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Evaluation of immune-determinants associated with resistance/susceptibility to *Paracoccidioides brasiliensis*: a mouse model perspective

Avaliação dos determinantes imunes associados à resistência/susceptibilidade a *Paracoccidioides brasiliensis*: uma perspectiva do modelo de ratinho

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e do
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ABSTRACT

Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis, one of the most prevalent systemic mycosis in Latin America. Infection by this fungus is thought to be initiated by the inhalation of conidia produced by the saprophytic phase of the fungus. Although many aspects of the host immune response against this pathogen are still unknown, several studies have suggested that macrophages play a key role in the defence against *P. brasiliensis*. Therefore, our aim is to characterize the macrophage response against strains of *P. brasiliensis* with different degrees of virulence in order to define the mechanisms required to control infection. To this end, we have established an *in vitro* model of bone marrow-derived macrophages (BMDM) infection. These cells were infected with *P. brasiliensis* 18, a highly virulent strain, or *P. brasiliensis* ATCC60855, a low virulent strain. Here, we show that *P. brasiliensis* morphology, assessed by scanning electron microscopy, has an important impact in the ability of BMDM to internalize *P. brasiliensis*. Indeed, while *P. brasiliensis* ATCC60855*,* a small strain with a reduced bud number, is easily internalized by BMDM, *P. brasiliensis* 18, a larger strain with multiple bud cells, is not efficiently internalized.

To determine the ability of macrophages to control infection, we assessed the ability of BMDM to control *P. brasiliensis* growth. We found that, after infection, stimulation of BMDM with interferon (IFN)-γ was not sufficient to activate the production of nitric oxide (NO), a key antimicrobial mediator, and to control *P. brasiliensis* growth. To define the mechanisms underlying this deficient activation in response to this pathogen, we measured the levels of several cytokines known to activate macrophages and we found that there was a reduced production of tumour necrosis factor (TNF), which was more evident after *P. brasiliensis* 18 infection. Indeed, it was only after stimulation of BMDM with both IFN-γ and lipopolysaccharide (LPS), a potent inducer of TNF, that these cells were able to produce large amounts of nitric oxide and to control infection. Therefore, our data suggest that *P. brasiliensis* modulates the signalling pathway that leads to the production of TNF to inhibit the activation of the macrophages fungicidal mechanisms. We also found that the reduced production of TNF by BMDMs is not mediated by interleukin (IL)-10, as both *P. brasiliensis* strains induced the same levels of IL-10. However, *in vivo* studies suggest that high levels of IL-10 during infection can be detrimental to the host by modulating the dynamics of the inflammatory response, stressing the importance of the regulation of this cytokine in the balance between protection and pathology during *P. brasiliensis* infection.

Altogether, our data support a model whereby the different induction of TNF by *P. brasiliensis* strains with different degrees of virulence may underlie the different forms or severity of the disease.

RESUMO

Paracoccidioides brasiliensis é o agente etiológico da paracoccidioidomicose, uma das micoses sistémicas mais prevalentes na América Latina. Pensa-se que a infecção por este fungo é iniciada aquando da inalação de conídios produzidos pela fase saprófita do fungo. Embora vários aspectos relacionados com a resposta imune do hospedeiro ainda sejam desconhecidos, estudos sugerem que os macrófagos têm um papel essencial na defesa contra *P. brasiliensis*. O nosso objectivo é caracterizar a resposta dos macrófagos contra estirpes de *P. brasiliensis* com diferentes graus de virulência de forma a definir os mecanismos necessários para controlar a infeção. Para isso, estabelecemos um modelo *in vitro* de infecção de macrófagos derivados da medula-óssea (BMDM). Estas células foram infectadas com *P. brasiliensis* 18, uma estirpe altamente virulenta, ou com *P. brasiliensis* ATCC60855, uma estirpe considerada menos virulenta. Através de uma análise por microscopia eletrónica de varrimento, observámos que a morfologia de *P. brasiliensis* tem um grande impacto na internalização deste fungo pelos BMDM. De fato, enquanto que *P. brasiliensis* ATCC60855, uma estirpe com células de dimensão reduzida e rodeadas por poucas células-filha, é mais facilmente internalizada, a estirpe *P. brasiliensis* 18, que é caracterizada por células de maiores dimensões e rodeadas numerosas células-filhas, dificilmente é internalizada. Para determinar a capacidade dos macrófagos para controlar a infecção, avaliamos a capacidade dos BMDM para controlar o crescimento de *P. brasiliensis*. Descobrimos que, após a infeção, a estimulação destes macrófagos com interferão (IFN)-γ não foi suficiente para activar a produção de óxido nítrico, um mediador chave na resposta microbicida dos macrófagos, nem para controlar o crescimento de *P. brasiliensis*. Para definir os mecanismos subjacentes a esta activação deficiente, medimos várias citoquinas que são produzidas em resposta â activação de macrófagos e observamos uma diminuição na produção de fator de necrose tumoral (TNF) que foi mais evidente após a infecção com *P. brasiliensis* 18. Assim, só depois da estimulação dos BMDM com IFN-γ e LPS, um potente indutor de TNF, é que estas células foram capazes de produzir grandes quantidades de óxido nítrico e de controlar a infecção. Desta forma, os nossos dados sugerem que *P. brasiliensis* modula a via de sinalização que leva à produção de TNF de forma a inibir a activação dos mecanismos fungicidas dos macrófagos. Também observamos que a produção de TNF não é mediada pela interleuquina (IL)-10, já que ambas as estirpes produzem as mesmas quantidades de IL-10 Contudo, estudos *in vivo* mostram que a IL-10 tem um impacto negativo na dinâmica da resposta inflamatória, o que demonstra a importância da regulação desta citoquina no balanço entre a inflamação e a patologia. Estes dados suportam um modelo no qual a indução de TNF

por estirpes de *P. brasiliensis* com diferente virulência pode ser a base das diferentes formas ou severidade da doença.

TABLE OF CONTENTS

FIGURE INDEX

ABBREVIATIONS

˚C- Degree Celsius
µg- Microgram
µL- Microliter
AG- Aminoguanidine hemisulfate
APC- Antigen presenting cell
ATCC- American Type Culture Collection
ATP- Adenosine-5'-triphosphate
BHI- Brain heart infusion
BMDM- Bone marrow-derived macrophages
BSA- Bovine serum albumin
cDNA- Complementaty deoxyribonucleic acid
CLR- C-type lectin receptor
CO_2 – Carbon dioxide
DCs- Dendritic cells
DC-SIGN- Dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)
DMEM- Dulbecco's Modified Eagle Medium
DNA- Deoxyribonucleic acid
ECM- Extracellular matrix
ELISA- Enzyme-Linked Immunosorbent Assay
FBS- Fetal bovine serum
FITC- Fluorescein isothiocyanate
FoxP3- Forkhead box P3
GTP- Guanosine-5'-triphosphate
H&E- Hematoxylin and eosin
H_3PO_4 – Phosphoric acid
HIV- Human immunodeficiency virus
HRP- Horseradish peroxidase
Hsp- Heat shock protein
IFN-γ- Interferon-gamma
IL- Interleukin
iNOS- Inducible nitric oxide synthase
IRF- Interferon regulatory factor
LCCM- L-929 conditioned medium
LPS- Lipopolysaccharide
MHC- Major histocompatibility complex
MIP2- Macrophage inflammatory protein 2-alpha
mL- Milliliter
mM- Millimolar
MOI – Multiplicity of infection
mRNA- Messenger ribonucleic acid
MT- Sheep metallothionein
MyD88- Myeloid differentiation primary-response protein 88
NF-κB- Nuclear factor kappa-light-chain-enhancer of activated B cells
NK- Natural killer
ng- Nanogram
NO- Nitric oxide
NOS2- Nitric oxide synthase II
PAMP- Pathogen-associated molecular patterns
PAS- Periodic acid-Schiff stain
PBS- Phosphate buffered saline
PCM- Paracoccidioidomycosis
PCR- Polymerase chain-reaction
PE-Phycoerythrin
PFA - Paraformaldehyde solution
pg - Picogram
PMNs- Polymorphonuclear neutrophils
PRR- Pattern recognition receptor
RNA- Ribonucleic acid
RNI- Reactive nitrogen intermediate
ROS- Reactive oxygen species
RT- Room temperature
TGF-β- Transforming growth factor β
Th - T-helper
TIR - Toll interleukin receptor
TLR- Toll-like receptor
TMB- 3,3′,5,5′-tetramethylbenzidine
TNF- Tumour necrosis factor
Treg- Regulatory T-cell
WT- Wild type
Zn- Zinc

CHAPTER I

INTRODUCTION

1.1 *PARACOCCIDIOIDES BRASILIENSIS* BIOLOGY

1.1.1 Phylogeny and cryptic speciation

Morphological and phylogenetic studies together with the recent development of molecular tools place *Paracoccidioides brasiliensis* in the phylum Ascomycota, order Onygenales and family Ajellomycetacea, a fungal group common to most agents of systemic mycosis, including *Bastomyces dermatitidis, Coccidioides immitis, Coccidioides posadassii* and *Histoplasma capsulatum* (Bagagli, Theodoro et al. 2008).

In classic systematics, *P. brasiliensis* is considered to be an imperfect fungus due to lack of sexual structures and a teleomorphic phase, despite the high genetic variability among different isolates (Montoya, Moreno et al. 1997, Bagagli, Theodoro et al. 2008). Indeed, through the analysis of eight regions from five nuclear coding genes, Matute *et al.* found that *P. brasiliensis* is stratified in at least three distinct phylogenetic species: i) species 1 (S1) is paraphyletic and found in Brazil, Argentina, Paraguay, Peru and Venezuela; ii) phylogenetic species 2 (PS2) is monophyletic and found in Brazil and Venezuela; and iii) phylogenetic species 3 (PS3), also monophyletic, is found in Colombia (Figure 1). Isolates belonging to S1 and PS2 are sympatric and recombinant, whereas those belonging to PS3 are allopatric and clonal (Matute, McEwen et al. 2006). Teixeira *et al.* suggested the existence of 17 genotypically similar isolates, including *P. brasiliensis* 01, which were distinct from the three phylogenetic species described above. In this regard, these authors proposed a new "Pb01-like-cluster" as a new *Paracoccidioides* species named *Paracoccidioides lutzii*. Isolates of the *P. lutzii* species are endemic to the Brazilian Central-Western region and differ from *P. brasiliensis* species in its virulence, resistance to fungicides and proliferation (Teixeira, Theodoro et al. 2009).

1.1.2 Morphological characteristics

The morphological transformation of *P. brasiliensis* is based in the existence of two distinct morphological forms. At temperatures below 25°C (in the environment), the most common form is the mycelium/conidial (Figure 2A), whereas at temperatures around 37°C the mycelium/conidial form develops into the yeast form (Figure 2B), being the latter the pathogenic form. The mycelium/conidial form is composed by two layers: the outer layer, which is rich in β-1,3-glucan fibrils, and the inner layer, rich in rigid chitin fibrils (Brummer, Castaneda et al. 1993). In conditions of nutrient deprivation and low temperatures, different types of propagules, including arthroconidia or conidia, are formed

(McEwen, Restrepo et al. 1987, Edwards, Salazar et al. 1991). These propagules are thought to be responsible for initiating infection after being inhaled by the host (Brummer, Castaneda et al. 1993).

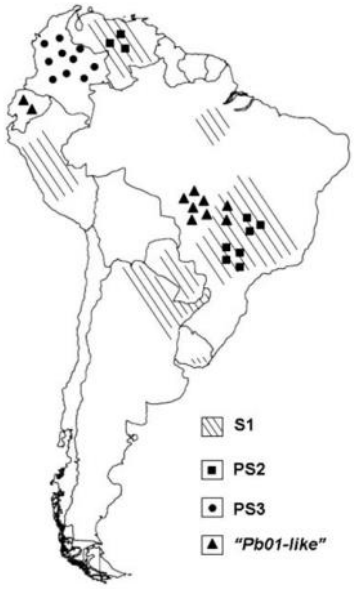

Figure 1. Geographic distribution of the phylogenetic species of *Paracoccidioides* genus. Adapted from Teixeira et al., 2009

During mycelial-to-yeast transition, which is triggered by the temperature, there is a switch in glucan polymer linkage in the cell wall β-1,3-glucan to α-1,3-glucan. Indeed, the yeast form has a cell wall composed by α-1,3-glucan and small amounts of α-1,3- or α-1,6-glycosidic linkages (Kanetsuna, Carbonell et al. 1969). The most characteristic feature of the yeast form is the "pilot wheel" appearance (Figure 2C) that results from multiple budding cell divisions. However, single cells with only one bud, odd-like morphologies and transitional forms (cells arranged in short chains, balloon-shaped or broken cells) are also common. This form is multinucleated, with variable sizes ranging from 4 to 40 μm (Figure 2D) (Brummer, Castaneda et al. 1993).

1.1.3 Ecology

Although the environmental niches of *P. brasiliensis* are still largely unknown, the ecological characteristics of the endemic regions of the Colombian and Brazilian areas suggest that humid environments with rivers and rain forests are ideal for the maintenance of this pathogen in the environment (Bagagli, Franco et al. 2003). *P. brasiliensis* was firstly isolated by Adolfo Lutz from nine-banded armadillo (*Dasypus novemcinctus*), a wild mammal usually common in South America. This pathogen was identified in several organs of these animals, including lymph nodes, liver and lungs,

suggesting active disease (Bagagli, Franco et al. 2003). However, the nature of the relationship between these two organisms is not yet completely understood (Naiff, Ferreira et al. 1986). Recently, *P. brasiliensis* was also detected in the naked-tailed armadillo (*Cabassous centralis*) and feces from fruit-eating bats (*Artibeus lituratus*) from Colombia and penguin excreta from Antarctica (Grose and Tamsitt 1965, Ferreira, Freitas et al. 1990, Giannini, Bueno et al. 1990, Corredor, Peralta et al. 2005).

Although advances have been made to define the ecological niche of *P. brasiliensis*, several environmental conditions, including temperature and the interaction with other soil organisms, remain largely unknown. It is therefore critical to define these conditions, as it will help determining how *P. brasiliensis* grows and disseminates in the host.

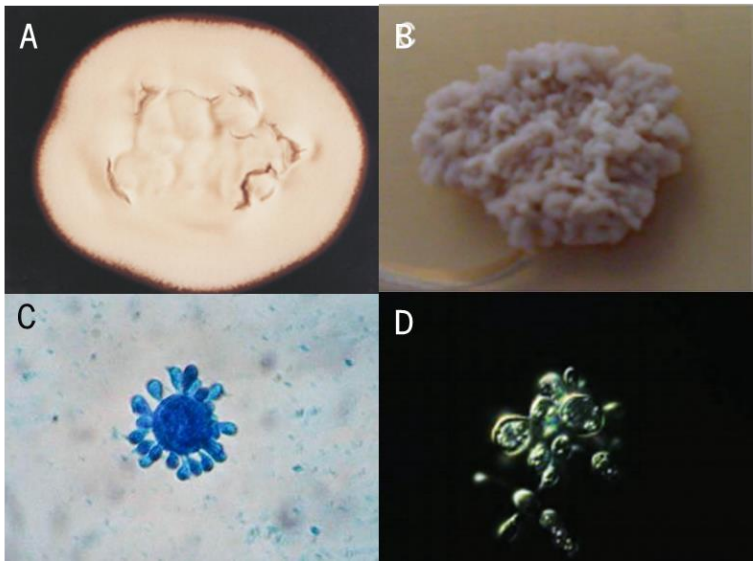

Figure 2. *Paracoccidioides brasiliensis* mycelia and yeast forms. Macroscopic characteristics of mycelial (A) and yeast form (B); Multiple budding cells observed in the yeast form (C); and Differential Interference Contrast (DIC) observation of yeast cells (D). Adapted from Menino, Saraiva et al. 2013

1.2 PARACOCCIDIOIDOMYCOSIS

Paracoccidioidomycosis (PCM) is a systemic endemic mycosis caused by *P. brasiliensis.* During the initial phase of infection, the pathogen affects the lungs subsequently disseminating to the mucosal membranes, skin, and many other organs including the liver and the spleen (Bonifaz, Vazquez-Gonzalez et al. 2011).

1.2.1 Epidemiology: Demographics-Geographical Distribution

PCM is frequently reported in South- and Central-American countries. This disease is considered to be autochthonous from southern Mexico to northern Argentina with higher incidence in Brazil (accounting for more than 80% of the cases), Venezuela and Argentina (Figure 3). However, several cases have also been reported in Chile, Guyana, Belize and Caribbean Islands (Brummer, Castaneda et al. 1993). It has been reported an annual incidence rate of 1-3 per 100000 habitants and a mean mortality of 1.45 per million inhabitants, being the eighth cause of death from chronic infections and parasitic diseases (Calle, Rosero et al. 2001).

PCM affects mainly adults from ages between 30 and 60 years. In children and young adults, PCM is uncommon and indeed only 8% of patients are less than 20 years of age (Benard, Orii et al. 1994, Paniago, Aguiar et al. 2003). In a recent study of 5500 PCM patients, 5045 were male and only 455 were female (male-to-female ratio of 11.1 to 1), showing that gender is an important factor in this fungal disease (Shankar, Restrepo et al. 2011). In this regard, studies performed with experimental models of PCM infection showed that estrogens (17β-estradiol) inhibit *P. brasiliensis* mycelium-to-yeast transition, thus preventing infection (Shankar, Restrepo et al. 2011).

PCM infection is often (approximately 60% of the cases) observed in farmers or rural workers, and therefore it is thought to be associated with inhalation of dust (Calle, Rosero et al. 2001). Indeed, other occupations that are a risk factor for PCM are masonry, bricklaying and mining (Conti-Diaz, Calegari et al. 1979).

Although in some cases the propagules may become dormant, comorbidities including alcoholism, malnutrition, smoking or immunosuppressive diseases, including human immunodeficiency virus, may lead to active disease (Benard and Duarte 2000, Restrepo, Benard et al. 2008).

Figure 3. Geographic distribution of Paracoccidioidomycosis. Blue areas represent regions with high incidence, including Brazil, Venezuela and Colombia. Duane et al., 2013

1.2.2 Pathobiology and clinical forms

According to *in vivo* infections performed by McEwen *et al.,* it is thought that infection by *P. brasiliensis* is initiated by the inhalation of fragments of mycelia or conidia, which are then phagocytosed by alveolar macrophages. The morphological transition to the pathogenic yeast form is started within only a few hours (12-18) followed by dissemination and induction of a progressive disease (McEwen, Bedoya et al. 1987). After this, and depending on host factors and virulence of the strain, the infectious process gives rise to asymptomatic infection or active disease.

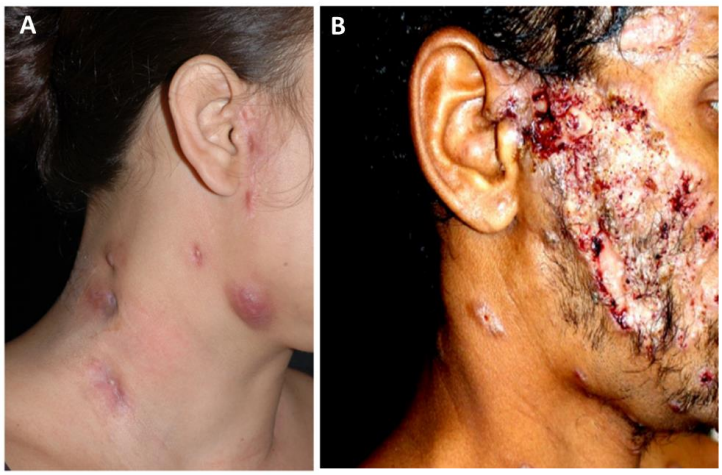

Figure 4. Acute form of PCM. Lymphadenopathy and scars of previous suppurative lymph nodes on the cervical region (A). Ulcerative lesions on face (B). Adapted from Marques, 2012.

The asymptomatic infection (subclinical infection) is only detected by skin-tests and has no clinical manifestations. On the other hand, the active disease is characterized by pulmonary granulomatous lesions, where viable *P. brasiliensis* cells persist (named latent foci). During active disease, dissemination of the fungus can lead to the formation of extrapulmonary latent foci in the liver, spleen, lymph nodes, skin, and adrenal glands (Restrepo, Benard et al. 2008).

The two most common forms of the active disease are the juvenile form and the chronic form. The juvenile form is considered the most severe form of the disease representing only 3 to 5% of all cases. This form is characterized by a rapid course of infection (weeks to months) leading to reticuloendothelial system organ hypertrophy and bone marrow dysfunction, which results in a septicemic episode (Brummer et al., 1993). Fever, weight loss, lymph node enlargement and hepatosplenomegaly are the most common signs and symptoms (Figure 4). There are no clinical or radiological manifestations of this form of the disease in the lungs (de Mattos, Mendes et al. 1991).

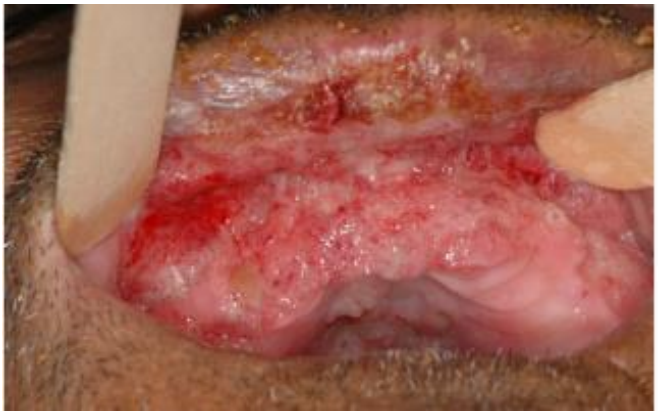

Figure 5. Chronic form of PCM. Ulcerative and infiltrative lesions with multiple hemorrhagic dots and crusts on the upper gingiva and lip. From Marques, 2012.

The chronic form of the disease is more common occurring in more than 90% of the patients, most of them adult males. The disease progresses slowly, taking months or years to become fully established. Unlike the juvenile form, in the chronic form, pulmonary manifestations are evident in 90% of the patients. This form of disease can involve a single organ or system (unifocal) or several organs (multifocal) including lungs, oropharynx, skin, lymph nodes, adrenal glands and the upper respiratory tract. Other organs such as the eyes, central nervous system, bones and testicles are less frequently affected (Figure 5) (Colombo, Faical et al. 1994).

1.2.3 Virulence factors

Virulence factors are described as specific characteristics of a pathogenic microorganism that improve its ability to invade the host, leading to infection (Mendes-Giannini, Taylor et al. 2000).

Over the past few years, several virulence factors of *P. brasiliensis* have been described, including the fungus dimorphism, the adherence to host cells and the cell wall components. San-Blas showed that the cell wall polysaccharides play a crucial role in the protection of the fungus against host mechanisms (San-Blas, 1985). This author observed that, during mycelia-to-yeast transition, the chitin content of the cell wall increases 3-fold, being α-1,3-glucan the most abundant glucoside in the yeast form, while in the mycelial form, the most common glucoside is β-1,3-glucan. Since the reduction of β-glucan was associated with a reduction in virulence, these data suggest an important role of this polysaccharide in the virulence of *P. brasiliensis* (San-Blas, 1985).

The 43 kDa immunodominant glycoprotein Gp43 is expressed by most *P. brasiliensis* strains and has also been described as an important virulence factor (Torres, Hernandez et al. 2013). This protein is considered to be an adhesin that binds to laminin and fibronectin (Vicentini, et al. 1994). Several studies suggest that Gp43 is capable of inhibiting macrophage phagocytosis leading to *P. brasiliensis* dissemination (Popi, Godoy et al. 2008).

Another important virulence factor that has been extensively studied is Cdc42, a protein that belongs to a small Rho-like GTPase family responsible for important biological processes in several organisms (Osmani, Peglion et al. 2010). This protein has a key role as a polarity cue molecule during *P. brasiliensis* growth and morphogenesis. Almeida *et al.* suggested that an 88% reduction in PbCDC42 expression led to a more homogenous cell shape and size, and more importantly, to a significant reduction in *P. brasiliensis* virulence. This was observed upon increased survival after intravenous infection of mice as well as more efficient phagocytosis by bone-marrow derived macrophages (BMDM) (Almeida, Cunha et al. 2009). While these data point to an important role of *P. brasiliensis* cell size and shape in the virulence of the fungus, it has been shown that this pathogen has also developed mechanisms to protect itself against oxidative burst of the host macrophages. Indeed, the production of melanin, a multifunctional polymer ubiquitous to all *P. brasiliensis* strains, protects this pathogen from nitrogen and oxygen intermediate radicals (Taborda, da Silva et al. 2008).

1.2.4 Diagnosis and treatment

The most effective and inexpensive diagnosis method of PCM is the direct examination of clinical samples from bronchoalveolar lavage fluids or the outer edge of ulcers in tissue biopsies. In these samples, *P. brasiliensis* is stained with potassium hydroxide or calcofluor. Other forms of diagnosis are based on the histological examination of tissue after silver methenamine or periodic acid-Schiff staining (PAS) (Del Negro, Pereira et al. 2000). The isolation and growth of *P. brasiliensis* from PCM patients is done in Sabouraud medium, supplemented with antibiotic drugs and mold inhibitors, with an incubation time of 20 days (Brummer et al., 1993).

During the last decade, several methods have been developed to detect *P. brasiliensis*, including polymerase chain reaction (PCR) and real-time PCR. These methods are used to identify DNA in clinical and environmental isolates (Motoyama, Venancio et al. 2000), and serologic reagents to detect Gp43, Gp70 and Hsp70 from *P. brasiliensis* (de Camargo and de Franco 2000).

Until 1946, this disease was considered incurable. However, new therapeutic drugs were discovered and used in patients with PCM, including sulphonamide and amphotericin B. The use of sulphonamide started in 1946 and has several advantages, including low cost and low toxicity. This drug can be administrated for a long period of time (up to 5 years), with an efficacy of about 25%. Amphotericin B, introduced in 1958, is more frequently used due to its efficiency and duration of treatment (6 months to 2 years) (Brummer, Castaneda et al. 1993).

1.3 IMMUNTITY TO *P. BRASILIENSIS* INFECTION

The mammalian immune system is composed by the innate system and the adaptive system (Mogensen 2009). The innate immunity confers the first line of defense to infection and is initiated by the interaction between pathogens and pattern recognition receptors (PRRs). The adaptive immunity, in turn, is specific since it activates effector mechanisms in an antigen-specific manner (Medzhitov 2007).

1.3.1 Innate immunity

Innate immunity is the first line of defense, comprising both chemical elements and cells components of the immune system responsible for defending the host from invading pathogens (Calich, da Costa et al. 2008).

The first line of innate mechanisms are the physical barriers, including skin, mucous membranes and ciliated epithelium of the respiratory tract, that prevent pathogens from entering the organism (Beutler 2004, Calich, da Costa et al. 2008). However, if a pathogen breaches these barriers, the innate immunity provides a non-specific response that is characterized by the presence of cellular components such as phagocytes (neutrophils, macrophages and dendritic cells), innate lymphoid cells (natural killer (NK) cells and NKT cells), mast cells and eosinophils (Blanco and Garcia 2008, Mogensen 2009). These cellular components are capable of detecting invading organisms due to the presence of conserved, transmembrane or intracytoplasmatic receptors generally known as pattern recognition receptors (PRRs). PRRs recognize specific molecular structures shared by groups of microorganisms called pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov 2002). Upon recognition, adaptor molecules, such as myeloid differentiation primary-response protein 88 (MyD88) and TIR domain-containing adaptor inducing interferon (IFN)-β (TRIF), transmit the signal downstream in order to activate pathways including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs) (Akira, Uematsu et al. 2006, Kawai and Akira 2007). These cascades ultimately lead to the induction of antimicrobial responses (pro-inflammatory cytokines) and the activation of phagocytic cells (macrophages and polymorphonuclear cells) (Mogensen 2009).

Innate immunity to *P. brasiliensis*

In *P. brasiliensis* infection, there are two main groups of PRRs responsible for the recognition of this pathogen: Toll-like receptors (TLRs) and C-type lectin receptors. Among these, dectin-1, dectin-2,

mannose receptor, dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), TLR2, TLR4 and TLR9 were shown to be involved in *P. brasiliensis* recognition (Menino, Saraiva et al. 2013, Richardson and Moyes 2015). Specifically, recognition is responsible for the activation of cells, including polymorphonuclear neutrophils (PMNs) and macrophages.

In recent years, it has been shown that *P. brasiliensis* is able to invade host cells due to the presence of laminin receptors and the glycoprotein Gp43 that behaves as an adhesin. These molecules interact with components of the extracellular matrix (ECM), such as fibronectin, laminin and fibrinogen, allowing *P. brasiliensis* to manipulate the host cells environment to favour their own growth and survival (Fortes, Miot et al. 2011, Torres, Hernandez et al. 2013). Indeed, it has been shown that, during *P. brasiliensis* infection, PMNs lose their ability to produce reactive oxygen species (ROS), one of the most important antimicrobial functions of these cells, leading to *P. brasiliensis* dissemination (Meloni-Bruneri, Campa et al. 1996). Although the mechanisms required to control *P. brasiliensis* are not completely understood, it is thought that macrophages play a critical role during *P. brasiliensis* infection. These cells produce tumour necrosis factor (TNF), a key cytokine for the activation of important macrophage antimicrobial mechanisms, including the production of nitric oxide (NO), and the formation and organization of the granuloma (Brummer, Hanson et al. 1989, Moscardi-Bacchi, Brummer et al. 1994, Murray and Nathan 1999, Gonzalez, de Gregori et al. 2000, Bernardino, Pina et al. 2013). In *P. brasiliensis* granuloma, macrophages appear to surround the yeast cells. These macrophages are then submitted to a process of maturation and structural modifications mediated by cytokines leading to their differentiation into epithelioid and giant cells. Neutrophils, NK cells and B cells are also present in the vicinity of *P. brasiliensis* yeast cells. Fava Netto and co-workers classified these lesions into two types: (1) a benign localized infection, where granuloma is compact and presents few *P. brasiliensis* cells; and (2) disseminated infection, with loose granulomatous inflammation and extensive areas of necrosis. The granulomatous structures generated after *P. brasiliensis* infection are composed of epithelial tubercles with central areas of necrosis, aggregates of polymorphonuclear leukocytes, a lymphomononuclear halo and fibrosis (de Camargo and de Franco 2000). Although the granulomatous response is thought to be required to control extrapulmonary dissemination of infection, this also leads to the formation of tissue scarring, often resulting in fibrosis (Moreno and Guzman de Espinosa 1976, Cock, Cano et al. 2000). During pulmonary *P. brasiliensis* infection, the development of fibrosis is initiated at week 8 and culminates at week 12-16 with the consolidation of the damaged tissue. In most cases this contributes to the death of the patient (Vilani-Moreno, Fecchio et al. 1998).

As described above, TNF is also critical to activate the antimicrobial activity of macrophages (Riipi and Carlson 1990, Jain, Evans et al. 2006). Indeed, several studies have suggested that monocytes stimulated with TNF produced more nitric oxide and exhibited more effective fungicidal activity (Carmo, Dias-Melicio et al. 2006, Moreira, Dias-Melicio et al. 2008). Nitric oxide (NO) is a potent macrophage antimicrobial mediator that is generated from amino acid L-arginine by the inducible isoform of the nitric oxide synthase (iNOS). In *P. brasiliensis* infection, the role of nitric oxide is still not completely understood. Indeed, while some studies point to an important role of NO in the control of *P. brasiliensis* growth, other studies suggest that NO may induce immunosuppression by inhibiting lymphoproliferation, MHC class II molecule expression and TNF production (Nascimento, Calich et al. 2002). It is therefore crucial to understand the relationship between TNF and NO in *P. brasiliensis* infection and to determine how these mediators impact macrophage ability to control infection.

1.3.2. Adaptive immunity

The adaptive immunity is initiated with the presentation of antigens by antigen-presenting cells (APCs), to naïve T-cells. APCs are sentinels of the immune system and include dendritic cells (DCs), macrophages and B cells (Hamilos 1989).

Upon pathogen recognition, APCs are activated and internalize pathogens as well as their antigens, migrating to the draining lymph nodes. During this process, APCs upregulate the expression of molecules presented in the surface of these cells, including the major histocompatibility complex (MHC) class II and costimulatory molecules (CD40, CD80 and CD84). Moreover, these cells also secrete cytokines such as interleukin (IL)-12 and IL-23 that will promote the differentiation of $CD4^+$ T-cells into different subpopulations (Parker 1993, Richardson and Moyes 2015). The T helper (Th) subsets Th1, Th2 and Th17 cells are considered to be the most important Th cells during fungal infections (Porter, Roberts et al. 2011, de Castro, Ferreira et al. 2013).

IL-12 produced by APCs, in combination with IFN-γ, leads to the induction of a Th1 phenotype (Mosmann and Coffman 1989, de Castro, Ferreira et al. 2013). Th1 cells are characterized by the expression of IFN-γ, TNF and IL-2 and are critical to control intracellular pathogens. On the other hand, Th2 cells are differentiated in the presence of IL-4 and produce mainly IL-4, IL-5 and IL-13, which are important in immunity to extracellular pathogens (Noelle and Snow 1992).

Th17 cells are the newest subpopulation of T-cells with an important role in fungal diseases (de Castro, Ferreira et al. 2013). These cells, which are differentiated in the presence of IL-1, transforming growth factor (TGF)-α and IL-6, are characterized by the production of IL-17A, IL-17F, and IL-22 (Mosmann and

Coffman 1989). Once activated, Th17 cells stimulate epithelial cells, macrophages and endothelial cells to produce cytokines and chemokines (CXCL8, IL-1β, IL-23, and IL-6), and also antimicrobial peptides (such as defensins) (Richardson and Moyes 2015). Although these $CD4^+$ T-cell phenotypes are important to control different infections, exacerbated Th1 responses induce immunopathology whereas excessive Th2 responses cause allergic inflammatory diseases (Zhu and Paul 2008). Nevertheless, the activity of these Th phenotypes is tightly regulated by regulatory T-cells (Treg). This specific subpopulation is characterized by the expression of the transcription factor Foxp3, and produce IL-10 and transforming growth factor (TGF)-β (Silva, Sotto et al. 2013).

Adaptive immunity to *P. brasiliensis*

To date, many aspects of the adaptive immune response against *P. brasiliensis* are still unknown. However, strong evidences suggest that, in humans, Th1 responses are associated with resistance to *P. brasiliensis* infection, whereas Th2 responses are associated with susceptibility (de Castro, Ferreira et al. 2013). Similarly, it has been shown that, in mice, resistance to *P. brasiliensis* infection is associated with high IL-2 and IFN-γ responses while susceptibility is associated with high secretion of IL-5, which inhibits IFN-γ production. Moreover, it has also been suggested that IL-10 is equally produced by both resistance and susceptible mice strains, suggesting the important role of this cytokine in regulating inflammation (Kashino, Fazioli et al. 2000).

The different clinical forms of this disease are also associated with different Th-subsets. The juvenile form, the severest form of the disease, presents a predominant Th2 response with increased production of IL-4, IL-10, IL-5 and specific antibodies (such as IgG4, IgE and IgA). On the other hand, the sub-clinical form is characterized by a Th1 response, with the production of inflammatory cytokines such as IFN-γ, IL-12 and TNF. Patients presenting the chronic form of the disease develop a heterogeneous immunological response, as they produce cytokines associated with both Th1 and Th2 response, including TNF, IFN-γ, IL-12, IL-4 and IL-10 (Benard and Duarte 2000, Mamoni and Blotta 2006) (Figure 6).

In addition to Th1 and Th2 cells, Th17 cells are a new subpopulation of effector $CD4^+$ T-cells that play a key role during *P. brasiliensis* infection (de Castro, Ferreira et al. 2013). During *C. albicans* infection, several studies suggested that the activation of dectin-1 results in the induction of Th17 cells and the production of IL-23 and IL-17. These cytokines are responsible for mediating neutrophil recruitment to the peripheral inflammatory sites resulting in fungal clearance (Hernandez-Santos and Gaffen 2012). In PCM, dectin-1 is also activated and IL-17 and IL-23 are produced leading to the recruitment of

neutrophils to the site of infection and improved *P. brasiliensis* control (Loures, Araujo et al. 2014). However, it has been shown that an exacerbated Th17 response can also result in tissue damage and fibrosis, highlighting the existence of regulators that control the balance between protection and immunopathology (de Castro, Ferreira et al. 2013).

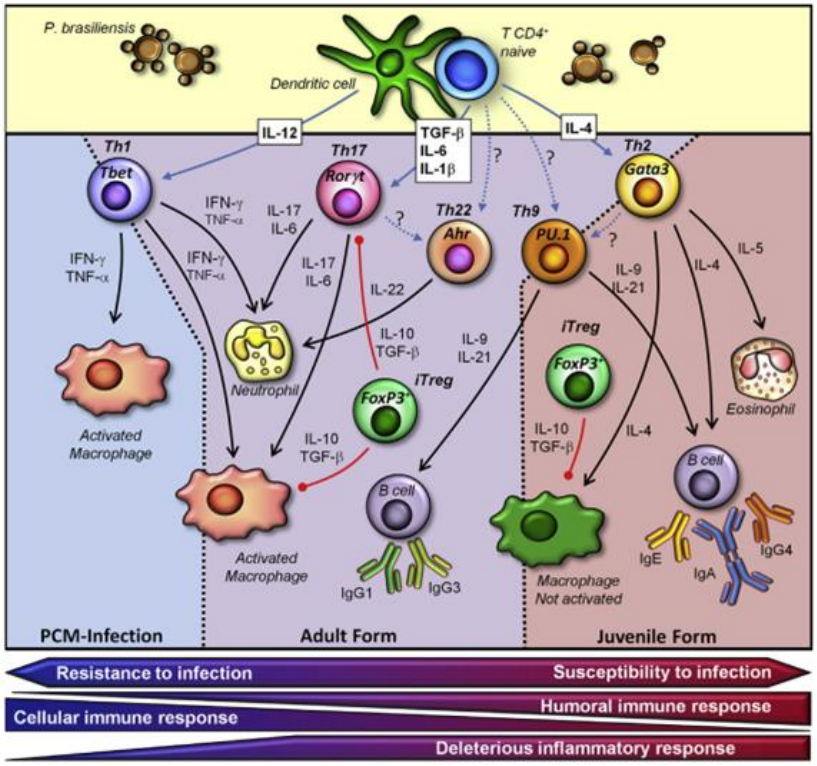

Figure 6. Proposed model to explain the immune responses observed in the different forms of PCM. Different forms of PCM present distinct immune responses elicited by *P. brasiliensis* infection. Adapted from de Castro, Ferreira et al. 2013.

1.4 MODULATION OF THE IMMUNE RESPONSE: THE ROLE OF INTERLEUKIN (IL)-10

When inflammation is exacerbated, the tissue is damaged resulting in serious consequences to the host. Therefore, it is essential to maintain a balance between inflammation and immunopathology. In this context, IL-10 has a crucial role in controlling the inflammatory reaction since it down-regulates the inflammatory response by developing protective immunity.

1.4.1 IL-10 biology

IL-10 is an anti-inflammatory cytokine that is recognized by several cell subsets, including macrophages, dendritic cells (DCs), neutrophils, B cells, eosinophils and NK cells. This cytokine is recognized through a specific surface receptor (IL-10R). IL-10R is composed by two subunits, IL-10R1 and IL-10R2. This interaction results in the activation of the Janus kinase/signal transducers and activators of transcription (JAK-STAT) signalling pathway and STAT transcriptional factors (Moore, de Waal Malefyt et al. 2001, Sabat, Grutz et al. 2010).

The anti-inflammatory nature of IL-10 is associated with the ability of this cytokine to inhibit the activity of the APCs, which include macrophages and DCs. In the early phase of infection, this cytokine impacts monocytes/macrophages effector functions by impairing the expression of MHC-II proteins, costimulatory molecules (CD80/CD86) and inflammatory cytokines, such as IFN-γ, IL-12, TNF and IL-1β. Therefore, by inhibiting the production of IL-12, this cytokine compromises the differentiation of specific naïve T-cells, including Th1-cells (Moore, de Waal Malefyt et al. 2001).

Despite its negative impact on inflammation, IL-10 has a positive effect mainly in the prevention of apoptosis of B cells and in the recruitment of NK cells and $CD8^+$ T-cells. Moreover, IL-10 prevents exacerbated responses since it re-establishes the balance between protection and immunopathology (Sabat, Grutz et al. 2010, Costa, Bazan et al. 2013).

1.4.2 IL-10 in Paracoccidioidomycosis

The role of IL-10 in fungal infections appears to be controversial. While it has been reported the beneficial role of this cytokine in *Trypanosoma cruzi* and *Toxoplasma gondii* infections, infections caused by *C. albicans,* and *H. capsulatum* are better controlled in the absence of IL-10 (Scharton-Kersten, Wynn et al. 1996, Hunter, Ellis-Neyes et al. 1997, Vazquez-Torres and Balish 1997, Guimaraes, Frases et al. 2009).

Specifically in *P. brasiliensis* infection, the role of IL-10 is still poorly understood. Nevertheless, *in vitro* and *in vivo* studies have suggested that IL-10 has a detrimental role in *P. brasiliensis* infection. Indeed, IL-10-deficient macrophages exhibited an improved fungicidal activity as a result of an increased production of NO, TNF, IFN-γ and MCP-1, resulting in a reduction in the number of viable yeast cells (Costa, Bazan et al. 2013).

In vivo studies also suggest a detrimental role of IL-10 in infection. In fact, IL-10-deficient mice showed lower fungal burden in the lungs and also a reduction in *P. brasiliensis* dissemination to other organs, including liver and spleen. Interestingly, improved control of *P. brasiliensis* was not associated with immunopathology (Costa, Bazan et al. 2013).

As previously referred, IL-10 seems to have a negative effect in the host immune response against *P. brasiliensis.* Therefore, we questioned whether a deregulation in the levels of IL-10 in different phases of infection could be responsible for the dissemination of the fungus and consequently severity of the disease.

CHAPTER II

AIMS

P. brasiliensis is a thermodimorphic fungus and the etiological agent of PCM, a human systemic mycosis that affects more than 10 million individuals. Although some important advances have been accomplished to better understand the host immune response against this fungus, there is still a lack of knowledge regarding the interaction between the host and the fungus and the role of specific immunological elements such as NO and cytokines. Therefore, we are interested in understanding the mechanisms whereby macrophages control *P. brasiliensis* infection and also which mediators participate in this process. Moreover, we are also interested in understanding whether a deregulation in IL-10 levels could impact the balance between protection and pathology.

To address the impact of macrophage effector functions in *P. brasiliensis* infection, we established an *in vitro* model of BMDM infection. We also begin to establish an *in vivo* model of *P. brasiliensis* infection to study the mechanisms involved in the early and late phases of infection.

The main goals of this work are:

(i) To define the mechanisms whereby macrophages control infection by *P. brasiliensis*, and determine whether the virulence of different strains has an impact in modulating the macrophage response.

(ii) Establish an *in vivo* model of *P. brasiliensis* infection to characterize the innate and acquired immune response to this pathogen.

CHAPTER III

MATERIALS AND METHODS

3.1 MICROORGANISMS AND CULTURE MEDIA

P. brasiliensis strains ATCC60855 and 18 were used in this study. *P. brasiliensis* ATCC60855 was registered at the American Type Culture Collection (Rockville, MD) whereas *P. brasiliensis 18* was provided by the Corporación para Investigaciones Biológicas (Medellín, Colombia) Culture Collection and isolated from Brazilian patients with PCM. Yeast cells were maintained at 37˚C by subculturing in Brain Heart Infusion (BHI) (Conda) solid media supplemented with 1% glucose and gentamicin (50 µg/mL). For *in vitro* and *in vivo* assays, strains were cultured in BHI liquid medium supplemented with 1% glucose and gentamicin (50µg/mL) for 7-10 days at 37˚C with aeration on a mechanical shaker (200rpm). For inoculum preparation, sterile glass beads were added to the cell suspension and vortexed in 3 cycles of 10 seconds. After, glass beads were removed; the cells suspension was centrifuged for 5 minutes at 3500 rpm and washed twice with lipopolysaccharide (LPS)-free phosphate buffered saline (PBS). Cells were resuspended in 10 mL of LPS-free PBS and allowed to sediment for the required time determined for each strain. The upper part of the suspension containing the less complex cells was collected, counted using a Neubauer's chamber and resuspended to the desired concentration and consequently diluted in cDMEM or LPS-free PBS for *in vitro* and *in vivo* infections, respectively. The viability of the cell suspension was determined using 0.4% Trypan blue solution.

3.2 ANIMALS

C57BL/6 (WT) mice were purchased from Charles River Laboratory and kept and bred in the Life and Health Sciences Research Institute (ICVS) animal housing facilities, at the School of Health Sciences, University of Minho. IL-10$^{-/-}$ animals (on a C57BL/6 background) were kindly provided by Anne O'Garra and kept and bred in the same conditions as WT mice. Mice were housed with food and water *ad libitum*.

pMT-10 animals (on a C57BL/6 background) were generated by Paulo Vieira and António Gil Castro at the Gulbenkian Institute of Science (IGC). A p169ZT vector carrying the sheep metallothionein (MT) Ia promoter was used to clone the mouse IL-10 cDNA. The resulting vector was injected in C57BL/6 eggs and transgenic mice were confirmed by PCR using specific primers to MT and IL-10. IL-10 overexpression was induced by administrating 2% sucrose solution with 50 mM of zinc sulfate to animals *ad libitum*. Since the presence of zinc activates IL-10 expression considerably fast, IL-10 overexpression in mice was detected 3 days after administration and this was confirmed by measuring the serum levels of this cytokine. Experiments were performed with 8-12 weeks old males.

All mouse protocols were performed according to the European Union Directive 86/609/EEC, and previously approved by the national authority Direcção Geral de Alimentação e Veterinária.

3.3 CULTURE AND INFECTION OF BONE MARROW-DERIVED MACROPHAGES (BMDM)

BMDM were generated from 8-12 weeks-old WT and IL-10$^{-/-}$ mice. Initially, mice were euthanized with CO_2 asphyxiation and the femur and tibiae removed aseptically and flushed using a 25G needle with complete Dulbecco's Modified Eagle Medium (cDMEM, DMEM supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 1% HEPES 1M, 1% L-glutamine and 1% sodium pyruvate 100mM (Invitrogen)). Cells were counted using a Neubauer's chamber and seeded during 7 days in Petri dishes (Sterilin) at a final concentration of 1x10^6/mL in 8 mL of DMEM supplemented with 20% of L-929 conditioned medium (LCCM), obtained from L-929 cell cultures in cDMEM, at 37˚C in 5% of CO_2 humidified air chamber. At day 7, macrophages were harvested from the Petri dishes using a cell scraper, counted and plated at a concentration of 1x10^6/mL in 24-well plates and incubated for 2 hours at 37˚C. After, supernatants were removed and BMDM infected with *P. brasiliensis* at a multiplicity of infection of 1:2 (yeast/macrophage ratio) and incubated at 37˚C. After 6 hours of infection, cells were washed with LPS-free PBS (6 times) to remove the non-internalized yeast cells and 1mL of cDMEM supplemented with IFN-γ (100U/mL), LPS (5ng/mL) or both was added to the respective groups and incubated for 1, 2 or 4 days. After the respective time periods, plates were centrifuged for 6 minutes at 1200 rpm and supernatants were filtered and stored at -80˚C for late cytokine and Nitric oxide (NO) analyses. 0.2 mL of sterile water were added to the wells for 30 minutes to allow macrophage lysis. Wells were then scraped to make sure that all BMDM were detached and the suspension was collected for yeast cell counting.

3.4 *P. BRASILIENSIS* PHAGOCYTOSIS ASSAYS

To determine the total percentage of phagocytosis (% of phagocytosis), BMDM were infected with *P. brasiliensis* at a MOI of 1:1 or 1:2 for 3 and 6 hours. After this period, wells were washed 6 times with LPS-free PBS to eliminate the non-internalized yeast cells and 0.2 mL of sterile water were added for 30 minutes to allow BMDM lysis. Yeast cells were then collected and counted using a Neubauer's chamber and the cell viability determined using 0.4% trypan blue solution.

3.5 IMMUNOFLUORESCENCE

For immunofluorescence staining, *P. brasiliensis* cells were incubated with fluorescein isothiocyanate (FITC) for 1 hour at room temperature (RT). Cells were then washed twice with LPS-free PBS and adjusted at a concentration of $1.25x10^6$/mL. BMDM were seeded on poly-D lysine-coated coverslips and infected with the labelled yeast cells for 6 hours. After this period, coverslips were washed 6 times and non-phagocytosed yeast cells stained with calcofluor for 10 minutes at RT. Cells were washed twice with LPS-free PBS and fixed for 30 minutes with paraformaldehyde (PFA) (4%). For latter macrophage staining, coverslips were incubated with blocking solution (PBS, 0.1% Triton X-100, 0.1% Tween-20, BSA 5% (p/v)) for one hour to allow blocking of unspecific binding sites to cells. CD11b-PE (clone M1/70, eBioscience) was diluted in blocking solution (1:100) and incubated for one hour at RT. Coverslips were washed 3 times for 5 minutes with washing solution (PBS, 0.1% Triton X-100, 0.1% Tween-20). Finally, the secondary antibody (Alexa fluor 594 goat anti-rat - Life Technologies) was added to bind to the primary antibody for one hour at RT and washed again 3 times before mounting coverslips with vectashield in microscope slides. Coverslips were observed with Olympus BX61 microscope and images were recorded with Olympus DP70 camera.

3.6 HEMATOXYLIN AND EOSIN STAINING

BMDM at a final concentration of $1x10^6$/mL were plated on poly-D lysine-coated coverslips in 24-well plates and infected for 3 and 6 hours at a MOI of 1:1 or 1:2. After these time periods, coverslips were washed 6 times with LPS-free PBS to eliminate extracellular yeast cells and fixed with 4% PFA for 30 minutes. Then, coverslips were stained with haematoxylin and eosin (H&E) and mounted in microscope slides.

3.7 ELISA

Enzyme-linked immunosorbent assay (ELISA) sets for IL-10 (88-7104), IL-6 (88-7064) and TNF (88-7324) were obtained from eBioscience. This assay was performed to measure the levels of these cytokines in the supernatants collected from infected cell cultures. Firstly, 96-well plates were coated with cytokine-specific capturing antibody overnight. Then, non-specific ligations were blocked and the supernatants and the cytokine standard concentration solutions were added to the wells and incubated at RT for 2h. The specifically bound protein was detected with cytokine-specific biotin-labelled detection antibodies upon incubation for 1h at RT. Avidin-horseradish peroxidase (-HRP) was added to bind to the

detection antibodies for 30 minutes at RT. Finally, 3,3′,5,5′-tetramethylbenzidine (TMB) was added, resulting in a coloured end-product since this reagent interacts with HRP being metabolized by this last one. Cytokine concentration was determined by spectrophotometry using a plate reader (Bio-Rad-Microplate reader 680), at 450nm. The optical density was then converted to concentration values using the Microplate Manager 5.2.1.

3.8 MEASUREMENT OF NITRIC OXIDE (NO) PRODUCTION

The concentration of NO in the supernatants of BMDMsinfected with *P. brasiliensis* was determined by the colorimetric Griess reagent. Supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamine, 0,1% nathylethylenediamine, 2,5% H_3PO_4) and incubated for 10 minutes in the dark. NO concentration was determined by spectrophotometry using a plate reader (Bio-Rad- Microplate reader 680), at 550nm. The optical density was then converted to concentration values using the Microplate Manager 5.2.1.

3.9 *IN VIVO* INFECTIONS

Intraperitoneal infection: for intraperitoneal infection, inoculum was prepared as previously described and WT and pMT-10 mice were infected with $1x10^6$ *P. brasiliensis* 18 cells, resuspended in 0.2 mL of LPS-free PBS, by intraperitoneal route. At weeks 1, 4 and 8, animals were euthanized by CO_2 asphyxiation and the lungs, liver and spleen removed for flow cytometry and histological analysis.

Intranasal infection: for intranasal infection, animals were anesthetized and, with a loading tip, $1x10^6$ *P. brasiliensis* 18 yeast cells were carefully delivered to the respiratory tract in a total volume of 20µL. The suspension was administrated drop by drop in the nostril until the animals inhaled all the volume. Animals were monitored daily and at weeks 1, 2, 8 and 20 were sacrificed and their lungs, liver and spleen collect for histological analysis and flow cytometry analysis.

Intratracheal infection: WT mice were first anesthetized and placed in an angled platform hanging by its incisors on a wire and gently restrained with a rubber band. The mice tongue was pull out with forceps and a sterile bent gavage needle inserted until the bend of the needle was by the front incisors with the help of a laryngoscope. After the needle was in the correct position, $1x10^6$ *P. brasiliensis* ATCC60855 yeast cells were injected into the trachea. Finally, animals were held upright for a few seconds to allow the inoculum inhalation into the lungs. Control of infection was performed at day 25 post-infection. Animals were monitored daily and mice showing severely impaired mobility or

respiratory problems in the first 48 hours were sacrificed and theirs lungs removed for histological analysis.

3.10 HISTOLOGICAL ANALYSIS

Lungs, liver and spleen from sacrificed animals were harvested and fixed in 4% PFA for 7 days at 4˚C. Later, these organs were embedded in paraffin and tissue sections were stained with hematoxylin and eosin (H&E). Sections were observed under the microscope and the presence of granuloma or necrotic areas were assessed using Olympus BX61 microscope and images were recorded with Olympus DP70 camera with a magnification of 10x or 40x.

3.11 FLOW CYTOMETRY ANALYSIS

For extracellular staining, 2-3x10^6 cells from the lung or the spleen were washed with FACS buffer (PBS containing 2% of FBS, 0.01% of azide and 0.5% saponine) and stained for 30 minutes at 4˚C. The following antibodies were used: MHC II-FITC (M5/114.15.2. Biolegend), CD11b-PE (clone M1/70, eBioscience), Ly6C-PerCP/Cy5.5 (clone AL-21, Pharmingen), CD62L-PE/Cy7 (clone MEL-14, Biolegend), CD11c-BV421 (clone N417, Biolegend), Ly6G-APC (clone 1A8, Biolegend), F4/80-APC/Cy7 (clone BM8, Biolegend); CD8A-FITC (clone 5H10-1, Biolegend), CD44-PerCP/Cy5.5 (clone MI7, Biolegend), CD3-PE clone 17A2, Biolegend), CD4-BV421 (clone GK1.5, Biolgend), CD19-APC/Cy7 (clone HIB, Biolegend). After, cells were washed twice with FACS buffer and fixed in 100 ml of 4% paraformaldehyde (PFA) for 30 minutes at RT. Samples were acquired on a LSRII flow cytometry with Diva software. Data was analysed using FlowJo version 7 software. The number of cells was assessed taking into account the number of cells in the organ determined by Countess® Automated Cell Counter.

3.12 RNA EXTRACTION AND QUANTIFICATION

RNA from samples was extracted with NZYol Reagent (NZYTech) according to the manufacturer's instructions. Initially, glycogen (20µg/µL, Roche) was added to each samples and incubated for 5 minutes at RT. After, 50 µL of chloroform were added to each samples and mixed by vortexing for 15 seconds and incubated on ice for 15 minutes. Samples were then centrifuged for 15 minutes at 13000 rpm at 4˚C and the RNA-containing aqueous phase collected. This phase was then mixed with an equal

volume of isopropyl alcohol (Sigma-Aldrich) in order to precipitate the RNA. Samples were incubated for 4 hours at -20˚C and centrifuged for 15 minutes at 13000 rpm. Supernatant was removed and 800µL of ethanol (Carlo Erba reagents) were added to the pellet to wash the RNA. Ethanol was removed by centrifugation for 5 minutes at 9000 rpm and the RNA was resuspended in RNase-free water (Gibco). RNA concentration was determined at 260nm and the purity determined according to the A_{260}/A_{280} and A_{260}/A_{230} ratios (Nanodrop ND-1000 Spectophotometer).

3.13 COMPLEMENTATY DNA (cDNA) SYNTHESIS

cDNA was synthesized using NZY First-Strand cDNA Synthesis Kit (NZYTech). Briefly, RNA samples were adjusted to 100ng/µL. A reaction mix containing 10µL of NZYRT 2x Master Mix and 2µL of NZYRT Enzyme Mix was mixed with 10µL of RNA sample. cDNA synthesis was performed in a thermocycle (Eppendorf) with the next conditions: 10 minutes of incubation at 25˚C followed by an incubation period of 30 minutes at 50˚C and a period of 5 minutes at 85˚C to inactivate the reaction. cDNA was then used for gene quantification by real-time polymerase chain reaction (RT-PCR).

3.14 REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR)

IL-10, TNF, IL-6 and ubiquitin expression was measured using SsoFast EvaGreen SuperMix (Bio-Rad). Briefly, 1µL of cDNA was mixed with 1 µL of 0.4µM sense and antisense specific primers (Table 1), 1µL of water and 5µL of SsoFast EvaGreen SuperMix. RT-PCR was performed in CF*96TM Real-time system (Bio-Rad) using the next conditions: 95˚C for 15 seconds, followed by 40 amplification cycles of 95˚C for 15 seconds, 58˚C for 20 seconds and 70˚C for 15 seconds.

3.15 STATISTICAL ANALYSIS

The results are given as means± standard deviation (SD) of at least 3 replicates per experimental group in *in vitro* infections, and 4 animals per experimental group in *in vivo* infections. Statistical analyses were performed using the GraphPad Prism 6 software. Student's t-test, one-way analyses of variance (ANOVA) or two way analyses of variance (ANOVA) and the post-test Bonferroni were used to evaluate significant differences between different conditions. Values were considered significant when $p<0.05$.

Table 1. Sequences of primers used for measuring the expression of IL-6, Ubiquitin, IL-10 and TNF by RT-PCR.

Protein	Primer	Sequence
IL-6	Sense	5'- ACA CAT GTT CTC TGG GAA ATC GT -3'
	Antisense	5'- AAG TGC ATC ATC GTT GTT CAT ACA -3'
Ubiquitin	Sense	5'- TGG CTA TTA ATT ATT CGG TCT GCA -3'
	Antisense	5'- GCA AGT GGC TAG AGT GCA GAG TAA -3'
IL-10	Sense	5'- TTT GAA TTC CCT GGG TGA GAA -3'
	Antisense	5'- GCT CCA CTG CCT TGC TCT TAT T -3'
TNF	Sense	5'- GCC ACC ACG CTC TTC TGT CT -3'
	Antisense	5'- TGA GGG TCT GGG CCA TAG AAC -3'

CHAPTER IV

RESULTS

4.1 PREPARATION OF *P. BRASILIENSIS* INOCULUM FOR *IN VITRO* AND *IN VIVO* INFECTIONS

Different strains of *P. brasiliensis* can display different morphologies, including single cells, multiple budding cells and transitional forms (Brummer, Castaneda et al. 1993). These morphological differences have been suggested to be associated with virulence (Almeida, Cunha et al. 2009).

To determine the morphology of *P. brasiliensis* 18, a highly virulent strain, we begin by performing scanning electron microscopy. As represented in Figure 7, we found that, in addition to its large size that varies between 10 and 15 µm, this strain also presented several multiple budded cells (Figure 7A) that formed large aggregates after *in vitro* growth (Figure 7B).

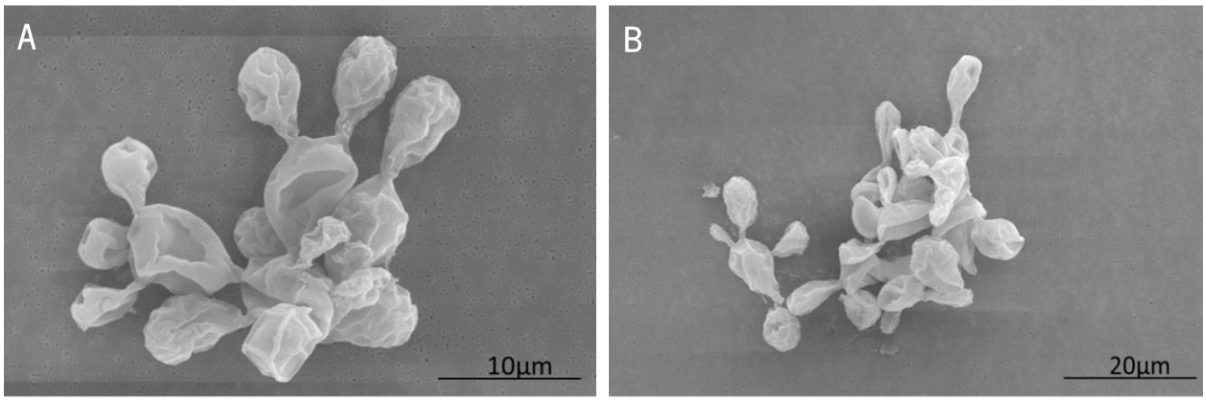

Figure 7. *P. brasiliensis 18* presented a large size and multiple budding-cells. Scanning electron microscopy showed that *P. brasiliensis* 18 had a spherical shape with larger and multiple budded cells (A). Cells tended to aggregate leading to the formation of clumps (B).

Taking into consideration the complexity of this *P. brasiliensis* strain, we first wanted to standardize the preparation of the inoculum for *in vitro* and *in vivo* infections. To this end, *P. brasiliensis* 18 was grown in Brain Heart Infusion (BHI) liquid medium supplemented with 1% glucose and gentamicin (50µg/mL) until the exponential growth phase (7-10 days). Next, the entire suspension was collected, glass beads were added and the suspension was vortexed in 3 cycles of 10 seconds to dissociate the clumps. Glass beads were then removed and the suspension was centrifuged for 5 minutes at 3500 rpm. The supernatant was discarded and the pellet was washed twice with lipopolysaccharide (LPS)-free phosphate buffered saline (PBS). At this point, sedimentation of larger cells and clumps was allowed for 5, 10, 15 and 20 minutes to determine the time required to obtain a homogeneous suspension (Figure 8, panels A-E).

As represented in Figure 8, we found that for *P. brasiliensis* 18, 20 minutes was the time required to obtain a homogenous population of single, smaller and less complex cells. We also assessed the *P. brasiliensis* cell complexity by flow cytometry analysis with similar data (Figure 8F).

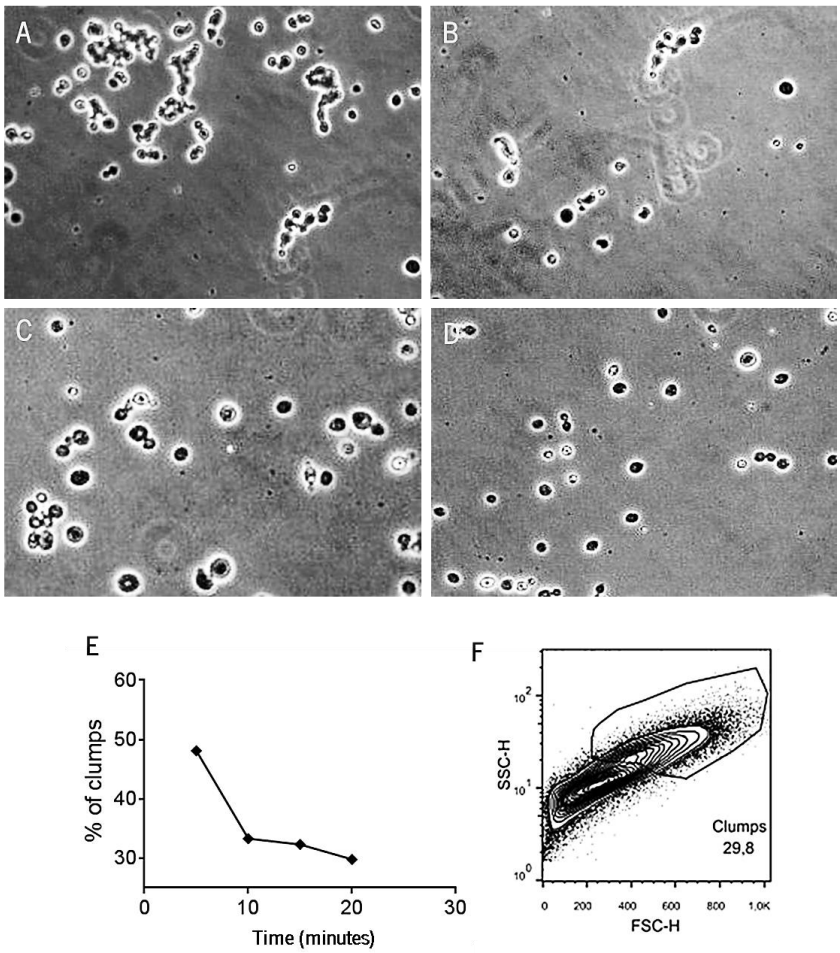

Figure 8. Longer periods of sedimentation resulted in a less complex inoculum. Cells were washed twice with LPS-free PBS and sedimentation of clumps and larger cells was allowed for 5 (A), 10 (B), 15 (C) and 20 (D) minutes. The percentage of clumps was reduced throughout time (E). After 20 minutes, 29,8% of the inoculum was only composed by clumps (F). Results were from one representative experiment out of four independent experiments. Cells were photographed by phase contrast microscopy (DIC). Magnification 20x

Overall, these data showed that this approach allowed for the preparation of a homogeneous suspension of *P. brasiliensis* yeast cells that were used to infect BMDM and mice.

4.2 TUMOR NECROSIS FACTOR (TNF) IS AN ESSENTIAL MEDIATOR FOR *P. BRASILIENSIS* CLEARANCE

After the entry of *P. brasiliensis* into the respiratory airways, it is thought that resident macrophages are the first cells to contact with this fungus (Tavares, Silva et al. 2007). It is therefore important to define the macrophage's mechanisms required to control *P. brasiliensis*.

To this end, we established an *in vitro* model of BMDM infection. We begin by infecting BMDM from WT mice with *P. brasiliensis* at a multiplicity of infection (MOI) of 1:1 or 1:2 (yeast: macrophage), followed by 3 or 6 hours of incubation, to define the optimal conditions to perform this infection (Figure 9A). In addition, infected BMDM were fixed with 4% paraformaldehyde (PFA) and stained with hematoxylin and eosin (H&E) to determine whether *P. brasiliensis* was phagocytosed or just adherent to macrophages (Figure 9B).

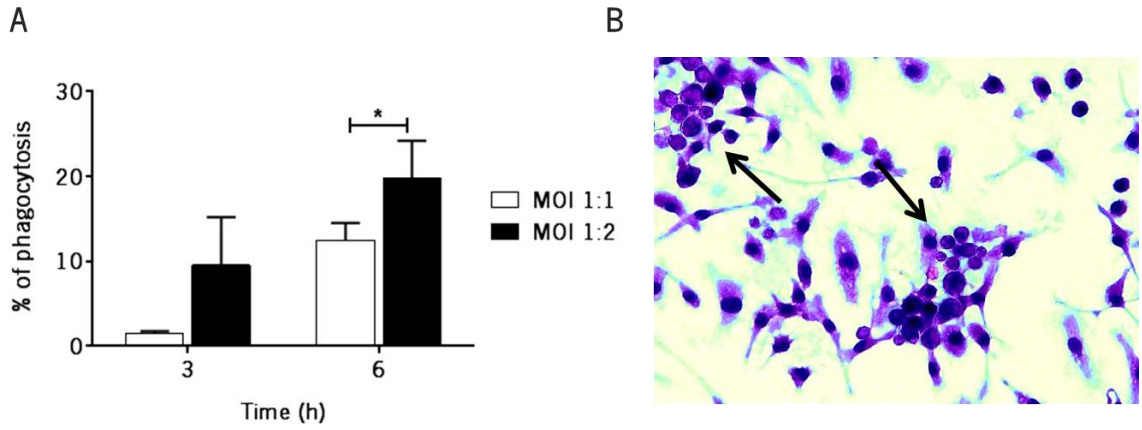

Figure 9. BMDM internalized a higher percentage of yeast cells at a MOI of 1:2 and a period of incubation of 6 hours. BMDM were infected with *P. brasiliensis* 18 at different MOIs and times of incubation. With a MOI of 1:2 and an incubation period of 6 hours, BMDM internalized a considerably higher percentage of yeast cells (A). By staining these cells with H&E, we observed that larger yeast cells remained attached to BMDM (B). The data points represent the means ± SD (n= 3). Statistical analysis between the different groups was determined by the Student's T test (*P<0.05). Amplification 40x.

As showed by figure 9A, we found an increased uptake of *P. brasiliensis* by BMDM at a multiplicity of infection (MOI) of 1:2 and a period of incubation of 6 hours. In addition, *P. brasiliensis* was found associated with BMDM, showing that it is recognized by these cells (Figure 9B). However, with this approach, we were not able to determine whether *P. brasiliensis* cells were intracellular or just adherent to BMDM. Therefore, yeast cells were labelled before infection with fluorescein isothiocyanate (FITC) for

1 hour at room temperature (RT) and, upon infection, extracellular yeast cells were labelled with calcofluor for 10 minutes at RT.

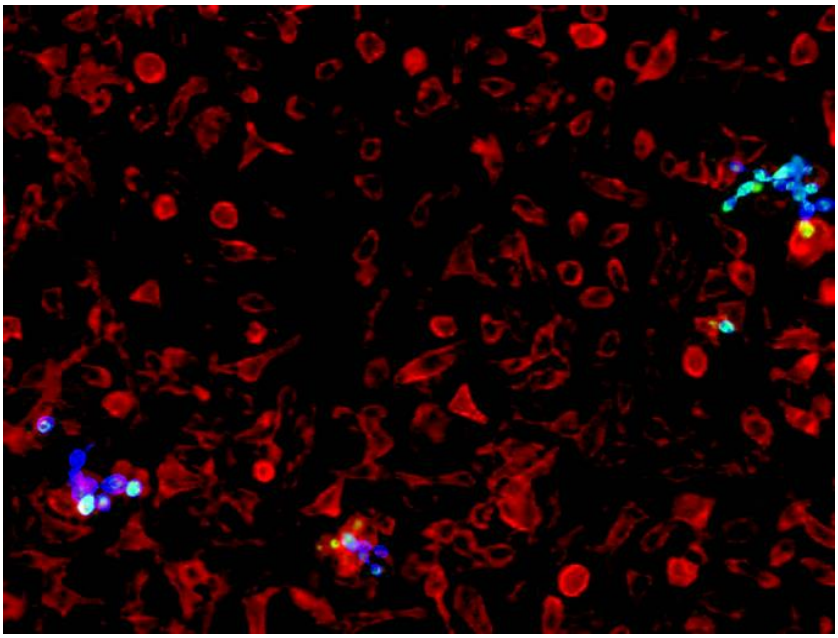

Figure 10. BMDM presented reduced ability to internalize larger *P. brasiliensis* cells. Inoculum was initially labelled with FITC (green) for 1h at RT and used for infection. After 6 hours of infection, extracellular yeast cells were labelled with calcofluor (blue) for 10 minutes at RT. While smaller cells were internalized by BMDM, larger cells and clumps remained attached to BMDM. Red –BMDMs; Blue–Extracellular *P. brasiliensis* cells; Green- Intracellular *P. brasiliensis* cells.

As represented in Figure 10, this approach showed that only smaller *P. brasiliensis* cells were internalized by BMDM, whereas larger and more complex cells remained adherent to BMDM.

With the optimal experimental conditions established, we next infected BMDM using *P. brasiliensis* 18 to determine the ability of macrophages to control *P. brasiliensis* growth. This was evaluated by counting the number of viable fungal cells at the established time-points.

As observed in Figure 11, we found that non-stimulated BMDM were not able to control *P. brasiliensis* growth as there was an increased number of viable yeast cells throughout infection. Surprisingly, when BMDM were stimulated with interferon (IFN)-γ, there was also no control of fungal growth. These data suggested that IFN-γ alone was not sufficient to activate macrophages and to control infection.

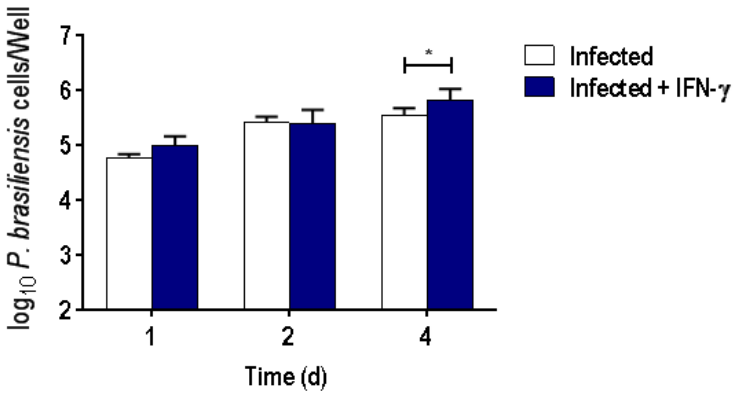

Figure 11. IFN-γ-activated infected BMDM did not control infection. BMDM were infected with *P. brasiliensis* 18 and then treated or not with IFN-γ. The number of viable yeast cells was determined at days 1, 2 and 4 post-infection. Unstimulated-infected BMDM did not control infection. Surprisingly, IFN-γ-activated infected BMDM were also not able to control infection. Results are from one representative experiment out of three independent experiments. The data points represent the means ± SD (n= 6). Statistical analysis between the different groups was determined by the Student's T test (*P<0.05).

The production of NO is one of the key macrophage antimicrobial mechanisms to control infection (Gonzalez, de Gregori et al. 2000). We therefore measured the levels of NO produced both by non-infected and *P. brasiliensis* infected BMDM. As expected, non-infected BMDM, whether or not stimulated with IFN-γ, did not produce NO. Surprisingly, we found that neither unstimulated nor IFN-γ-activated *P. brasiliensis*-infected BMDM produced NO (Figure 12).

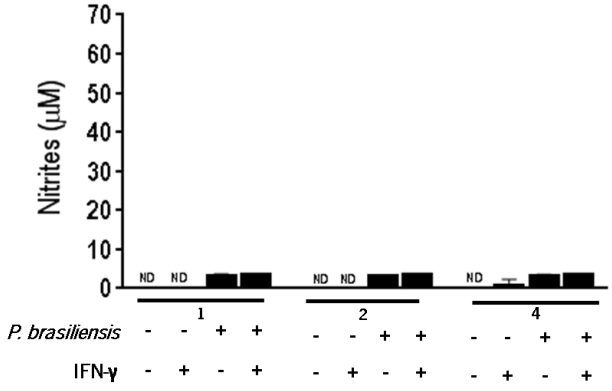

Figure 12. IFN-γ-activated infected BMDM did not produce NO. Both unstimulated and IFN-γ-activated BMDM were infected with *P. brasiliensis* 18 and NO measured at days 1, 2 and 4. Unstimulated and IFN-γ-activated infected BMDM did not produce NO, suggesting that these macrophages were not activated. Results are from one representative experiment out of three independent experiments. The data points represent the means ± SD (n= 6). Statistical analysis between the different groups was determined by the two-way ANOVA test and the post test of Bonferroni. ND-Non detected values.

Several studies reported that the production of NO by BMDM is only achieved after induction with IFN-γ and TNF (Mosser and Edwards 2008). Therefore, we questioned whether *P. brasiliensis* was also inducing low levels of TNF in these cells. TNF production is initiated by the interaction of different pattern recognition receptors (PRRs) with pathogen-associated molecule patterns (PAMPs) presented in infectious agents, such as lipopolysaccharide (LPS). This interaction initiates a cascade of events leading to the production of pro-inflammatory cytokines, including TNF, IL-1 and IL6 (Chow, Young et al. 1999). To determine the extent to which *P. brasiliensis* infection induced TNF production by BMDM, we measured the levels of TNF in the culture supernatants.

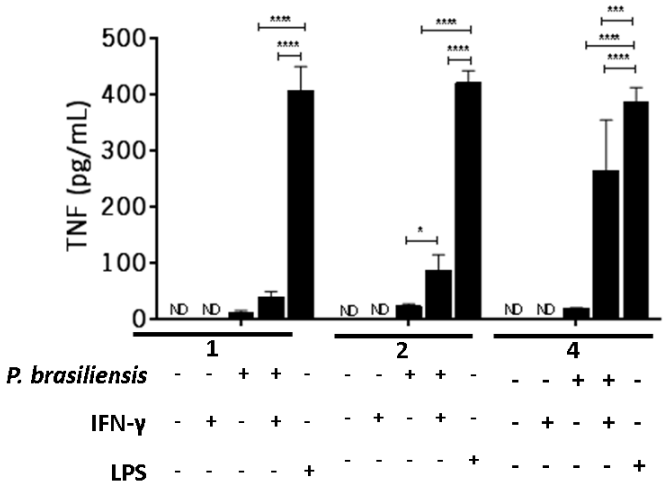

Figure 13. IFN-γ-activated infected BMDM produced lower levels of TNF. BMDM were infected with *P. brasiliensis* 18 for 6 hours and then stimulated with IFN-γ. When compared to the positive control (LPS-activated non-infected BMDM), IFN-γ-activated infected BMDM did not produce high levels of TNF suggesting that *P. brasiliensis* did not induce BMDM to produce TNF. Results are from one representative experiment out of three independent experiments. The data points represent the means ± SD (n= 6). Statistical analysis between the different groups was determined by the one-way ANOVA test and the post test of Bonferroni (*P<0.05, ***P<0.001, ****P<0.0001). ND-Non detected values

As observed in Figure 13, we found an increased production of TNF by IFN-γ-activated infected BMDM when compared to unstimulated infected-BMDM. However, when we compared these levels with the positive control, LPS-activated non-infected BMDM, we observed that, even if activated by IFN-γ, *P. brasiliensis*-infected BMDM did not produce enough amounts of TNF to activate BMDM.

To determine whether the low production of TNF was the cause for the reduced production of NO by IFN-γ-activated infected BMDM, we induced the expression of TNF in these BMDM by stimulating these cells with LPS after infection.

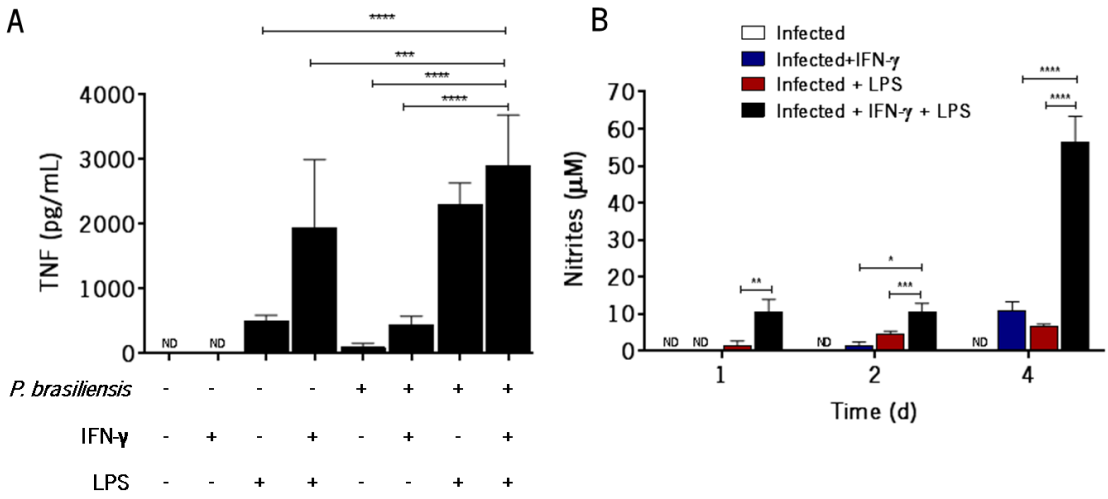

Figure 14. IFN-γ- and LPS- activated BMDM produced high levels of TNF and NO. When stimulated with both IFN-γ and LPS, BMDM showed high levels of TNF (A) and NO (B), suggesting activation. BMDM were infected with *P. brasiliensis* 18 for 6 hours. cDMEM supplemented with IFN-γ, LPS or both was then added to the cultures for the established days. TNF was measured by ELISA and the results are from day 4 but representative throughout infection. NO production was measured in culture supernatants by Griess reagent (B). For TNF levels, statistical analysis between the different groups was determined by one-way ANOVA. For NO production, statistical analysis between the different groups was determined by two way ANOVA and post-test Bonferroni. Results are from one representative experiment out of three independent experiments. The data points represent the means ± SD (n= 6). ND-Non detected values (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

As represented in Figure 14, when *P. brasiliensis*-infected BMDM were stimulated with IFN-γ and LPS, increased production of TNF was observed (Figure 14A). Moreover, when we measured the production of NO, we also found that only IFN-γ- and LPS-activated *P. brasiliensis*-infected BMDM produced high levels of NO (Figure 14B), which was in accordance with our hypothesis.

Since we wanted to determine whether IFN-γ- and LPS-activated BMDM were able to better control *P. brasiliensis* 18, we measured the number of viable yeast cells. As shown in Figure 15, unstimulated, IFN-γ-activated or LPS-activated BMDM did not control *P. brasiliensis* growth. Indeed, we observed an increased number of yeast cells from day 1 to day 4. On the other hand, the activation of BMDM with both IFN-γ and LPS led to control of infection with 1 $\log_{10}$ reduction in fungal burden.

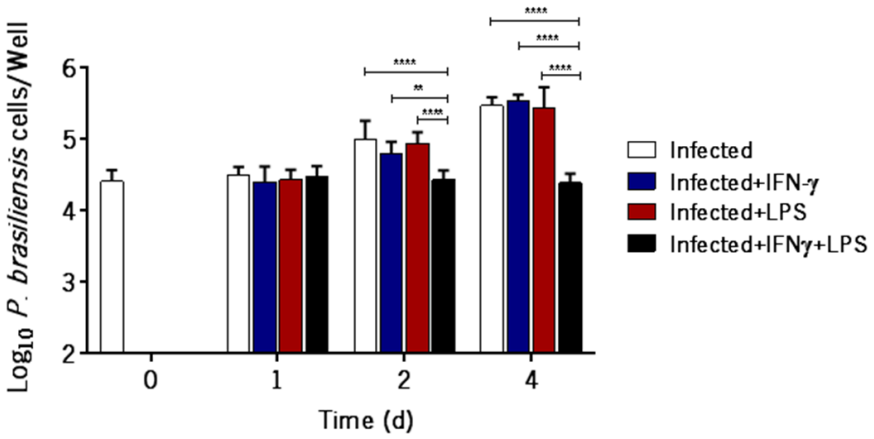

Figure 15. IFN-γ- and LPS-activated infected BMDM exhibited improved fungicidal activity. BMDM were infected with *P. brasiliensis* 18 and then stimulated with IFN-γ, LPS or both. Unlike unstimulated, IFN-γ-activated and LPS-activated BMDM, IFN-γ- and LPS-activated BMDM were able to control infection. Statistical analysis between the different groups was determined by two-way ANOVA and post-test Bonferroni. Results are from one representative experiment out of three independent experiments. The data points represent the means ± SD (n= 6). (**P<0.01, ***P<0.001, P<0.0001)

In summary, our results showed that *P. brasiliensis* 18 induced reduced production of TNF by BMDM which, in turn, is required for BMDM to produce NO. Accordingly, when TNF was induced by LPS, IFN-γ-activated BMDM produced high levels of NO resulting in improved fungicidal activity.

4.3 BMDM WERE BETTER ABLE CONTROL *P. BRASILIENSIS* ATCC60855 INFECTION

Taking into consideration that *P. brasiliensis* 18 is a highly virulent strain, we next wanted to determine whether the reduced production of TNF, and consequently NO, in response to this strain was a characteristic of virulent strains, or a more generalized mechanism used by all *P. brasiliensis* strains. To address this, we used our BMDM model of infection to determine the extent to which these cells control infection by the less virulent strain *P. brasiliensis* ATCC60855.

Previous studies performed in our laboratory suggested an association between *P. brasiliensis* morphology and its virulence (Almeida, Cunha et al. 2009). Indeed, it was shown that mice infected with less complex cells exhibited improved resistance to infection (Menino, Osorio et al. 2012).

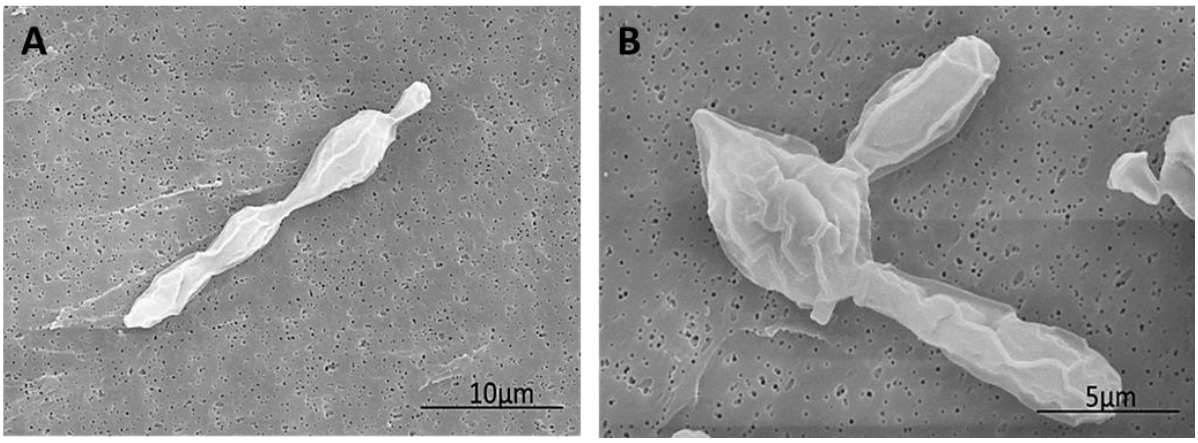

Figure 16. *P.brasiliensis* ATCC60855 presented smaller and less complex cells. Scanning electron microscopy showed that *P. brasiliensis* ATCC60855 presented smaller and longer cells when compared to the spherical shape of *P. brasiliensis* 18 cells (A). Nevertheless, multiple-budded cells were also observed since this is a typical characteristic of *P. brasiliensis* morphology (B). Magnification 10000x.

To define *P. brasiliensis* ATCC60855 morphology, we performed scanning electron microscopy and we found that, contrarily to *P. brasiliensis* 18, *P. brasiliensis* ATCC60855 cells were smaller in size, varying from 5 to 10 µm (Figure 16A), with reduced bud number and size (Figure 16B).

The inoculum preparation was performed as described for *P. brasiliensis* 18 with minor modifications, including longer sedimentation times (10, 15, 20, 30 and 40 minutes), since we observed that *P. brasiliensis* ATCC60855 suspension had a higher density at the end of the log phase of growth. Indeed, for this strain we found that only after 40 minutes of resting, the inoculum was homogeneous and composed of small and less complex cells (Figure 17).

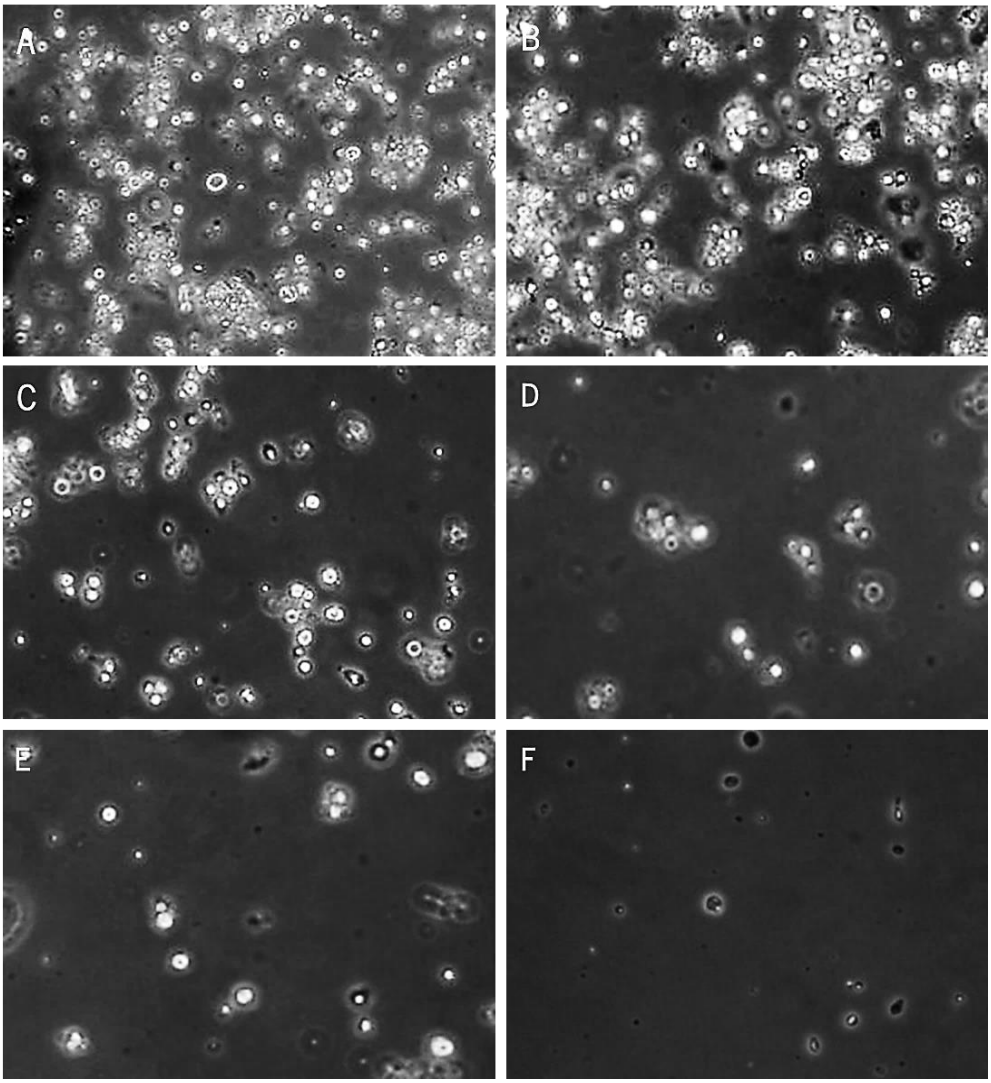

Figure 17. The complexity and heterology of the *P. brasiliensis* ATCC60855 inoculum was reduced during sedimentation. For inoculum preparation, yeast cells, grown for 7-10 days in BHI medium, were washed twice with LPS-free PBS and sedimentation was allowed for 10 (A), 15 (B), 20 (C), 25 (D) 30 (E) and 40 (F). After 40 minutes, inoculum presented small and individual cells. Cells were photographed by phase contrast microscopy (DIC). Amplification 20x.

We also performed a phagocytic assay to determine the ability of BMDM to phagocyte *P. brasiliensis* ATCC60855. Our results showed that there were no differences in the total percentage of internalized yeast cells between the MOIs tested (Figure 18). We also observed that the percentage of internalized yeast was considerably higher for *P. brasiliensis* ATC60885 when compared to *P. brasiliensis* 18, suggesting that *P. brasiliensis* ATCC6055 morphology may impact internalization by BMDM.

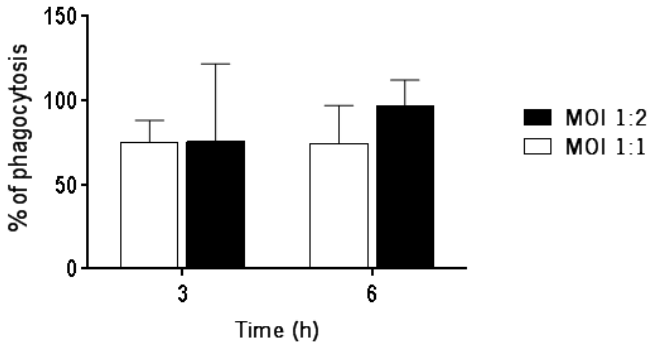

Figure 18 *P. brasiliensis* ATCC60855 was easily internalized by BMDM. BMDM from WT mice were infected with *P. brasiliensis* ATCC60855 for 6 hours, washed to remove extracellular yeast cells and lysed with sterile water. Percentage of cells was determined by dividing the number of counted yeast cells after lysis by the initial number of cells. Contrarily to *P. brasiliensis* 18, the total percentage of phagocytosis was higher. Statistical analysis between the different groups was determined by Student's T test. Results are from one representative experiment out of three independent experiments. The data points represent the means ± SD (n= 3). (**P<0.01, ***P<0.001, P<0.0001)

As described above, we showed that, after *P. brasiliensis* 18 infection, BMDM produced low levels of TNF and NO and they failed to control *P. brasiliensis* growth. Indeed, only with IFN-γ and LPS stimulation BMDM were able to control infection. We therefore infected BMDM with *P. brasiliensis* ATCC60855 under the conditions described for *P. brasiliensis* 18, to determine whether this less virulent strain was able to activate BMDM.

Our results showed that unstimulated, IFN-γ-stimulated or LPS-stimulated infected BMDM did not produce significant levels of NO upon infection with *P. brasiliensis* ATCC60855, while the combination between IFN-γ- and LPS led to the production of high levels of this mediator (Figure 19A).

Interestingly, contrarily to *P. brasiliensis* 18 infection, unstimulated, IFN-γ-activated and LPS-activated BMDM were able to control *P. brasiliensis* ATCC60855 growth over time since the number of viable yeast cells remained sustained throughout infection. Moreover, in IFN-γ- and LPS-activated infected BMDM, we observed a reduction in the number of viable yeast cells throughout time, which was not observed in *P. brasiliensis* 18 infection (Figure 19B). Surprisingly, when we measured the levels of TNF in BMDM infected with this strain, we observed that unstimulated *P. brasiliensis* ATCC60855-infected BMDM produced more levels of this cytokine when compared to unstimulated *P. brasiliensis* 18-infected BMDM (Figures 14A and 19C).

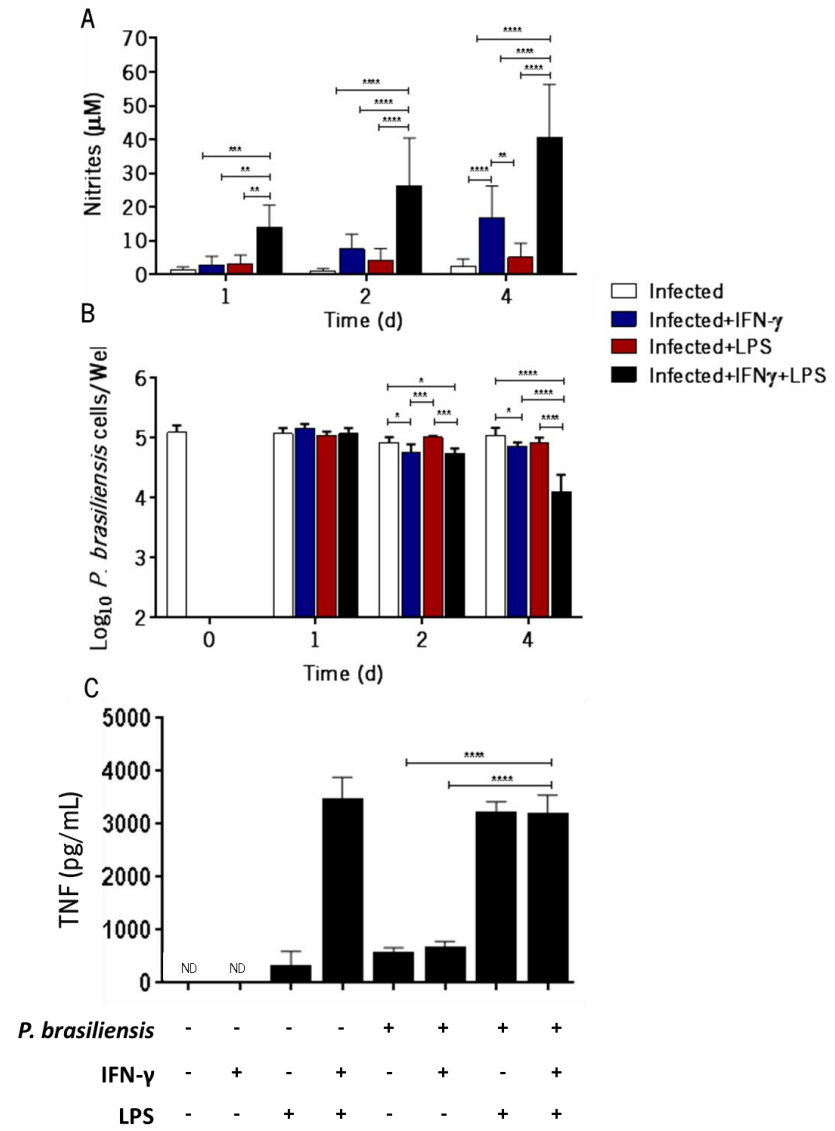

Figure 19. BMDM presented improved fungicidal activity against *P. brasiliensis* ATCC60855. BMDM were infected, supernatants removed for NO and TNF determination and BMDM lysed with sterile water to determine the number of viable yeast cells. IFN-γ- and LPS-activated BMDM produced higher levels of NO when compared to other treatments (A). Unstimulated, IFN-γ-activated and LPS-activated BMDM were better able to control *P. brasiliensis* ATCC60855 growth over time, which was not observed in *P. brasiliensis* 18 infection. IFN-γ- and LPS-activated BMDM controlled infection leading to a reduction in the number of yeast cells (B). Contrarily to *P. brasiliensis* 18, this strain induced more TNF in unstimulated-infected BMDM (C). NO production was measured by Griess method and TNF by ELISA. Statistical analysis between the different groups was determined by two-way ANOVA and the post-test Bonferroni. Results are from one representative experiment out of three independent experiments. The data points represent the means ± SD (n= 6) (*P<0.05, **P<0.01,***P<0.01, ****P<0.0001).

Considering the results obtained regarding the levels of TNF in BMDM infected with different strains, we next questioned whether TNF expression at 1, 3 and 24 hours post-infection was also different in *P. brasiliensis* 18 or *P. brasiliensis* ATCC60855 infected BMDM. Our results showed that unstimulated-

infected BMDM expressed low levels of TNF suggesting that neither *P. brasiliensis* 18 (Figure 20A) nor *P. brasiliensis* ATCC60855 (Figure 20B) induced the expression of this cytokine in the first hours post-infection. Interestingly, we observed that IFN-γ- and LPS-activated *P. brasiliensis* 18-infected BMDM expressed more TNF at 3 hours post-infection (Figure 20A).

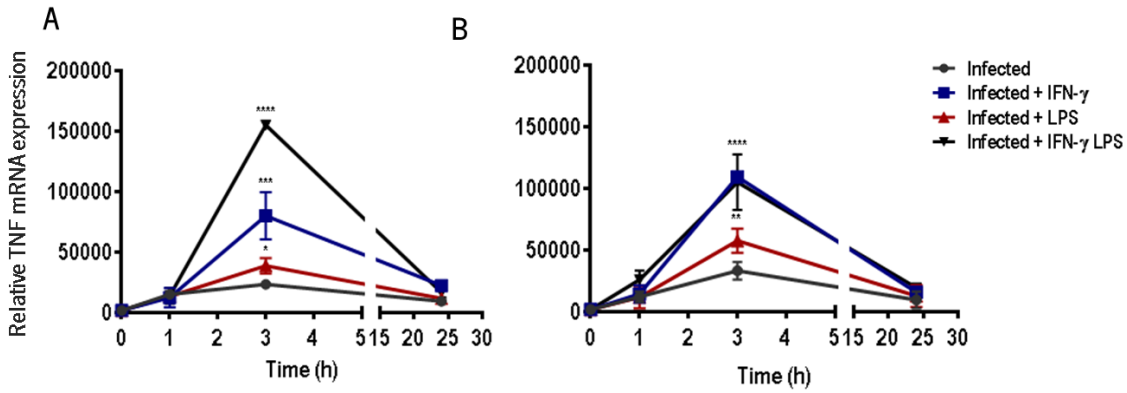

Figure 20. No differences were observed in TNF expression from 1hour to 24 hours post-infection by BMDM infected with *P. brasiliensis* 18 or *P. brasiliensis* ATCC60855. BMDM were left untreated or treated with IFN-γ, LPS or both, and infected with *P. brasiliensis* 18 (A) or *P. brasiliensis* ATCC60855 (B) for1, 3 and 24 hours. Supernatants were collected and RNA extracted. After 3 hours of infection, INF-γ- and LPS-activated BMDM infected with *P. brasiliensis* 18 expressed more TNF than BMDM infected with the less virulent strain. Expression values were normalized to ubiquitin. Statistical analysis between the different groups was determined by two-way ANOVA and post-test Bonferroni. Results are from one experiment. The data points represent the means ± SD (n= 3) (*P<0.05,**P<0.01,***P<0.001).

Overall, our results suggested that *P. brasiliensis* 18 and *P. brasiliensis* ATCC60855 were differently controlled by BMDM. While *P. brasiliensis* 18 infection was only controlled under stimulation with IFN-γ and LPS, the control of *P. brasiliensis* ATCC60855 infection by BMDM did not require stimulation with IFN-γ or LPS. However, although the number of *P. brasiliensis* ATCC60855 cells remained sustained over time in BMDM stimulated with IFN-γ or LPS alone, a reduction in the number of viable yeast cells was only observed with the combination of both stimuli. Moreover, we showed that TNF is required for BMDM activation and *P. brasiliensis* control.

4.4 IL-10 DID NO IMPACT THE FUNGICIDAL ACTIVITY OF BMDM AGAINST *P. BRASILIENSIS* INFECTION

Considering that *P. brasiliensis* ATCC60855- infected BMDM produced more TNF than *P. brasiliensis* 18-infected BMDM (Figure 14A and 19C), we questioned whether IL-10, an anti-inflammatory cytokine and a regulator of inflammation, could be responsible for these differences. To address this, we determined the levels of IL-10 in BMDM infected with *P. brasiliensis* 18 or *P. brasiliensis* ATCC60855 at different time-points.

As represented in Figure 21, no statistical differences were observed between the unstimulated BMDM infected with *P. brasiliensis* 18 or *P. brasiliensis* ATCC60855. Therefore, these data suggested that, contrarily to TNF, different *P. brasiliensis* strains have the same impact in IL-10 production by BMDM.

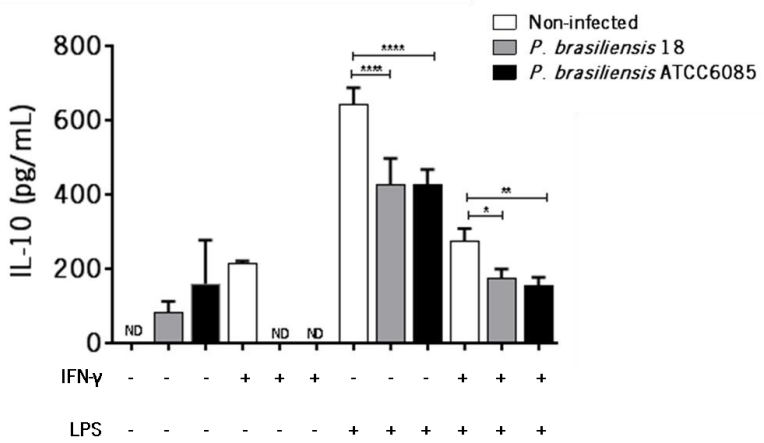

Figure 21. IL-10 production was not affected by distinct *P. brasiliensis* strains. BMDM were left unstimulated or stimulated with IFN-γ and LPS, alone or in combination, and infected with *P. brasiliensis* 18 or *P. brasiliensis* ATCC60855 for 1, 2 and 4 days. At these time-points, supernatants were removed and IL-10 levels measured by ELISA. The results, which are from day 4 but representative throughout infection, showed that IL-10 production was not affected by *P. brasiliensis* infection. Statistical analysis between the different groups was determined by two way ANOVA test and post-test Bonferroni. Results are from one experiment. The data points represented the means ± SD (n= 6) (*P<0.05,**P<0.01,***P<0.001). ND-Non detected values.

To understand whether activated IL-10$^{-/-}$ BMDM were able to control *P. brasiliensis* growth, we infected these cells with *P. brasiliensis* ATCC60855 and stimulated them with IFN-γ and LPS, as previously performed. Our results showed that both WT (Figure 22A) and IL-10$^{-/-}$ (Figure 22B) BMDM exhibited the same fungicidal ability. Since we only infected BMDM with *P. brasiliensis* ATCC60885, we are currently testing this for *P. brasiliensis* 18.

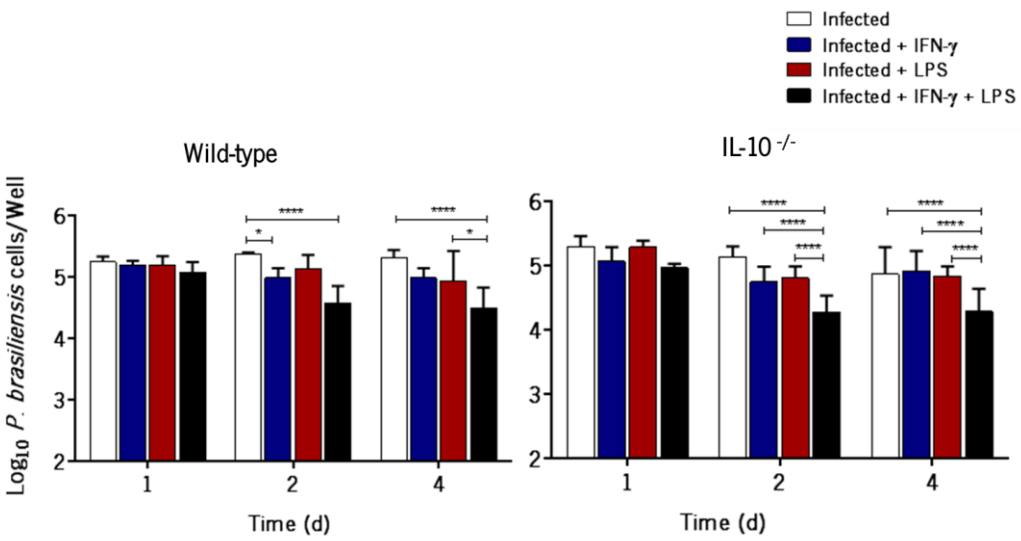

Figure 22. WT and IL-10$^{-/-}$ BMDM exhibited the same ability to control *P. brasiliensis* ATCC60855 infection. Both WT and IL-10$^{-/-}$ BMDM were infected for 6 hours with *P. brasiliensis* ATCC60855. After this incubation period, BMDM were lysed and viable yeast cells counted. IFN-γ- and LPS-activated BMDM from both WT and IL-10$^{-/-}$ BMDM controlled *P. brasiliensis* infection. Statistical analysis between the different groups was determined by two-way ANOVA and post-test Bonferroni. Results are from one representative experiment out of two independent experiments. The data points represent the means ± SD (n= 6) (*P<0.05, ****P<0.0001).

Altogether, *in vitro* infections showed that IL-10 did not impact macrophage fungicidal activity in the early phase of infection. Indeed, we observed no differences between the number of viable yeast cells in WT and IL-10$^{-/-}$ BMDM.

4.5 IL-10 OVEREXPRESSION IMPACTED CELL RECRUITMENT IN *P. BRASILIENSIS* INFECTED MICE

Considering that the deletion of IL-10 did not impact BMDM ability to control *P. brasiliensis* ATCC60855 in the early phase of infection, we next investigated the impact of high levels of IL-10 in the late phase of infection. To this end, we infected pMT-10 and WT mice by intraperitoneal infection with *P. brasiliensis* 18.

At week 1 post-infection, we observed no inflammatory reactions in the lungs and liver of both pMT-10 mice overexpressing IL-10 and control groups. Moreover, we also did not observe differences in cell recruitment in animals exposed to zinc, suggesting that, at this phase of infection, high levels of this cytokine did no interfere with the inflammatory response against *P. brasiliensis*. At week 4 post-infection, histological sections showed the formation of inflammatory infiltrates in the liver, although no significant differences were observed in cell activation (data not shown).

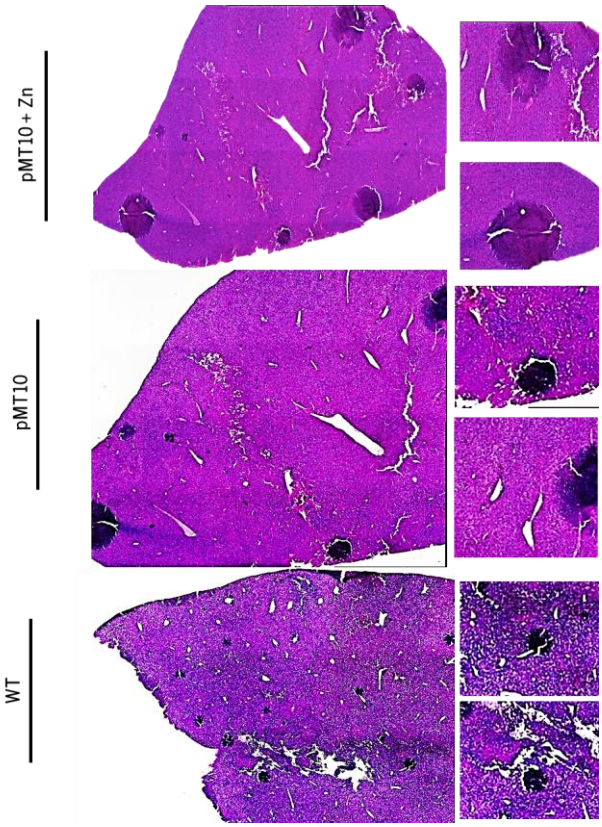

Figure 23. pMT-10 mice overexpressing IL-10 developed a more severe inflammatory reaction at week 8 post-infection. Mice were infected by intraperitoneal infection with $1x10^6$ *P. brasiliensis* ATCC60855 yeast cells. Infected pMT-10 mice were exposed to zinc for 7 days before the sacrifice. Histological sections showed that pMT-10 mice overexpressing IL-10 developed a more severe inflammatory reaction in the liver. Results are from one experiment. Sections were embedded in paraffin and tissue sections were stained with hematoxylin and eosin (H&E) Amplification 4x and 10x.

However, this inflammatory reaction became more pronounced at week 8 post-infection. At this phase, we observed lesions in both pMT-10 mice overexpressing IL-10 and control animals, suggesting a possible establishment of an inflammatory response against *P. brasiliensis*. Interestingly, we observed that animals overexpressing IL-10 showed more lesions in the liver (Figure 23). Although at week 8 post-infection the lungs of the animals showed no severe lesions, we observed that pMT-10 mice overexpressing IL-10 presented increased number of inflammatory monocytes and neutrophils when compared to non-infected animals (Figure 24).

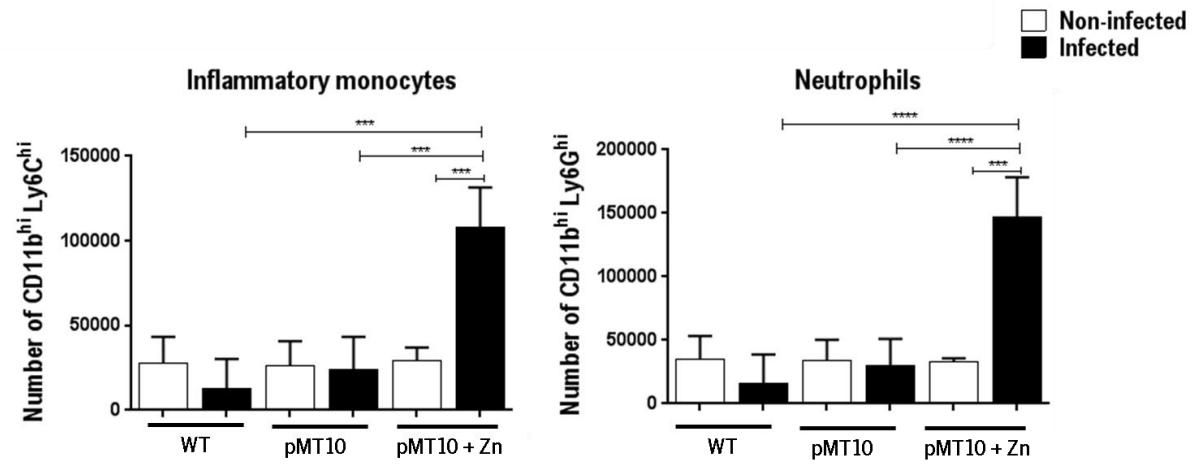

Figure 24. IL-10 overexpression in *P. brasiliensis*- infected mice resulted in the recruitment of inflammatory monocytes and neutrophils. Animals were infected with *P. brasiliensis* 18 for 8 weeks and, one week prior to sacrifice, were exposed to zinc. Lungs were collected and homogenized and the cells stained with myeloid lineage markers. Animals overexpressing IL-10 showed recruitment of neutrophils and inflammatory monocytes. Statistical analysis between the different groups was determined by two-way ANOVA test and post-test Bonferroni. The data points represent the means ± SD(n= 4). Results are representative of one experiment (***P<0.001, ****P<0.0001).

Upon activation, monocytes upregulate the expression of MHC-II molecules, which can be inhibited by IL-10 signalling (Moore, de Waal Malefyt et al. 2001, Richardson and Moyes 2015). However, the lungs of infected animals showed that IL-10 overexpression upregulated MHC-II expression through an unknown mechanism. This effect was not observed in the spleen of these animals since no sign of infection was observed in this organ (Figure 25).

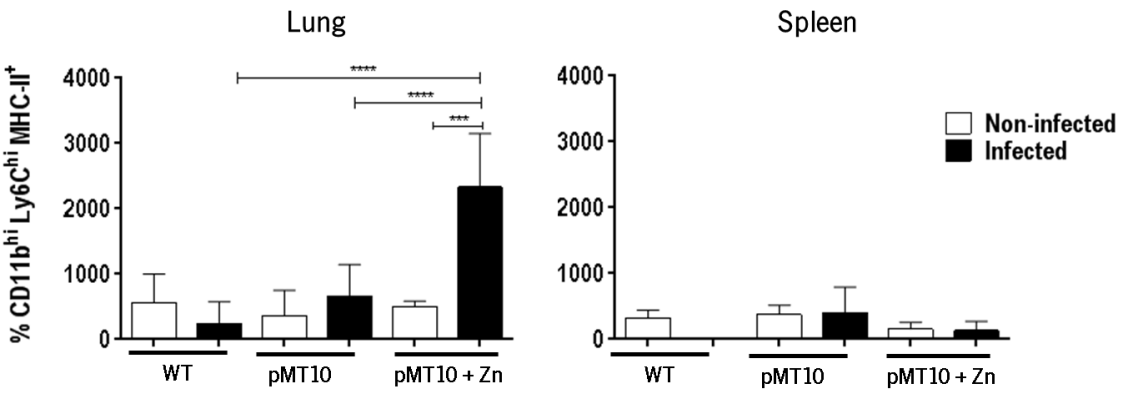

Figure 25. IL-10 overexpression resulted in the accumulation of MHC-II$^+$ inflammatory monocytes. In the lungs of animals overexpressing IL-10, a higher number of inflammatory monocytes expressing MHC-II was observed. Statistical analysis between the different groups was determined by two-way ANOVA test and post-test Bonferroni. Results are representative of one experiment. The data points represent the means ± SD (n= 4) (***P<0.001, ****P<0.0001).

Altogether, we conclude from the *in vitro* and *in vivo* infections that the impact of IL-10 was different throughout infection. Indeed, while in the early phase of infection, the deletion of IL-10 did not impact the outcome of infection since *P. brasiliensis* infection was equally controlled by WT and IL-10$^{-/-}$ BMDM, in the late phase of infection, high levels of this anti-inflammatory cytokine led to severe lesions followed by the recruitment of inflammatory monocytes and neutrophils.

4.6 ESTABLISHING AN INTRANASAL MODEL OF INFECTION: MIMICKING THE NATURAL ROUTE OF *P. BRASILIENSIS* INFECTION

Although other *in vivo* infections have been performed by our group, the intranasal model of infection has never been established. Considering that this is the natural route of infection, we set out to establish this model of infection. Natural infection is initiated by the inhalation of conidia, the saprophytic phase of this fungus (Cano, Gomez et al. 1994). Therefore, we begin by optimizing the production and isolation of conidia. Although conidia have been successfully isolated from mycelial fragments before, it is known that this is a complex procedure (Restrepo, Salazar et al. 1986). In this study, we successfully produced conidia but we did not obtain sufficient quantities of isolated conidia to infect a set of animals. Taking this into consideration, we infected WT mice by intranasal infection with $1x10^6$ *P. brasiliensis* ATCC60855 yeast cells.

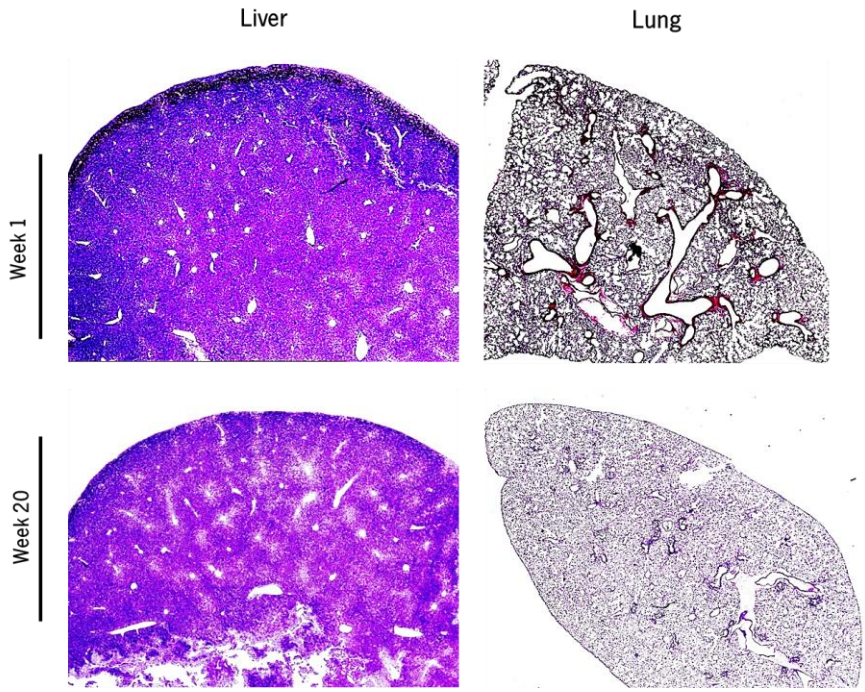

Figure 26. Histological sections of the liver and lung from WT mice showed no inflammatory reactions. Animals were infected by intranasal infection and their liver and lungs were removed at weeks 1 and 20 for histological analysis. Organs sections were stained with H&E and observed in an optical microscope. Even after 20 weeks of infection, no signs of infiltrations or granuloma formation were observed. Magnification 4x

To confirm infection, animals were euthanized at weeks 1, 2, 4 and 20 post-infection and the lungs and liver removed for histological analysis. As represented in Figure 26, at weeks 1 and 20 post-infection, animals showed no inflammatory reactions in the lungs and liver suggesting that infection was not

successful. Since this infection was not successful, we are currently investigating new approaches to establish an *in vivo* model of infection, including the establishment of an intratracheal model of infection.

CHAPTER V

DISCUSSION

Fungal pathogens are increasingly becoming a threat in the world, especially to immune compromised patients (Rizzetto, Giovannini et al. 2013). Therefore, it is extremely important to understand the nature of fungal pathogenesis and to develop new strategies capable of controlling infection. To this end, exploring the host immune responses against fungi is crucial to understand which mechanisms are involved in fungal clearance.

Paracoccidioidomycosis, a systemic mycosis prevalent in Latin America, is caused by *P. brasiliensis,* a thermodimorphic ascomycete. This fungus grows as a mycelium at environmental temperatures whereas at host temperatures it shifts to the pathogenic yeast form (Brummer, Castaneda et al. 1993). It is thought that, once inside the lungs, conidia or fragments of mycelium are phagocytosed by alveolar macrophages which then convert into the yeast form producing an infection that can also disseminate to other organs (Fortes, Miot et al. 2011). This disease is characterized by a diversity of clinical manifestations that varies from asymptomatic infection to the most severe and disseminated forms (Costa, Bazan et al. 2013). It is thought that the different forms of PCM depend on several factors, including the type of immune response triggered by the host. Indeed, a recent estimative points out that 10 million individuals are infected by *P. brasiliensis*, although only 2% develop active disease, suggesting that the majority of the population that contact with this fungus is able to mount a protective immune response (Taborda, da Silva et al. 2008). Although several studies have elucidated important aspects of the host immune response against *P. brasiliensis*, there is still a lack of knowledge mainly in the interaction between the host and fungus and the role of immunological elements (such as nitric oxide (NO) and cytokines) in the balance between inflammation and immunopathology. In this regard, we aimed at characterizing the mechanisms involved in the host defense against *P. brasiliensis* and which mediators participate in these events.

As previously referred, after entering into the lungs, *P. brasiliensis* cells face a sequence of innate mechanisms that aim at defending the host from this pathogen. Since this infection is acquired through the respiratory route, it is likely that macrophages are the first cells to interact with this fungus (Gonzalez, de Gregori et al. 2000, Blanco and Garcia 2008, Pina, Bernardino et al. 2008, Fortes, Miot et al. 2011). Taking this in consideration, we established an *in vitro* model of bone marrow-derived macrophages (BMDM) infection with *P. brasiliensis* to investigate the mechanisms underlying the macrophage response against this infection. Our data show that *P. brasiliensis* 18 induce a reduced activation of BMDM. In fact, we observed that infected BMDM did not control *P. brasiliensis* growth even when stimulated with interferon (IFN)-γ, an essential cytokine for the activation of the macrophage

effector functions. Besides IFN-γ, TNF is also an important cytokine for the activation of macrophages. Indeed, it is the synergistical effect of IFN-γ and TNF that induce the production of NO, a key molecule for macrophages to control pathogens (Mosser and Edwards 2008). When we measured the production of TNF by BMDM, we observed very low levels of this cytokine and therefore it was not surprising that these BMDM did not produce NO in response to *P. brasiliensis,* even when activated with IFN-γ. However, after stimulating these cells with both IFN-γ and LPS, a potent inducer of TNF, we observed that BMDM produced high levels of NO. More importantly, these high levels led to an improved fungal control.

Considering that *P. brasiliensis* 18 is a highly virulent strain, we questioned whether the reduced production of TNF, and consequently NO, by infected BMDM is a mechanism used by virulent strains or a more generalized mechanism common to all *P. brasiliensis* strains. To address this, we used our BMDM model of infection to determine the extent to which these cells control infection by the low virulent strain *P. brasiliensis* ATCC60855. Unlike infection of BMDM with *P. brasiliensis* 18, *P. brasiliensis* ATCC60855 did not grow in unstimulated, IFN—γ-activated or LPS-activated BMDM suggesting that these cells are very able to control infection by this strain. Accordingly, we also found that unstimulated *P. brasiliensis* 18- infected BMDM produced lower levels of TNF when compared to unstimulated *P. brasiliensis* ATCC60855-infected BMDM. Altogether, these data point to a critical role of the TNF pathway in the control of *P. brasiliensis.*

The deficient macrophage effector function is not only unique to *P. brasiliensis* infection. In fact, it was also suggested that, in infections caused by *C. albicans* and *Leishmania major*, macrophage activation with IFN-γ resulted in reduced production of NO and pro-inflammatory cytokines, leading to a less macrophage ability to kill these pathogens (Vazquez-Torres and Balish 1997, Mosser and Edwards 2008). Our results suggest that this is not the case for *P. brasiliensis* infection. Indeed, the deficient macrophage activation by IFN-γ in response to infection is a consequence of the reduced production of TNF by BMDM. Nevertheless, the role of IFN-γ in *P. brasiliensis* infection is still not well understood. While some reports suggest that IFN-γ exerts a protective role in pulmonary *P. brasiliensis* infection by activating macrophages to control fungal growth, others suggest that both unstimulated and IFN-γ-activated macrophages fail to control *P. brasiliensis* (Brummer, Hanson et al. 1988, Cano, Kashino et al. 1998, Calvi, Peracoli et al. 2003). These evidences are likely a result of the ability of *P. brasiliensis,* or at least some strains of *P. brasiliensis,* to modulate the TNF pathway.

It was been previously shown that Gp43, the main antigen secreted by *P. brasiliensis*, binds to TLR-2 or mannose receptor (MR), inhibiting macrophage effector functions such as phagocytosis and the production of NO and hydrogen peroxide. Moreover, it was also suggested that Gp43 can modulate TNF production (Almeida, Unterkircher et al. 1998, Flavia Popi, Lopes et al. 2002, Nakaira-Takahagi, Golim et al. 2011). Taking our data and these data into consideration, it is possible that Gp43 may play a critical role in the virulence of *P. brasiliensis* by modulating TNF production thus preventing macrophage activation. In this regard, it would be interesting to investigate the role of both TLR-2 and MR receptors, and PAMPs produced by *P. brasiliensis* that signal through these receptors, in the modulation of TNF production and fungal clearance. TNF inhibition by molecules secreted by virulent pathogens was also previously reported during *Mycobacterium ulcerans* infection. Indeed, it was shown that the levels of TNF produced by BMDM were strongly associated with the production of a lipidic toxin by *Mycobacterium ulcerans* known as mycolactone, In fact, while strains of *Mycobacterium ulcerans* that did not produce mycolactone induced high levels of TNF production by BMDM, mycolactone produced by virulent strains inhibited the production of TNF (Torrado, Adusumilli et al. 2007). Another aspect to take in consideration for the induction of TNF by *P. brasiliensis* is the morphology of the strain. Indeed, we show that, while *P. brasiliensis* 18 that display larger and more complex cells, induces low amounts of TNF, *P. brasiliensis* ATCC60855, that display smaller and less complex cells, induce higher amounts of TNF, suggesting that the morphology of the strain may also impact the ability of macrophages to produce TNF (Figures 14 and 19).

Altogether, these data suggest that the modulation of the TNF pathway by *P. brasiliensis* is an important virulence mechanism, but a complex one, as it is likely that this modulation is accomplished by multiple mechanisms. Hereafter, we will take advantage of the *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocol, previously optimized in our laboratory, to generate avirulent *P. brasiliensis* strains to better define the impact of *P. brasiliensis* morphology in virulence (Almeida, Carmona et al. 2007).

Considering the differences observed in TNF production and BMDM ability to control *P. brasiliensis* 18 and *P. brasiliensis* ATCC60855, we next questioned whether IL-10 could be responsible for these differences. However, we found that BMDM induce the same levels of IL-10 in response to *P. brasiliensis* 18 or *P. brasiliensis* ATCC60855. Furthermore, both WT and IL-10$^{-/-}$ BMDM were equally able to control *P. brasiliensis* ATCC60855, suggesting that induction of IL-10 is not a mechanism whereby *P. brasiliensis* modulates macrophage activation. In this regard, much effort has been made to

clarify the role of IL-10 in fungal infections. However, a consensus has not been reached. Several studies have pointed to the deleterious role of IL-10 in fungal infections (Gazzinelli, Wysocka et al. 1996, Costa, Bazan et al. 2013). However, others suggested the beneficial role of IL-10 in controlling the inflammatory response and fungal growth (Romani and Puccetti 2006). Interestingly, a recent study by Costa *et al.* showed that mice deficient in IL-10 are better able to control *P. brasiliensis* without the development of pathological consequences to the host (Costa, Bazan et al. 2013). Taking this and our results in consideration, we suggest that macrophages are not the main source of IL-10 during *P. brasiliensis* infection.

With the above data in mind, we next investigated the mechanisms underlying the IL-10-mediated susceptibility during *P. brasiliensis* infection *in vivo.* To this end, we use the pMT-10 mice that overexpress IL-10 when exposed to zinc. Our results show that animals overexpressing IL-10 exhibit more pronounced lesions in the liver at week 8 post-infection. Unexpectedly, we observe an increased recruitment of inflammatory monocytes and neutrophils in the lungs of these mice. Inflammatory monocytes exhibit a high antimicrobial ability to phagocyte pathogens and to secrete important inflammatory cytokines including TNF, IL-1β and IFN-γ. Moreover, these cells selectively circulate to the sites of infection to produce inflammatory cytokines contributing to a systemic inflammation (Yang, Zhang et al. 2014). Thus, due to this property to circulate to the sites of infection, inflammatory monocytes can also contribute to fungal dissemination. Taking this in consideration, we suggest that IL-10 overexpression promotes a more severe infection in the late phase of infection by enhancing the recruitment of inflammatory monocytes to the *P. brasiliensis*-infected lungs. It is possible that, by circulating to other organs, inflammatory monocytes might transport *P. brasiliensis* from the lungs to other organs. Altogether, our results provide evidences to the important role of IL-10 as a target molecule to control *P. brasiliensis*. To address these hypotheses we are establishing an intranasal model of *P. brasiliensis* infection to mimics the natural route of infection that will allow us to elucidate these aspects and also extend our knowledge. Since our laboratory has never performed intranasal infection of mice, we are currently optimizing this infection. We first started by optimizing the conidia isolation protocol but we did not obtain a high yield, which may be explained by the difficulty in separating the mycelium from the propagules (Restrepo, Salazar et al. 1986, del, Restrepo et al. 2004). Although we were not able to set up a new model, we are currently testing new approaches to establish a model of infection.

Altogether, the work herein presented improves our knowledge on the host immune response against *P. brasiliensis*. Indeed, we provide evidences suggesting TNF as a key mediator for macrophage response against this infection. In addition to this, for the first time we found differences in the macrophage response against *P. brasiliensis* strains with different degrees of virulence, which can explains the wide spectrum of clinical forms of the disease (Figure 27). Moreover, with the relevant evidences found during the *in vivo* infections, we add additional knowledge on the role of IL-10 in *P. brasiliensis* infection. The findings here presented should be taken in consideration since it could be important for the future development of new treatments for PCM.

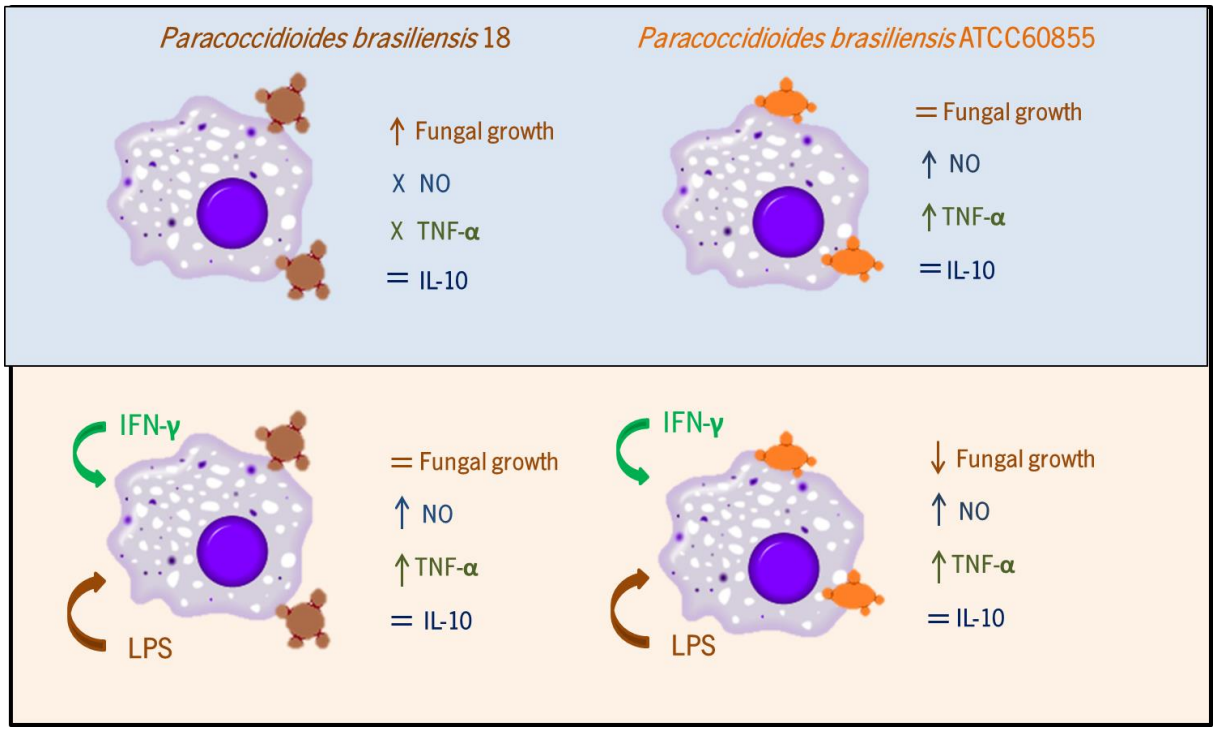

Figure 27. Different *P. brasiliensis* strains induce distinct macrophage responses. Without exogenous stimulation, BMDM are not capable of controlling *P. brasiliensis* 18. However, the number of viable *P. brasiliensis* ATCC60855 yeast cells remains sustained over time. TNF is differently produced by *P. brasiliensis* and *P. brasiliensis,* suggesting the importance of this mediator in the immune response against *P. brasiliensis*.

CHAPTER VI

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Fungi are heterotrophic eukaryotes ubiquitous in the environment. Most fungi interact with humans without causing disease (Romani 2004). Nevertheless, some fungi such as *H. capsulatum* and *P. brasiliensis* are opportunistic triggering an exhaustive infection and consequently disease in immunocompromised hosts. Since the percentage of immunosuppressed individuals has increased, fungi became a threat to humans (Pappas 2010).

In this work, we clarify some aspects of the host immune response against *P. brasiliensis*. Indeed, we suggest that TNF pathway is crucial for the fungicidal activity of macrophages and that *P. brasiliensis* interferes with this pathway to survive inside the host. Taking this in consideration, our work suggests that TNF is a possible target molecule to control this infection. We also found an association between morphology and virulence which can explains the different macrophage responses against strains with different degrees of virulence. Since we only investigated the macrophage response against two *P. brasiliensis* strains, it would be interesting to study the impact of other strains, including clinical isolates. In this regard, the development of avirulent strains by a silencing CDC42, a gene involved in the regulation of *P. brasiliensis* morphology, would be a useful tool for both *in vitro* and *in vivo* infections.

We also found new evidences for the impact of IL-10 during *P. brasiliensis* infection. While in our *in vitro* model of infection, IL-10 did not impact macrophage effector functions, the *in vivo* model of infection showed that high levels of IL-10 resulted in a more severe inflammatory reaction in the lungs with the accumulation of inflammatory monocytes. We hypothesize that these cells may contribute to the dissemination of the fungus. We will investigate this negative role of inflammatory monocytes during infection by depleting inflammatory monocytes in pMT-10 mice exposed to zinc.

In summary, our work provides important information regarding the host immune response against *P. brasiliensis*. The establishment of the *in vitro* model of infection provided important clues regarding the ability of different *P. brasiliensis* strains to modulate the macrophage response. We will take advantage of this model to further dissect how *P. brasiliensis* modulates the macrophage response. This information may help us define novel fungal or immunological therapeutic targets.

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