged mice. Asterisks represent significant differences between WT and TLR9$^{-/-}$ mice (**P<0.01). There is also a trend towards higher TNF-α levels in TLR9$^{-/-}$ mice, though no statistically significant differences were detected. Bars represent means and standard deviations.

To further validate if the absence of TLR9 correlated with an exacerbated immune response to *P. brasiliensis*, and to study differential patterns of cellular recruitment in WT versus TLR9$^{-/-}$ mice, we used a model of i.p. infection The analysis of the peritoneal exudates revealed that the cytokine expression was increased in the absence of TLR9 (Fig. 5A-E). Of notice, a marked increased expression of both MIP-2 and IL-17, known to be associated with neutrophil recruitment (Ohtsuka, Lee et al. 2001; Kolls and Linden 2004; Burdon, Martin et al. 2005), was found in the absence of TLR9 (Fig. 5C, D). Consistently, in TLR9$^{-/-}$ mice we found an increased recruitment to the peritoneal cavity of both mononuclear cells (macrophages and dendritic cells) and neutrophils, with a special significance of the latter ones (Fig. 5F-H). Thus, the data obtained for peritoneal infection recapitulates that obtained for intravenous infection.

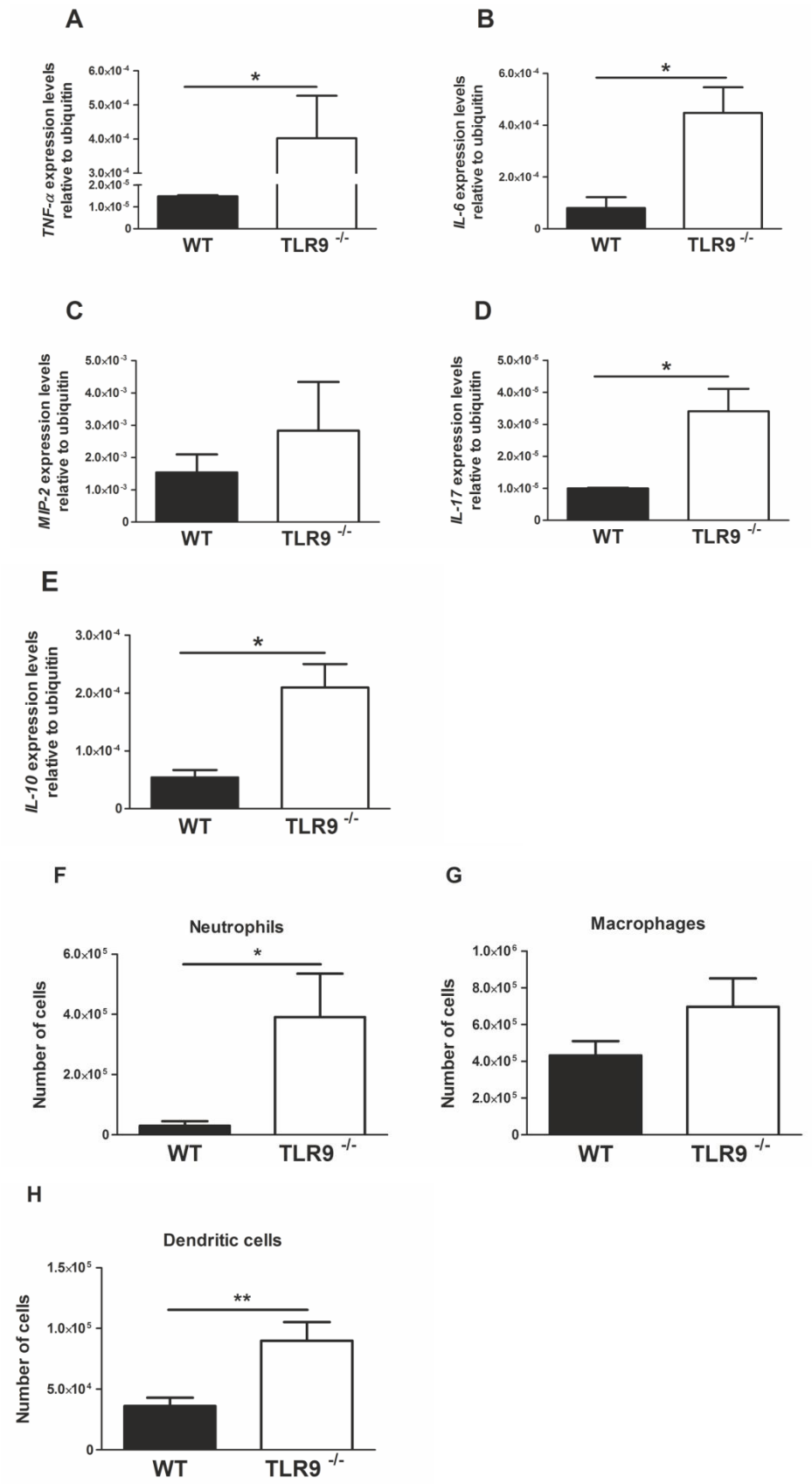

Figure 5 – Outcome of intraperitoneal infection of WT and TLR9$^{-/-}$ C57BL/6 mice (n=10) with 1x10^6 *P. brasiliensis* yeast cells grown to the exponential phase. (A-E) - Expression profile of TNF-α, IL-6, MIP-2, IL-17, and IL-10 at mRNA

level in cells collected from the peritoneal cavity of WT and TLR9$^{-/-}$ mice. Peritoneal lavages were performed 6 h after infection and gene expression levels were accessed by RT-PCR. Asterisks represent significant differences between WT and TLR9$^{-/-}$ mice (*P<0.05); (F-H) - Influx of neutrophils and mononuclear cells to peritoneal cavities of WT and TLR9$^{-/-}$ mice. Peritoneal lavages were performed 6 h after infection, and total and differential leukocyte counts were done. An average of $2.1x10^6 \pm 1.4x10^6$ cells was collected. Asterisks represent significant differences between WT and TLR9$^{-/-}$ mice (*P<0.05, **P<0.01). Bars represent means and standard deviations.

NEUTROPHILIA MEDIATES THE EARLY DEATH OF *P. BRASILIENSIS*-INFECTED TLR9$^{-/-}$ MICE

Considering the high influx of neutrophils to the peritoneal cavity of TLR9$^{-/-}$ mice, which correlated with the high expression of MIP-2 and IL-17 found in the liver and peritoneal cavity, we next evaluated if this could be the detrimental factor during the infectious process of TLR9$^{-/-}$ hosts. For this purpose, we depleted neutrophils in WT and TLR9$^{-/-}$ mice prior to infection with *P. brasiliensis*, using a specific monoclonal antibody (MAb RB6-8C5). I.p. injection of MAb RB6-8C5 abrogated neutrophils from 6 h to up to 48 h post-injection (data not shown, (Appelberg, Castro et al. 1994)). As shown in figure 6, depletion of neutrophils reverted the highly susceptible phenotype observed in TLR9$^{-/-}$ mice during the first two days of infection. Depletion of neutrophils in WT mice had no influence on the mice survival during the first 48 h post-infection (Fig. 6). Therefore, a high neutrophil recruitment to the site of infection appears to be responsible for the death observed in the absence of TLR9 during the early stages of *P. brasiliensis* infection.

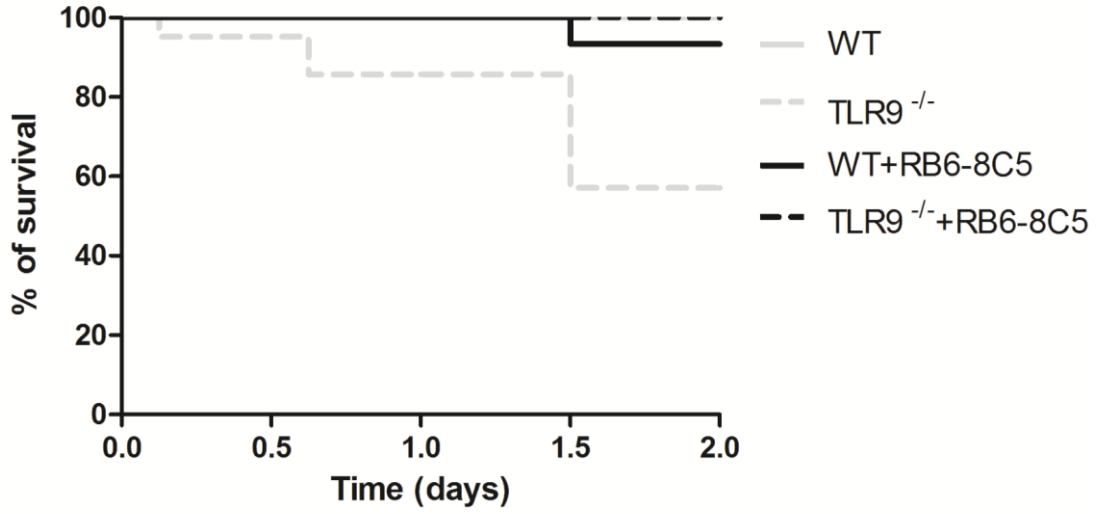

Figure 6 – Outcome of *in vivo* intravenous infection of WT and TLR9$^{-/-}$ neutropenic mice with *P. brasiliensis*. Representative survival curves of an experimental intravenous infection carried out in WT and TLR9$^{-/-}$ C57BL/6 mice

(n= 21) with $1x10^6$ wild-type *P. brasiliensis* ATCC 60855 yeast cells grown to the exponential phase. Mice were treated i.v. with the neutrophil-depleting MAb RB6-8C5 6 h pre-infection. Grey lines represent the survival curve of the experimental intravenous infections carried out in WT and $TLR9^{-/-}$ C57BL/6 mice (n= 21) with $1x10^6$ wild-type *P. brasiliensis* ATCC 60855 yeast cells grown to the exponential phase.

DISCUSSION

Since the discovery of TLRs and their major role in host recognition of conserved molecular structures from microorganisms, particularly those of invading pathogens, enormous advances have been made in comprehending how the immune system responds to pathogenic organisms. It is well established that the first phase of an immune response involves innate immune mechanisms (Beutler 2004), namely those triggered by TLR activation (Janeway and Medzhitov 2002; Levitz 2010; Romani 2011). These receptors are present in neutrophils, monocytes and macrophages, cells that besides their phagocytic activity are crucial for the signaling and amplification of the response against the pathogen (van de Veerdonk, Kullberg et al. 2008).

The knowledge on PRR recognition and activation of an efficient immune response against *P. brasiliensis* has progressively increased, mainly in what concerns TLR2, TLR4, and Dectin-1 (Calich, Pina et al. 2008; Bonfim, Mamoni et al. 2009). However, a role for TLR9 during *P. brasiliensis* infection has only been addressed in the context of vaccination (Amaral, Garcia et al. 2005), despite the evidences for the involvement of this TLR in other fungal infections (Nakamura, Miyazato et al. 2008; Ramirez-Ortiz, Specht et al. 2008; van de Veerdonk, Netea et al. 2008; Wang, Lee et al. 2011). Considering the high DNA content and number of unmethylated CpG oligonucleotides of this multinucleated fungus (Souza, Correa et al. 2001), a role for this receptor during *P. brasiliensis* infection is likely. We herein demonstrate that *P. brasiliensis* purified DNA activated TLR9 in macrophages, leading to the expression of cytokines. This is in line with previous studies showing that fungal DNA is recognized by TLR9. *A. fumigatus* DNA stimulates the production of pro-inflammatory cytokines in mouse and human dendritic cells (Ramirez-Ortiz, Specht et al. 2008). Similarly, murine dendritic cells express IL-12p40 and CD40 upon stimulation with DNA from *C. neoformans* (Nakamura, Miyazato et al. 2008). Human monocytes and macrophages from $TLR9^{-/-}$ mice were described to produce less IL-10 than cells from control mice when stimulated with *C. albicans* (van de Veerdonk, Netea et al. 2008).

Despite TLR9 activation by purified *P. brasiliensis* DNA, upon macrophage stimulation with *P. brasiliensis* yeast cells grown to the exponential phase, TLR9 was activated in a lesser extent.

This finding is likely due to the low exposure of DNA in this case, as when yeast cells on late stationary phase or lysed cultures were used, TLR9 recognized *P. brasiliensis* more prominently, leading to the production of pro-inflammatory cytokines by macrophages. Even though intact *P. brasiliensis* yeast cells did not fully activate TLR9 to induce the production of pro-inflammatory cytokines, in the absence of this receptor macrophages showed a decreased ability to phagocyte *P. brasiliensis*. Our findings are in line with previous studies showing that *P. brasiliensis* DNA activates macrophages, promoting their capacity to phagocyte *P. brasiliensis* (Souza, Correa et al. 2001). Altogether, our data indicate that TLR9 triggering affected the overall responsiveness of macrophages to *P. brasiliensis*. Furthermore, during infection, the release of DNA from *P. brasiliensis* is expected to occur following fungal cell death, thus suggesting that TLR9 activation *in vivo* is very likely to happen.

To assess the role of TLR9 in *P. brasiliensis* infections, we used *in vivo* models of infection. As our results show, TLR9 is crucial for mice survival in early times of infection (first 48 h). Remarkably, we found that, in mice showing severe signs of disease, lack of TLR9 increased the expression of pro-inflammatory cytokines in the liver. Earlier studies on immune responses to other organisms have revealed that an excessive inflammatory response, mainly due to high levels of TNF, can lead to premature death of the host cells (Cerami 1993; Kulkarni, Huh et al. 1993; Rink and Kirchner 1996; Rock and Kono 2008). In the context of *P. brasiliensis* infection, it was demonstrated that an excessive inflammatory response may be detrimental rather than protective. A more severe disease development in mice susceptible to *P. brasiliensis* was associated with the presence of increased IL-12 and IFN-γ levels in the lungs, suggesting that the production of pro-inflammatory mediators does not always correlate with immunoprotection (Gonzalez, Sahaza et al. 2003). In addition to high TNF expression, we also found increased expression of MIP-2 and IL-17 in the liver and cells from the peritoneal cavity of *P. brasiliensis*-infected TLR9$^{-/-}$ mice. A detrimental role of IL-17 during *P. brasiliensis* infection was previously described as TLR2 and TLR4 deficiency associate with an increase of Th17 responses, lung pathology and more severe forms of infection (Loures, Pina et al. 2009; Loures, Pina et al. 2010). Both IL-17 and MIP-2 have been previously associated with neutrophil recruitment (Kolls and Linden 2004; Burdon, Martin et al. 2005; Henningsson, Jirholt et al. 2010; Pelletier, Maggi et al. 2010). In line with this, we observed a high influx of granulocytes/neutrophils into the peritoneal cavity of TLR9$^{-/-}$ i.p. infected animals. This enhanced neutrophil recruitment could thus be contributing to the detrimental response observed in *P. brasiliensis*-infected TLR9$^{-/-}$ animals.

Indeed, upon transient neutrophil depletion, TLR9$^{-/-}$ mice survived during the first 48 hours post-infection, resembling the phenotype of WT mice. Therefore, the susceptibility profile observed for TLR9$^{-/-}$ mice early after infection with *P. brasiliensis* likely associates with an exacerbated neutrophil recruitment to the site of infection and/or with a particular detrimental phenotype of neutrophils. Although neutrophils are crucial during acute inflammatory response and subsequent resolution of fungal infection, in some situations, due to excessive release of oxidants and proteases, these cells may be responsible for injury to organs and fungal sepsis (Bellocchio, Moretti et al. 2004; Zelante, De Luca et al. 2007). In addition to fungal infection, neutrophilia can also be harmful to the host in the context of other infections, such as *M. tuberculosis* and *P. aeruginosa* (Ras, Theron et al. 1992; Eruslanov, Lyadova et al. 2005; Cruz, Fraga et al. 2010; Lowe, Redford et al. 2012).

Our data implicating TLR9 in the phagocytic activity of macrophages raises the hypothesis that, in the absence of TLR9, less *P. brasiliensis* cells are phagocytized. Thus, in the absence of this receptor, the extracellular *P. brasiliensis* cells may contribute to an exacerbated recruitment of neutrophils, which can result in a deregulated immune response. Since a significantly higher recruitment of dendritic cells and neutrophils is observed upon infection of TLR9$^{-/-}$ hosts, it is possible that the higher cytokine expression observed in this scenario results from the fact that more producing cells are present. Therefore, a fine tuned balance is required during infection with *P. brasiliensis*, in order to protect the host from infection. Several mechanisms must operate to achieve this balance, as is the case reported for TLR2/TLR4 activation (Loures, Pina et al. 2009). It is also important to refer that *P. brasiliensis* can be triggering TLR9-independent mechanisms in macrophages, as supported in the literature (Takaoka, Wang et al. 2007; Ishii, Kawagoe et al. 2008). One cannot rule out the hypothesis of a parallel activation of other receptors together with TLR9. Several studies refer that Dectin-1 interaction with TLR9 results in a sinergistyc induction of IL-10, TNF-α, IL-2, IL-6 and IL-23 and down-regulation of IL-12 (Dennehy, Ferwerda et al. 2008; Gerosa, Baldani-Guerra et al. 2008). Studies with *A. fumigatus* reported a link between TLR2-mediated recognition and the phagocytic response (Luther, Torosantucci et al. 2007), whereas internalization of TLR2 with *A. fumigatus* phagossome was demonstrated (Chai, Kullberg et al. 2009). As it has been shown for TLR2/TLR4, it is also possible that TLR9 signaling is involved in the instruction of appropriate regulatory T cell responses, a hypothesis currently under investigation.

Overall, this study highlights the relevant role of TLR9/neutrophils for the nature of the immune response to *P. brasiliensis*, and paves the way to the development of new preventive/therapeutical strategies, as resistance patterns of the host can be of great value on the comprehension of susceptibility and pathogenesis of *P. brasiliensis* infections.

CHAPTER 5

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Fungi are a group of microorganisms that, in a vast majority, interact with humans without causing any kind of disease. However, there are certain groups of fungi that are opportunistic pathogens (e.g. *Candida* and *Aspergillus* species), usually causing disease in immunocompromised hosts, and those that are naturally pathogenic, causing disease even in immunocompetent host (e.g. *Histoplasma capsulatum*, *Coccidioides immitis* and *Paracoccidioides brasiliensis*). Among this last group of organisms, some share important features. Fungi such as *H. capsulatum*, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* possess particular morphological characteristics and an inherent ability to change from the multicellular filamentous form to a unicellular form during the infectious process.

P. brasiliensis is a thermodimorphic fungus and a causative agent of paracoccidioidomycosis, an endemic mycosis affecting population from Latin America countries such as Brazil, Colombia and Venezuela (Brummer, Castaneda et al. 1993; Restrepo, McEwen et al. 2001). The infective process comprises a temperature-dependent morphological switch of the fungus from the conidia/mycelial phase at environmental temperatures (below 26°C) to the pathogenic yeast phase at the mammalian host temperature (around 37°C) (Brummer, Castaneda et al. 1993). The pathogenic yeast phase is characterized by its polymorphic nature, highly heterogeneous in and among the different clades (Brummer, Castaneda et al. 1993). This morphological heterogeneity can be an important aspect for determining the virulence profile of *P. brasiliensis* species, a question that was never addressed. On the other hand, the existing data on the processes governing the fungus dimorphism result mainly from transcriptome studies, whereas a functional approach targeting genes that could control dimorphic transitions in *P. brasiliensis* was never performed. This fact is mainly due to the absence of molecular tools to create knock-out mutants in *P. brasiliensis*, the most commonly used approach to study gene function in fungi. This has been a crucial limiting factor for a better understanding of the fungus biology, and for the uncovering of new virulence factors. Such data on *P. brasiliensis* genetics and biology is essential for the development of prophylactic or therapeutic strategies. In line with these observations, it is also imperative to further clarify how host immune-responses are triggered upon recognition of *P. brasiliensis*, an essential aspect for determining the outcome of infection.

Throughout the years, phylogenetic analyses have been used in order to discriminate *Paracoccidioides* clinical and environmental isolates, according to their genetic background, morphological and biochemical characteristics (Matute, McEwen et al. 2006; Teixeira, Theodoro

et al. 2009; Desjardins, Champion et al. 2011). However, despite the fact that the *P. brasiliensis* gene *CDC42* (a Rho-like GTPase encoding gene) is known to control the morphological behavior of the fungus (Almeida, Cunha et al. 2009), a systematic analysis of the morphological traits exhibited by *Paracoccidioides* isolates and their correlation with genetic determinants was never performed. In order to shed light on this matter, we carried out a detailed morphogenetic evaluation of the yeast-forms of different clinical and environmental *Paracoccidioides* isolates comprising all the phylogenetic lineages from the different species (S1, PS2, PS3 and *P. lutzii*) (Chapter 2). We show that there is a lack of a clear characteristic morphologic profile for any of the phylogenetic groups. Moreover, data is presented revealing that the bud size and shape in all isolates is highly dependent on the mother cell morphology. Importantly, a strong correlation between expression of *PbCDC42* and both the shape of mother and bud cells and the size of the buds was demonstrated for all isolates, suggesting that *PbCDC42* expression can explain approximately 80% of mother and bud cell shape and 19% of bud cell size. The morphogenetic correlations herein presented can be of utmost importance, as previous studies reveal that induced-reduction of *PbCDC42* expression levels weakens the pathogenicity of the fungus (Almeida, Cunha et al. 2009). As a future approach, a more broader and detailed analysis on *PbCDC42* expression profiles of all the isolates, belonging to the different clades, can be interesting, on a perspective of stratification of *Paracoccidioides* strains according to their virulence and levels of *PbCDC42* expression.

In dimorphic fungi such as *H. capsulatum*, temperature and nutritional factors are accountable for triggering dimorphic transitions. However, in *P. brasiliensis*, temperature shift is the only known requirement for the dimorphic transition to the yeast pathogenic phase from the conidia/mycelial phase (Brummer, Castaneda et al. 1993). Studies on the genetic profiles exhibited by each of these morphological forms during the transition process indicate that several genes are differentially expressed (Felipe, Andrade et al. 2005; Nunes, Costa de Oliveira et al. 2005; Andrade, Paes et al. 2006). Moreover, the differential sulfur requirement between conidia/mycelium and yeast cells determines growth rate followed by each of this forms and the possibility to undergo transition. In order to unravel mechanisms other than temperature governing conidia/mycelium-to-yeast transition in *P. brasiliensis*, we addressed the role of *PbSCONC,* the negative regulator of the inorganic sulfur assimilation pathway (Marzluf 1997), in the dimorphism and virulence of this pathogen (Chapter 3). Our data further support that *P.*

brasiliensis auxotrophy for organic sulfur compounds in the yeast form is due to *SCONC*, a phenomenon that can be overcome by modulating its genetic expression. Furthermore, we provide evidences that by down-regulating *SCONC*, *P. brasiliensis* is able to metabolize inorganic sulfur compounds in the yeast phase. Taking advantage of the *Agrobacterium tumefaciens*-Mediated Transformation (ATMT) system, we successfully down-regulated *SCONC* by antisense-RNA in unrelated clinical isolates from *P. brasiliensis* species and in one isolate from *P. lutzii* species. We were able to promote mycelium-to-yeast transition in the absence of organic sulfur compounds in the *SCONC* down-regulated clones, whereas the wild-type counterparts were not able to accomplish transition in such conditions. As a consequence of down-regulating *SCONC* expression, the ATP and NADPH cellular pools decreased, probably due to their usage in the inorganic sulfur assimilatory pathway. These alterations led to a decreased availability of energy for cellular metabolic processes, and increased cellular oxidative stress levels. These imbalances impacted on the down-regulated clones' growth, since they significantly decreased their biomass yield in cultures with both organic and inorganic sulfur. As a consequence of these metabolic alterations, the virulence of the down-regulated clones decreased. The data herein presented associates sulfur metabolism with the dimorphic transition, and implicates SconC as a virulence factor, since that by modulating *SCONC* expression the fungus virulence was abrogated.

The understanding of the fungal nutritional requirements could further clarify how metabolic processes are triggered/blocked during the transition process. This would allow the identification of new genetic targets, to test using the available ATMT-based aRNA methodology. Further clarifications on the sulfur requirements of *P. brasiliensis* should be presented. The usage of radioactive-labeled sulfur isotopes can represent an interesting approach to evaluate the routes that inorganic sulfur follows either in the mycelial and yeast phases.

Besides characterizing *P. brasiliensis* morphology, and the impact of sulfur metabolism in the fungus dimorphism and virulence, we were also interested in further elucidating the host immune responses to *P. brasiliensis*. Considering the multinucleated nature of *P. brasiliensis*, and since upon cell death significant amounts of DNA are expected to be released, we addressed the role of TLR9, a pattern recognition receptor (PRR) known to recognize CpG motifs in DNA (Rutz, Metzger et al. 2004) (Chapter 4). Our results show that TLR9 is able to recognize DNA and the pathogenic yeast form of *P. brasiliensis*, leading to expression/production of pro-inflammatory cytokines, as demonstrated by an *in vitro* model of infection with bone marrow derived

macrophages. We further show that TLR9 signaling is involved in regulating phagocytosis, since lack of this receptor leads to a four-fold decrease in the phagocytic capacity of macrophages. Additionally, the role of TLR9 as an immuno-protective player in early stages of infection with *P. brasiliensis* was revealed, since TLR9$^{-/-}$ mice intravenously infected with *P. brasiliensis* yeast cells died at higher rates during the first 48 hours post-infection, when compared to wild-type mice. We were able to demonstrate that this impaired survival is accompanied by tissue damage and increased expression of several cytokines, such as TNF-α and IL-6. This same trend was observed upon intraperitoneal infection, where a high recruitment of neutrophils and mononuclear cells to the site of infection was detected. Our data support the hypothesis that the death profile presented by TLR9$^{-/-}$ mice in early-times of infection is mainly due to the host detrimental high recruitment of neutrophils to the site of infection, since neutrophil depletion previous to infection results in increased resistance of mice to *P. brasiliensis* infection.

It is of major importance to further dissect how the host develops an efficient immune response against *P. brasiliensis*, specifically by clarifying the role played by other TLRs during infection. A possible synergistic cooperation between TLR9 and other TLRs is an important subject to be addressed, in order to understand the mechanistic processes underlying exacerbated inflammatory responses, which can be detrimental to the host.

The development of case-control studies would also be interesting, in the perspective of looking for associations between host genetic factors and the disease, ultimately allowing the risk stratification of individuals to paracoccidioidomycosis.

In summary, the work herein presented improves our knowledge on the genetic and physiological determinants that govern morphological traits and dimorphic transition in *P. brasiliensis.* Yeast cell morphology is revealed to be correlated to expression of *PbCDC42,* while novel data is presented on how cellular sulfur metabolism can affect *P. brasiliensis* dimorphism, via what can be considered a novel virulence determinant, SconCp. Furthermore, data is presented unraveling the role of TLR9 during *P. brasiliensis* infection as part of the protective immune mechanisms, at least early after infection, developed by the host against infection with the fungus.

The work presented here adds knowledge on:

(i) the morphological trends presented by *P. brasiliensis* species;

(ii) the genetics underlying *P. brasiliensis* heterogeneity;

(iii) *P. brasiliensis* requirements to undergo dimorphic transitions;

(iv) the differential sulfur requirements of *P. brasiliensis*;

(v) the unraveling of new virulence determinants;

(vi) host responses to *P. brasiliensis*.

Taken together, this data further clarifies the physiology and genetics of *P. brasiliensis*, also elucidating host responses upon infection. This can be relevant for the uncovering of new putative genetic determinants accountable for *P. brasiliensis* pathogenicity, which could ultimately lead to the development of novel therapeutic strategies.

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