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Estudo dos mecanismos de ação do anticorpo monoclonal F1.4 gerado contra leveduras de Paracoccidioides brasiliensis

Tese apresentada ao Programa de Pós-graduação em Microbiologia do Instituto de Ciências Biomédicas da Universidade de São Paulo para obtenção do título de Doutora em Ciências (Microbiologia).

Orientador: Professor Dr. Carlos Pelleschi Taborda

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RESUMO

A paracoccidioidomicose (PCM) é uma micose sistêmica causada por fungos termodimórficos do gênero *Paracoccidioides*. É endêmica na América Latina, desde o Sul do Mexico até o Norte da Argentina, sendo o Brasil o país mais afetado pela doença. O principal órgão afetado pela PCM são os pulmões, embora possa se disseminar para outras partes, como fígado, baço, pele, linfonodos e mucosas. Sendo que a forma clinica mais comum da PCM é a forma crónica, a terapia usual da PCM consiste na administração, a longo prazo, de antifúngicos, cuja toxicidade é amplamente conhecida. Uma alternativa promissora para o tratamento de micoses sistêmicas é baseada na imunoterapia com anticorpos monoclonais. Nos propomos o estudo dos mecanismos de ação pelos quais o anticorpo monoclonal F1.4 (mAb F1.4), gerado contra um antígeno glicoproteico de parede celular de *P. brasiliensis,* pode ter efeito protetor no tratamento da PCM experimental. Neste projeto, analisamos os mecanismos de ação do mAb F1.4 e seu efeito na transferência passiva para camundongos infectados com *P. brasiliensis* (Pb18). Observamos *in vitro* um aumento na capacidade fagocítica e na concentração de nitrito de sódio induzida por macrófagos murinos. Além disso, detectamos uma redução significativa na viabilidade celular de leveduras de *P. brasiliensis* e *P. lutzii* fagocitadas. *In vivo*, uma redução significativa na carga fúngica pulmonar foi detectada quando o mAb F1.4 usado em combinação com SMX/TMP foram administrados simultaneamente. Essa redução das UFC foi correlacionada com um aumento nos títulos das citocinas IFN-γ, TNF- α, IL-17, bem como uma diminuição nos títulos de IL-10 e IL-4 sugerindo uma resposta imune do tipo Th1. A análise histopatológica dos tecidos pulmonares indicou melhora da infecção com redução do número de leveduras dentro dos granulomas, bem como granulomas organizados e bem definidos.

Palavras chave: anticorpos monoclonais, paracoccidioidomicose, imunoterapia, beta glucanas fúngicas, hibridoma.

* Normas ABNT: Associação Brasileira de Normas Técnicas.

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ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by thermal dimorphic fungi of the *Paracoccidioides* genus. It is endemic in Latin America, from southern Mexico to northern Argentina, with Brazil being the country most affected by the disease. The main organ affected by PCM are the lungs, although may disseminate to other parts, such as liver, spleen, skin, lymph nodes and mucous membranes. Because the most common clinical form of PCM is chronic, the usual therapy for PCM is the long-term administration of antifungal drugs, which toxicity is widely known. A promising alternative for the treatment of systemic mycoses is based on immunotherapy with monoclonal antibodies. We proposed the study of the action mechanisms by which the monoclonal antibody F1.4 (mAb F1.4), generated against a glycoproteic antigen from cell wall of *P. brasiliensis*, in association with an antifungal drug (SMX/TMP) may have a protective effect in the treatment of experimental PCM. In this project, we analyzed the action mechanisms of mAb F1.4 and their effect on passive transference to mice infected with *P. brasiliensis* (Pb18). We observed *in vitro* an increase in the phagocytic capacity and the nitric oxide concentration induced by murine macrophages. Also, we detected a significative reduction in cell viability of yeast of *P. brasiliensis* and *P. lutzii* phagocytized. *In vivo*, a significant reduction in the pulmonary fungal burden was detected when mAb F1.4 were used in association with TMP/SMX, simultaneously. This reduction in CFUs was correlated with an increase in titers of cytokines IFN- γ, TNF- α and IL-17, as well as a decrease in IL-10 and IL-4 titers, suggesting a Th1 type immune response. Histopathology analysis of lung tissue indicated improvement of the infection with reduction in the number of yeasts within the granulomas, as well as organized and well-defined granulomas.

Key words: monoclonal antibodies, paracoccidioidomycosis, immunotherapy, hybridoma cell lines.

* ABNT Norms: Associação Brasileira de Normas Técnicas.

Introduction

Literature Review

A. General aspects of Paracoccidioidomycosis

Pulmonary mycoses caused by endemic or by opportunistic fungi constitute an important public health issue (reviewed in MILLER; WILMOTT, 2019). Pulmonary fungal infections are usually self-limited, but some patients develop acute or chronic infection (reviewed in DI MANGO *et al*., 2019). Pulmonary mycoses are a major cause of death in immunocompromised patients; however, immunocompetent individuals can be affected by endemic mycoses such as blastomycosis, histoplasmosis, coccidioidomycosis and paracoccidioidomycosis (reviewed in BELLMANN; BELLMANN-WEILER; WEILER, 2008; DI MANGO *et al*., 2019).

Endemic mycoses are recognized by their capacity to cause illness in otherwise healthy people and by their particular distribution in specific geographic areas (reviewed in MILLER; WILMOTT, 2019). A great number of people are infected annually with endemic fungi, mainly those who live in endemic regions or in areas with a high rate of migration of people from endemic regions (reviewed in DI MANGO *et al*., 2019; MILLER; WILMOTT, 2019).

Thermal dimorphic fungi in the *Paracoccidioides* genus cause an endemic disease known as paracoccidioidomycosis (PCM), a systemic mycosis characterized by a granulomatous reaction (SHIKANAI-YASUDA *et al.*, 2017). Adolfo Lutz described the disease for the first time in 1908. However, it was until 1930 that the etiologic agent was characterized and named as *Paracoccidioides brasiliensis* (reviewed in TURISSINI *et al*., 2017).

Endemic to Latin America, PCM is prevalent in residents from southern Mexico to northern Argentina (reviewed in AMEEN; TALHARI; TALHARI, 2009; MARTINEZ, 2017).

The disease mainly affects people living in rural areas and agricultural workers. However, cases have also been reported in peri-urban areas (reviewed in AMEEN; TALHARI; TALHARI, 2009, MARTINEZ, 2017). Cases of human PCM have been reported in USA, Europe, Asia and Africa; however, all the cases were considered to be imported cases, since all of them were from people who lived and/or were involved with agricultural work in endemic areas (MARTINEZ, 2017; SHIKANAI-YASUDA *et al.*, 2017).

B. Epidemiology of PCM

According to a study by *Prado et al*., PCM is the most prevalent systemic mycosis in Latin America. Brazil is the most affected country, presenting about 80% of total cases, as well as the highest mortality rate (168 deaths / year) (PRADO *et al*., 2009).

The real impact of PCM on global public health cannot be accurately evaluated, due to a lack of information (MARTINEZ; BELLISSIMO-RODRIGUES; MACHADO, 2011). Some authors consider that epidemiologic records do not reflect the reality of PCM (SHIKANAI-YASUDA *et al*., 2006) because PCM is not a disease compulsory reported to government health authorities. Hence, PCM cases are underestimated in Brazil *(*COUTINHO *et al*., 2005; 2015) and its epidemiology has been estimated using regional records (MARTINEZ, 2017; MARTINEZ; BELLISSIMO-RODRIGUES; MACHADO, 2011).

According to a study performed from 1996 to 2006, PCM is the systemic mycosis that caused more deaths during the decade studied by authors (PRADO *et al.*, 2009; reviewed in MARTINEZ, 2017). This study found that a PCM lethality rate of 3 - 5% could be associated to between 3360 and 5600 cases of PCM per year in Brazil (reviewed in MARTINEZ, 2017). The greatest prevalence was reported in the Southwest Region of Brazil (with the states of São Paulo, Rio de Janeiro, Minas Gerais and Espiritu Santo being the most affected) and in the South Region (with the states of Paraná and Rio Grande do Sul being the most affected).

Brazil, Venezuela, Colombia, Argentina and Ecuador are the countries that

show highest prevalence of PCM (reviewed in BOCCA. *et al*., 2013; MARTINEZ, 2017). According to an estimative based on previous studies, the annual incidence in endemic areas varies from 1 to 4 cases per 100,000 inhabitants (reviewed in MARTINEZ, 2017, VIEIRA *et al*., 2014). In the hyper-endemic areas, such as the western Amazon region of the State of Rondônia- Brazil, the incidence of PCM reaches 9.4 cases per 100,000 inhabitants per year, and municipalities in the southern region of this state have about 40 cases per 100 000 inhabitants per year (VIEIRA *et al*., 2014). According to Vieira *et al*., the rise in the number of cases observed in the state of Rondônia was correlated with the process of deforestation to a create fields for agriculture or cattle breeding (VIEIRA *et al*., 2014).

Countries such as Chile, Suriname, Guyana, Belize and Nicaragua have not reported autochthonous cases yet (RESTREPO; MCEWEN; CASTAÑEDA, 2001; SHIKANAI-YASUDA *et al.*, 2017). In non-endemic regions outside the tropical and subtropical areas of Latin America, such as Europe, Asia and USA, imported cases of PCM have been reported. This number has increased in recent years due to migration from Latin America to Europe and USA and Canada (reviewed in BUITRAGO *et al*., 2011).

Based on the study published in 2015 by Coutinho and collaborators, the period from January 1998 to December 2006, registered a total of 6,732 cases. This study used data available from the Hospital Information System of the Public Health System (SUS) in Brazil. The annual average of cases was 748/year and the rate of hospital admissions for PCM was 4.3 admissions per 1 million inhabitants, with cases being reported in the 26 states of the country. According to the place of residence, the South region has the highest rate of PCM infections, accounting for 8% of cases throughout Brazil. The Northeast region of Brazil has the lowest rate of PCM (COUTINHO *et al.*, 2015).

Overall, the Midwest and North regions of Brazil have the highest rates of hospitalizations and mortality rates, demonstrating that they are important endemic areas of PCM in the country (PRADO *et al*., 2009; reviewed in MARTINEZ, 2017).

Armadillos are the most common wild host of *Paracoccidioides* spp. *Paracoccidioides brasiliensis* has been isolated from nine-banded armadillos (*Dasypus novemcinctus*) in diverse areas where PCM is endemic (BAGAGLI *et al.*, 2003; RESTREPO; MCEWEN; CASTAÑEDA, 2001). In a study performed by

Bagagli *et al., P. brasiliensis* was isolated in 75-100% of the *D. novemcinctus* captured in hyperendemic areas (BAGAGLI *et al.*, 2003). Because of their body temperature (32,5-37° C) and continuous interaction with soil, armadillos are optimal hosts for *Paracoccidioides* spp. and could maintain their cycle of infection in nature (BAGAGLI *et al*., 2006). *Paracoccidioides* spp. has been isolated from other animals such as dogs, penguins, sloths, dolphins, and bats (BAGAGLI *et al*., 2003; VILELA *et al*., 2016).

An important factor that impacts the dynamics of human infection with *Paracoccidioides* spp. is agricultural expansion, which causes massive deforestation and supports the contact between agricultural workers and other people exposed to the soil containing the fungi (reviewed in MARTINEZ, 2017).

Figure 1 shows how the infection is acquired through the inhalation of fragments of hyphae and / or chlamydoconidia present in aerosols generated when handling soil (BAGAGLI *et al*., 2006; SHIKANAI-YASUDA *et al.,* 2017). These infectious structures invade the airways and, once in contact with the pulmonary alveoli, a morphological change to the parasitic form of yeast occurs due to the tissue temperature (37 ºC), leading to primary infection in the lungs (reviewed in BONICHE *et al.*, 2020; BRUMMER; CASTANEDA; RESTREPO, 1993). The yeasts are then able to spread via lymph and blood, reaching other organs, mainly spleen, liver, bones and central nervous system (reviewed in DE OLIVEIRA *et al.*, 2015).

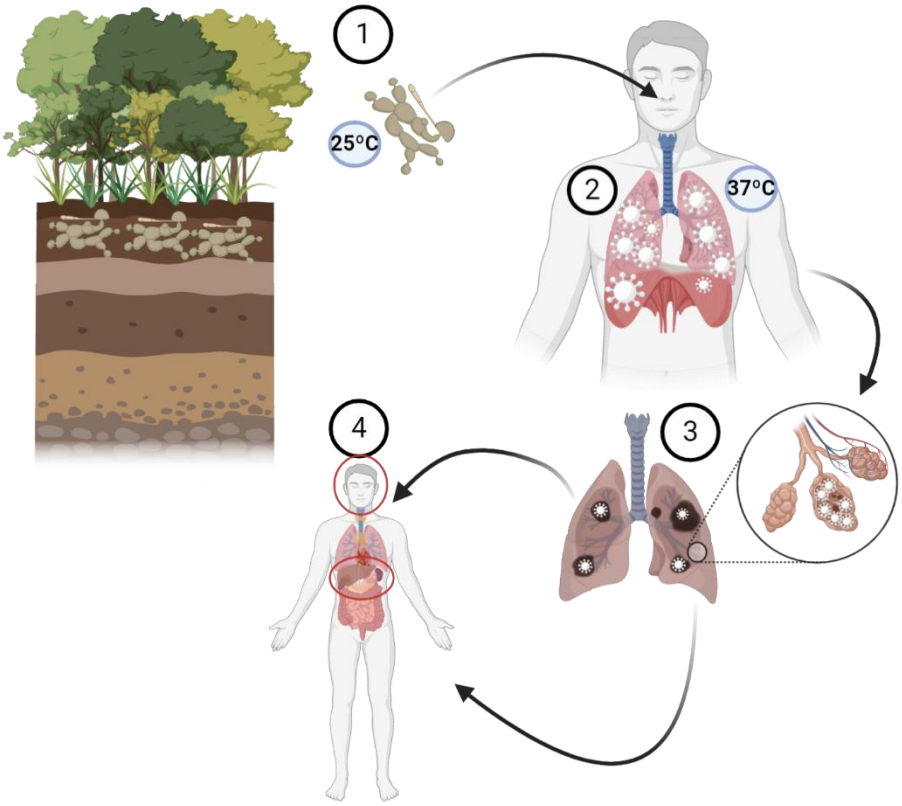

Figure 1. Cycle of infection of *Paracoccidioides* sp.**1:** The infection is acquired through inhalation of fragments of hyphae and / or chlamydoconidia, present in aerosols generated when handling soil. **2**: Infectious structures invade the airways and once in contact with the pulmonary alveoli a morphological change to the parasitic yeast form occurs due to the tissue temperature, leading to primary infection in the lungs. **3**: The yeasts are able to spread via lymphatic and hematogenous. **4:** The yeasts can reach other organs, mainly spleen, liver, bones and central nervous system. Created with Biorender.com

C. Etiological agents of PCM

The etiological agents that cause PCM belong to the genus of thermal dimorphic fungi *Paracoccidioides.* This genus belongs to the *Ajellomycetacea* family, order *Onygenalles*, a characteristic they share with other thermal dimorphic fungi of medical importance (UNTEREINER *et al*., 2004; reviewed in MARTINEZ, 2017). The genus was named in 1930 by Floriano Almeida after its marked differences with *Coccidioides immitis,* the etiological agent of a similar mycosis endemic in North America.

The genus was classified as one species known as *Paracoccidioides lutzii* and one species complex, the *Paracoccidioides brasiliensis*, containing four cryptic species *(MATUTE et al., 2006; TEIXEIRA et al*., 2014a, 2014b). Turissini

et al., (2017) propose the re-classification of the species in the *P. brasiliensis* species complex as: *P. brasiliensis* (previously S1), *P. americana* (previously PS2), *P. restrepiensis* (previously PS3) and *P. venezuelensis* (previously PS4) (TURISSINI *et al*., 2017). All the four cryptic species have different evolutionary features and a different geographic distribution. Turissini *et al*., (2017) demonstrated by an analysis of nuclear genes and mitochondrial DNA that despite the fact that the four species of the *Paracoccidioides brasiliensis* species complex have had the chance to interbreed, they are vastly differentiated at nuclear loci *(*TURISSINI *et al*., 2017).

P. brasiliensis (S1) is considered a recombinant, monophyletic group with a wide distribution in South America and it is believed to be the responsible for most of the PCM cases. *P. americana* (PS2), found in Brazil and Venezuela, is considered a recombinant paraphyletic group. *P. restrepiensis* (PS3) is a monophyletic clonal population reported exclusively in Colombia. *P. venezuelensis* (PS4) was described more recently and classified as a monophyletic group found in Venezuela (MATUTE *et al*., 2006; TURISSINI *et al.*, 2017).

P. lutzii is considered a monophyletic group, and is endemic to the North (State of Rondônia) and the Midwest regions (States of Mato Grosso and Goiás) of Brazil (TEIXEIRA et al., 2014a).

The genus *Paracoccidioides* has thin, branched, septate, aerial mycelia and chlamydoconidia at 25° C (reviewed in DE OLIVEIRA *et al*., 2015; SAN-BLAS, 1993). The fungus is found in soil from tropical and subtropical forest, with temperatures going from 17° C to 24° C, high rainfall and dense foliage (reviewed in BRUMMER; CASTANEDA; RESTREPO, 1993).

At 37 ° C, *Paracoccidioides* spp. have oval or rounded yeast shape of variable size (6 to 40 µm), usually with multiple buds some of them presenting the pathognomonic form of yeasts in the shape of a ship's wheel (RESTREPO *et al*., 2011).

During the transition of the mycelial phase to the pathogenic yeast, the cell wall of the fungus changes and expresses an increase in the alpha glucan content (SAN-BLAS, 1993). The individual characteristics of each species have different implications in the diagnosis, clinical manifestations, treatment and recovery from the disease (TEIXEIRA et al., 2014b).

D. Paracoccidioidomycosis

The broad spectrum of clinical and immunological symptoms allows to classify PCM into three major forms, (DE CASTRO *et al*., 2013). Progression to clinical manifestations of the infection mainly depends on the host immune response type. The type of immune response is influenced and immunomodulated by the individual living conditions as well as their demographic and genetic characteristics (reviewed in MARTINEZ, 2017).

The asymptomatic form of PCM may go unnoticed in most cases of infection (reviewed in BENARD, 2008) and is typically detected in healthy patients who exhibit positive results for cutaneous tests (DE CASTRO *et al*., 2013).

The acute / subacute form or juvenile form of PCM, represents 5-25% of PCM cases. Occurs in children, teenagers and young adults, being predominantly at puberty and affecting men and women equally (BLOTTA *et al.*, 1999). Juvenile form of PCM can be frequently observed in some endemic regions, like the states of Maranhão, Minas Gerais, Pará, Goiás and São Paulo in Brazil (SHIKANAI-YASUDA *et al.,* 2017). This form of PCM generally exhibits lymphadenomegaly and skin lesions as predominant manifestations. The juvenile type is characterized by high specific antibody levels, associated with a Th2 immune response. Usually, it takes weeks to months for the patient to fully recover (SHIKANAI-YASUDA *et al.*, 2006, 2017).

The chronic form or adult form has a higher prevalence between the third and sixth decade of life and is clinically more common, accounting for 75 to 96% of PCM cases (BELLISSIMO-RODRIGUES; MACHADO; MARTINEZ, 2011; SHIKANAI-YASUDA *et al*., 2017). This form occurs mainly in men (75% to 95% of total cases) (BLOTTA *et al.*, 1999). Women are infected with *Paracoccidioides* spp. as much as men, although they are less likely to develop PCM. Due to higher levels of circulating estrogen, women are able to inhibit the transformation of inhaled conidia into yeast, and modulate the cellular immune response against *Paracoccidioides* spp. (reviewed in MARTINEZ, 2017; SHANKAR *et al.*, 2011). An additional factor that may explain the higher proportion of cases in men than in women is the higher proportion of men involved in agricultural activities in endemic areas (BELLISSIMO-RODRIGUES *et al.*, 2013).

It is common for patients living in endemic areas, or ex-residents to develop the disease several decades after being infected, since the chronic form of PCM can appear years later. The latency period of chronic PCM is on average 15 years (reviewed in AMEEN; TALHARI; TALHARI, 2009; SHIKANAI-YASUDA *et al*., 2017) .

The classic symptoms of the chronic form include pulmonary (32% of cases) and mucosal involvement, hoarseness, cough and dyspnea. In adult patients the oral, pharyngeal and laryngeal mucosa are usually affected, causing granular-looking ulcers and hemorrhagic spots (reviewed in BENARD, 2008; MARQUES, 2013; SHIKANAI-YASUDA *et al.*, 2006). Chronic PCM has a recurrence rate of 30% (reviewed in AMEEN; TALHARI; TALHARI, 2009). Chronic PCM in immunosuppressed patients leads to a disseminated disease similar to the acute form, presenting an atypical form of the disease known as opportunistic (reviewed in BENARD; DUARTE, 2000).

There is one last form of the disease known as residual or sequel PCM. It is characterized by the clinical manifestations caused by the scars that are consequence of the prolonged treatment of the PCM (SHIKANAI-YASUDA *et al.*, 2017). The pulmonary fibrotic consequences of PCM remain even if the appropriate antifungal treatment reduces the progression of the disease. Relapses can happen long after the patient finishes the treatment, since *Paracoccidioides* spp. can re-activate after prolonged periods of dormancy (reviewed in SHIKANAI-YASUDA *et al.*, 2015).

The pattern of the immune response of each individual against *Paracoccidioides* spp. determines the progress of the disease and its clinical consequences (BONFIM *et al.,* 2009). It has been reported that reactive immunity against *Paracoccidioides* spp. differs between healthy individuals, those with chronic disease, or with acute / subacute form, and between people who healed from the mycosis spontaneously (reviewed in BENARD, 2008).

E. Immune response against PCM

In the infection with *Paracoccidioides* spp., the host's cellular immune response plays an important role in the inflammatory reaction against the fungus.

Monocytes and macrophages are the main effector cells against *Paracoccidioides* spp., causing the death of the yeasts by oxidative mechanisms and modulating the production of cytokines (reviewed in DE OLIVEIRA *et al*., 2015; FORTES *et al*., 2011; NASCIMENTO *et al*., 2002).

In order to control PCM efficiently, the host's immune system must develop a Th1-type immune response, in which the production of IFN-γ, TNF-α and IL-2 could be capable of activating and promoting monocytes, macrophages and lymphocytes T maturation. This process leads to phagocytosis stimulation and to granuloma formation, which are necessary to prevent the spread of *Paracoccidioides* spp. yeasts and block their replication within the lungs (reviewed in BENARD, 2008; FORTES *et al.*, 2011). In PCM, resistance to the fungus is dependent on the activity of T Helper $CD4^+$ lymphocytes and macrophages/monocytes, mediated by cytokines IFN-γ and TNF-α (reviewed in BENARD, 2008; FORTES *et al*., 2011).

The activation of the pulmonary macrophages is induced by the fungal antigens, since those are recognized by pattern recognition receptors (PRRs) as illustrated in **Figure 2**. Pulmonary macrophages are also induced by the increase in IFN-γ concentration, which stimulates the production of nitric oxide (NO) by activated macrophages, enhancing their microbicide activity (NAKAIRA-TAKAHAGI *et al.*, 2011). However, the interactions between toll like receptors and *P. brasiliensis* is considered an evasive mechanism established by the yeasts for survival inside phagocytic cells (reviewed in FORTES *et al.,* 2011).

The main antigen of *P. brasiliensis* is a 43 kDa glycoprotein known as gp43, predominantly found in the sera of patients with chronic PCM (SILVA *et al.*, 2017; TABORDA *et al.*, 1998; UNTERKIRCHER *et al.*, 1996). This secreted glycoprotein is also present in the cell wall of the yeasts and it is involved in the process of endocytosis, related to the adherence and absorption of *Paracoccidioides* spp. to macrophages (PUCCIA;TRAVASSOS, 1991; TABORDA *et al*., 2015).

Popi *et al*. (2002) demonstrated *in vitro* that gp43 inhibits the phagocytosis of *P. brasiliensis* yeasts by non-activated mice peritoneal macrophages. Additionally, gp43 inhibits the activation and the ability of pulmonary

macrophages to kill the yeasts (reviewed in DE OLIVEIRA *et al.*, 2015; TRAVASSOS; TABORDA, 2012).

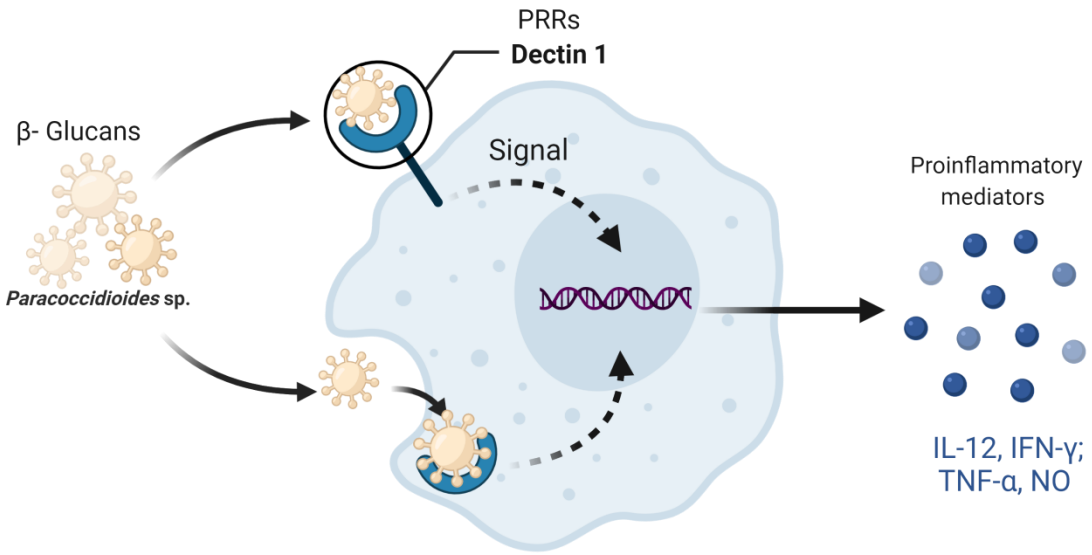

Figure 2. Illustration of macrophage activation via Dectin 1 by the panfungal antigen β- Glucan. Dectin-1, a PRR present on the surface of host phagocytic cells, identifies fungal cell wall β-(1,3)-glucan and induct phagocytosis, respiratory burst, and release of cytokines such as TNF-α and IL-12, amongst others (reviewed in CAMACHO; NIÑO-VEGA, 2017). Created with Biorender.com.

During the early stage of infection, neutrophil cells also play a protective role by producing reactive oxygen and nitrogen species (DIAS *et al*., 2008). Neutrophils exert their greatest protective capacity during the acute phase of infection (24 to 96 hours). However, a study performed by Puerta-Arias *et al*., determined that in the chronic phase of infection, neutrophil depletion resulted in a decreased number of CFU (PUERTA-ARIAS *et al.*, 2016). It has been described that neutrophils play an important role in the pathogenesis of PCM producing large quantities of chemokines such as leukotrienes and prostaglandin E2, which produce edema and promote inflammation (reviewed in FORTES *et al*., 2011; GONZÁLEZ, 2020).

Studies have shown that in experimental infections with *P. brasiliensis*, dendritic cells migrate to the lymph nodes, acting as the first contact in activating the Th1 type response (DOS SANTOS; SPADARI; DE ALMEIDA, 2011).

Regarding the humoral immune response to PCM, *Paracoccidioides* spp. is capable to activate B lymphocytes to generate antibodies against the fungus (LARA DE CARLI *et al*., 2015).

F. Diagnostic of PCM

Diagnosis of pulmonary systemic endemic mycoses is difficult, particularly in low-prevalence areas where medical doctors may not be aware of the clinical manifestations (reviewed in DI MANGO *et al*., 2019).

The gold standard for the diagnosis of PCM is based on the demonstration of the presence of pathognomonic yeasts in clinical samples by direct microscopy and/ or by culture of clinical samples such as bronchioalveolar lavage, sputum, expectorations, nasopharyngeal secretions, mucocutaneous biopsies (SHIKANAI-YASUDA *et al*., 2017). The Grocott-Gomori methenamine silver stain technique demonstrates the typical budding yeast structures of *Paracoccidioides* sp. (reviewed in DI MANGO *et al*., 2019). However, cultures of *Paracoccidioides* sp. from clinical samples are difficult because of its slow growth taking up to a month (reviewed in AMEEN; TALHARI; TALHARI, 2009). The diagnosis of the disease is supported by the detection of antibodies against *Paracoccidioides* spp. yeasts. In immunocompetent patients, serological techniques allow the detection and quantification of antibodies against *Paracoccidioides* spp., which are also used to monitor the evolution of the disease and the patient's response to the treatment. (reviewed in AMEEN; TALHARI; TALHARI, 2009; GEGEMBAUER *et al*., 2014). Among the different serological techniques used, double gel immunodiffusion (DID) is the most commonly used (DE CAMARGO; DE FRANCO, 2000). However, its sensitivity ranges from 80 to 95% due to the differences in the preparation of the diagnostic antigens used in the different laboratories (VIDAL *et al.*, 2014).

Additional tests commonly used for the diagnosis of PCM are indirect immunofluorescence, immunoenzymatic assays, immunoblots, and molecular assays such as PCR (SHIKANAI-YASUDA *et al*., 2017).

G. Treatment of PCM

Treatment of PCM with antifungal drugs depends on the severity of each case and must be administered for long periods of time (minimum periods of 1.5 years) to achieve a resolution of the infection (reviewed in AMEEN; TALHARI; TALHARI, 2009; SHIKANAI-YASUDA *et al.*, 2017). The options for mild to moderate clinical forms are sulfonamides (trimethoprim sulfamethoxazole) and azole derivatives (itraconazole, fluconazole and voriconazole) (SHIKANAI-YASUDA *et al.*; 2006, 2017).

In severe or widespread cases, the drug of choice used is amphotericin B (reviewed in BOCCA *et al.*, 2013; SHIKANAI-YASUDA *et al*., 2017). However, amphotericin B has disadvantages such as nephrotoxicity when the deoxycholate form of the drug is administered to the patients (TUON *et al*., 2013). Due to toxicity, the use of the liposomal form and the lipid complex of amphotericin B is preferred. However, as these preparations are less toxic and the drug reaches higher concentrations on tissues - including the central nervous system, they are expensive and less preferred (BELLMANN, 2007).

Patient lack of adherence to the PCM treatment is the most common reason of therapeutic failure as a consequence of the constant use of antifungal drugs for extended periods (SHIKANAI-YASUDA *et al*., 2017).

Antifungal drugs are the basis for the treatment of systemic mycoses in both immunocompetent and immunocompromised patients (reviewed in TABORDA; NOSANCHUK, 2017). Based on issues such as toxicity, the resistance of some isolates to available drugs, the high cost of less toxic antifungal drugs, the chances of relapse, and the prolonged treatment regimens, there is an urgent need to study and develop new drug options to treat invasive mycoses (reviewed in TRAVASSOS; TABORDA; COLOMBO, 2008; TABORDA; NOSANCHUK, 2017; TRAVASSOS; TABORDA, 2017).

H. Novel Antifungic Therapies: Monoclonal Antibodies

This subtopic was widely addressed in our published review “Immunotherapy against systemic fungal infections based on monoclonal antibodies” (**Annex 1**). This review aimed to updating the progress made in the

field of therapeutic antifungal vaccines based on mAbs against systemic mycoses. Also covered the recent progress on mAb therapies and the current challenges faced in therapeutic antifungal vaccines (BONICHE *et al.*, 2020).

The role of antibody mediated immunity in fungal infections was explained as a consequence of the developments made in the decade of 1970 by the scientists Cesar Milstein and Georges J. F. Köhler with the creation of the hybridoma technology (reviewed in LEAVY, 2016). Hybridomas enabled the generation of monoclonal antibodies (mAbs) in large amounts, but mainly, allowed the understanding of the immunological role that specific antibodies can exert in the adaptative and humoral immune responses (reviewed in BONICHE *et al*., 2020).

Monoclonal antibodies are complex and versatile molecule proteins produced by a single clone of B lymphocytes. Those molecules are composed of constant regions that interact with the immune system, and by variable regions that are in charge of the recognition of an exclusive and predetermined epitope in an antigen, as illustrated in **Figure 3**. (reviewed in LEAVY, 2016; STROHL, 2014).

The main challenge of monoclonal antibody production by hybridoma technology is the demand of purified antigens to generate a specific immune response in mice. Depending on the nature of the antigen, its purification could be demanding and sometimes, not cost- effective. Moreover, purified recombinant proteins in association with adjuvants can lead to the modification of the native conformation of the recombinant protein, resulting in unwanted immune responses in the immunized mice (reviewed in PARRAY *et al.*, 2020).

Regarding disadvantages, the use of monoclonal antibody therapies requires the manipulation of large cultures of mammalian cells, which is expensive and labor-intensive. The purification process should assure conditions of good manufacturing practices, which leads to higher production expenses and it is time consuming. Storing the mAbs is also more difficult than conventional antifungal drugs. Additionally, because mAbs are highly specific, they can be administered only if there is a precise diagnosis. And finally, the efficacy of mAbs may decrease rapidly as the infection develops with time (reviewed in BONICHE *et al*., 2020; CASADEVALL; DADACHOVA; PIROFSKI, 2004; CASSONE, 2008; ROMANI, 2011; SAYLOR; DADACHOVA; CASADEVALL, 2009; SPELLBERG,

2011; TABORDA; NOSANCHUK, 2017; LISTEK *et al*., 2020).

Because monoclonal antibodies are (generally) produced in mice, they are efficiently rejected by the body's immune system, which produce human anti mouse antibodies, known as HAMA (DENARDO *et al*., 2003; MIRICK *et al*., 2004). However, the application of humanized mAb therapies holds a great potential as a directed therapeutic option in many clinic areas, such as oncology, rheumatology and organ transplant (reviewed in BONICHE *et al*., 2020; BUGLI *et al*., 2013).

It is known that the mechanisms by which anti-tumor mAbs inhibit or kill cancer cells are varied, including direct induction of tumor cell apoptosis, antibody dependent cellular cytotoxicity, inhibition of growth factor receptor tyrosine kinases and complement dependent cytotoxicity (reviwewd in LIU *et al*., 2009; SCOTT; ALLISON; WOLCHOK, 2012).

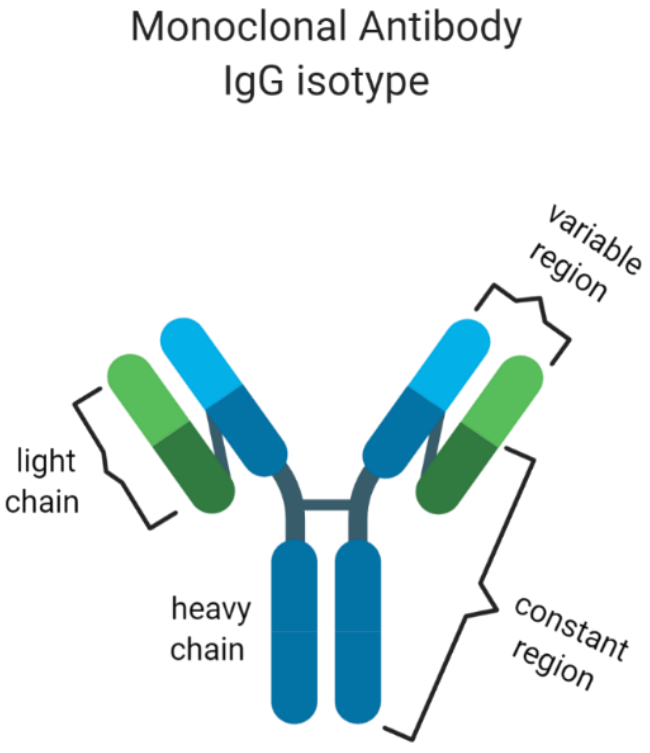

Figure 3. Structure of a monoclonal antibody of the IgG isotype. Created with Biorender.com

Most of the anti-tumor mAbs are used in combination with chemotherapy drugs to maximize their therapeutic efficacy (reviewed in LIU *et al*., 2009; SCOTT; ALLISON; WOLCHOK, 2012). Conditions such as immunosuppression or lack of

antibody production in some patients can interfere with chemotherapy efficiency, making those individuals key candidates for therapeutic antifungal vaccines (BONICHE *et al*., 2020; TABORDA; NOSANCHUK, 2017).

An antifungal therapy to treat human systemic mycosis that could enhance the immune system and also improve the protective effect of antifungal chemotherapy would allow the reduction of the time required for treatment, the reduction of the fibrotic consequences and would prevent the recurrence of PCM (reviewed in BONICHE *et al*., 2020; TRAVASSOS; TABORDA, 2012, 2017).

The use of monoclonal antibody therapies offers many advantages such as the evasion of toxicity risks since mAbs are directed specifically to pathogenic epitopes. Also, mAbs offer immediate immunity against pathogens, as opposed to traditional antifungal drugs, which require a prolonged period of time. Moreover, mAbs could reduce antifungal drug treatment periods by boosting their effectiveness when administered together. Other advantages are that mAbs could not alter the microbiota of the treated patients and their high specificity avoid the selection of drug-resistant fungal strains. Finally, mAbs can be produced against an wide variety of molecular epitopes (reviewed in BONICHE *et al*., 2020; CASADEVALL; DADACHOVA; PIROFSKI, 2004; CASSONE, 2008; ROMANI, 2011; SAYLOR; DADACHOVA; CASADEVALL, 2009; SPELLBERG, 2011; TABORDA; NOSANCHUK, 2017).

Recent progress made in the understanding of host-fungus interactions, which was enhanced by molecular technologies, allowed an increased recognition of monoclonal antibodies as an alternative option to treat systemic fungal infections (reviewed by BONICHE *et al*., 2020; SAYLOR; DADACHOVA; CASADEVALL, 2009).

Medically important fungi are capable of inducing a heterogeneous production of antibodies of different isotypes (FELDMESSER; CASADEVALL, 1998). Monoclonal antibodies with demonstrated protective activity could be used as a potent immune therapy against systemic mycoses (reviewed in TABORDA; NOSANCHUK, 2017). In individuals who are incapable of mounting an effective immune response, passive antibody transfers or administration of protective mAbs against the specific pathogen, would provide protection against the infection (BONICHE *et al*., 2020; TRAVASSOS; TABORDA, 2012a, 2012b).

Immunotherapy based on mAbs in fungal diseases is supported by the

antigenic contrast between fungi and mammals (reviewd in BONICHE *et al*., 2020; CASADEVALL; DADACHOVA; PIROFSKI, 2004; CASADEVALL; PIROFSKI, 2012; SAYLOR; DADACHOVA; CASADEVALL, 2009). Today, the majority of murine antibodies is developed using hybridoma technology or using gene libraries and the recombinant antibody fragments technology (LISTEK *et al*., 2020). Murine, humanized mAbs and genetically engineered antibody fragments have shown significant efficacy against fungi infections (reviewed in BONICHE *et al*., 2020; DATTA; HAMAD, 2015).

Many studies have shown that murine mAbs against fungi are protective in murine model infections. Furthermore, mAbs proved to be non-fungicidal or non- protective could be converted to fungicidal, for instance, by labelling with radiation emitters (radioimmunotherapy) or by associating them with nanoparticles containing a fungicidal drug (reviewed in BONICHE *et al*., 2020; HELAL *et al*., 2020; NOSANCHUK; DADACHOVA, 2012).

Currently, the clinically applied mAb therapies are chimeric, humanized or human IgG1, produced by hybridoma technology (reviewed in STROHL, 2014, 2018). Currently, there is no safe and effective vaccine available neither for therapeutic or prophylactic treatment of mycosis in humans or in veterinary (reviewed in BONICHE *et al*., 2020; DA SILVA; TABORDA; NOSANCHUK, 2020).

However, numerous studies are considering mAb therapies as main options in a new age of antimicrobial treatment. In the case of *Paracoccidioides* sp., the first study demonstrating protection mediated by monoclonal antibodies against *P. brasiliensis* was published in 2003 by Matos Grosso *et al.*

In this study the authors demonstrated that the passive transfer of mAbs of the IgG1 subclass named as B7D6 and C5F11 and produced against a 70 kDa glycoprotein (gp 70) of *P. brasiliensis* was able to protect infected mice in an experimental PCM model (DE MATTOS GROSSO *et al*., 2003). The major efficacy was achieved when the mAbs B7D6 and C5F11 were applied together, demonstrated by reduced fungal burdens within the lungs and the histopathology findings that showed lower numbers of granulomas and of yeast cells in pulmonary biopsies. The authors suggested that macrophages had an important role in the clearance of the yeasts, because the mAbs B7D6 and C5F11 could bind to the gp70 present on *P. brasiliensis* yeast surface to facilitate their killing by phagocytosis (reviewed in BONICHE *et al.,* 2020).

In 2007, Xandi *et al*., investigated a secreted protein of 75-kDa with phosphatase activity of *P. brasiliensis*. They infected BALB/c mice with *P. brasiliensis* which were previously treated with an IgM mAb (1G6) or with and an IgG2a mAb (5E7C) targeting the 75-kDa protein and every 3 days after infection until day 45. They discovered that the mAbs significantly decreased the colony-forming units (CFU), reduced the number and the proportions of the lung granulomas and modulated the inflammation (XANDER *et al*., 2007). The authors proposed the possibility that 1G6 and 5E7C mAbs are potent immunomodulators since they were able to interact with the effector cells to inhibit inflammatory responses (reviewed in BONICHE *et al.,* 2020).

Our group demonstrated the effect of mAbs against gp43 of *P. brasiliensis* in a study performed by Buissa-Filho *et al*. They proved that mAbs of the subclasses IgG2a and IgG2b could reduce the fungal burdens and the pulmonary inflammation, and also demonstrated a major increase of IFN-γ and IL-12 production in a murine model of PCM. From the panel of mAbs analyzed in this research, the mAb 3E (IgG2b) was highly effective in the treatment of the mice, while other mAbs were not able to modulate the disease (BUISSA-FILHO *et al*., 2008). *In vitro* assays revealed that mAb 3E stimulated yeast phagocytosis and increased NO production and expression in both, J774.16 and primary macrophages (reviewed in BONICHE *et al.,* 2020).

Polyclonal antibodies (pAbs) are a pool of antibody molecules secreted by different B cell clones that react against several epitopes of a specific antigen (reviewed in PARRAY *et al*., 2020). In another study performed by our group, Da Silva *et al*. used anti-melanin polyclonal antibodies to study the role of melanin during experimental infection of BALB/c mice with *P. brasiliensis*. This research confirmed the inhibitory effect of melanin on the phagocytic process of melanized yeast (DA SILVA *et al*., 2009). *In vitro* assays showed that J774.16 macrophages challenged with melanized yeast previously opsonized with the anti-melanin polyclonal antibodies improved their phagocytic capacity and the secreted ROS concentrations, increasing their fungicidal effect as a result (reviewed in BONICHE *et al.,* 2020).

A model of mAb therapy was proposed by our group using the mAbs 7B6 and 4E12 generated against heat shock protein 60 (HSP 60) from *H. capsulatum* (GUIMARAES *et al*., 2009) to modulate experimental PCM with a *P. lutzii* strain

(reviewed in BONICHE *et al.,* 2020). Thomaz *et al*. demonstrated that the mAbs 7B6 (IgG2b) and 4E12 (IgG2a) successfully reduced the pulmonary inflammation and the number of CFU, and reported compact granulomas, which is suggestive of a protective immune response and was confirmed by cytokine analyses (THOMAZ *et al*., 2014).

In another study, our group obtained the same results using polyclonal antibodies against acidic glycosphingolipids (GSL) from *P. brasiliensis* to treat experimental *P. brasiliensis* infections (reviewed in BONICHE *et al.,* 2020). The polyclonal antibodies administered to the infected mice resulted in the reduction of the number and size of the granulomas and lower pulmonary damage, associated with lower inflammation. The analysis of the cytokine response showed a mixed Th1 and Th2 immune response, which was capable to modulate the disease. Finally, *in vitro* assays showed an enhanced phagocytic capacity and fungicidal effect when macrophages were challenged with yeasts opsonized with anti-acidic GSL polyclonal antibodies (BUENO *et al*., 2016).

The cell wall is an essential structure for all fungi. It regulates the shape and preserve the cell from the environment (PINTO; BARRETO-BERGTER; TABORDA, 2008). Fungal antigens present in the cell wall are desirable targets for drug development, since they are vital for many processes such as growth, virulence and pathogenicity of the cells (reviewed in BONICHE *et al.,* 2020). The composition of the fungal cell wall can diverge substantially among species; however, the composition of the polysaccharide structures is very homogeneous (PINTO; BARRETO-BERGTER; TABORDA, 2008). Polysaccharides from the cell wall, such as β-Glucans has been proposed over 40 years as desirable targets to develop therapeutic mAbs to fungal diseases.

β-Glucans, also known as Beta Glycans, beta-1, 3-glucan, and beta-1, 3 /1, 6-glycan are soluble glucose polymers that form the cell walls of bacteria, fungi, yeasts, algae, lichens, and some plants (LIU *et al*., 2009; RAHAR *et al*., 2011). Moreover, beta-glucans are not expressed on mammalian cells; and glucan particles are recognized as pathogen-associated molecular patterns by pattern recognition receptors, being classified as biological response modifiers (BRMs) (reviewed in LIU *et al*., 2009).

The β-glucans detected in yeasts and mushrooms are characteristically a

linear glucose polymer with mainly 1, 3-glucan linkages and sporadically 1, 6 linkages. Typically has a superior biological activity than the β-glucans from plants, which contains mainly 1, 3 and 1, 4 linkages (KANG *et al*., 2018; RAHAR *et al*., 2011).

α-1,3-glucans constitute the *P. brasiliensis* yeast cell wall, nevertheless in the mycelial phase α-1,3-glucans are replaced by β-1,3-glucans, which are merely present in mycelial phase. However, in contradiction to that information, in *P. brasiliensis* and *P. lutzii*, one only β-1,3-glucan synthase gene was described, and reported to be overexpressed in the yeast phase of both species (ARANTES *et al*., 2015).

The mechanisms by which β-Glucans modulate the host immune system are diverse. Carbohydrate-binding receptors which are part of the larger pattern-recognition receptor family (PRR) are vastly expressed on phagocytic immune cells, such as macrophages and dendritic cells (reviewed in PINTO; BARRETO-BERGTER; TABORDA, 2008). Recognized PRRs for the detection of fungal cell wall associated polysaccharides include TLR2, TLR4, collectins, pentraxin-3, CR3 integrin, and C-type lectins (reviewed in PINTO; BARRETO-BERGTER; TABORDA, 2008). In fact, β-Glucans can prime cellular receptors CR3 expressed in neutrophils and other granulocytes to evoke dependent cellular toxicity (BROWN *et al*., 2003; reviewed in LIU *et al*., 2009). It was proved by many authors that glucan derived from yeast administered in association with anti- tumor mAbs resulted in higher efficacy than anti-tumor mAb therapies only, both in human and in murine models (reviewed in LIU *et al*., 2009).

It was proved for *C. albicans* that mAbs against cell wall polysaccharides (mannans) were protective against disseminated candidiasis in experimental model (DE MATTOS GROSSO *et al*., 2003; HAN; CUTLER, 1995; HAN; RIESSELMAN; CUTLER, 2000). Based on this evidence, our group produced a soluble glycoproteic extract (Polysoluble extract) originated from the yeast cell wall of *P. brasiliensis* (Pb18), using an alkaline extraction method with (KOH at 2%, 100 C, 2 hours; **Appendix 2**). The profile of this Polysoluble extract was characterized by proton nuclear magnetic resonance spectrum (NMR1H; **Appendix 3**) in order to characterize their composition, which was described as 79% Glucitol, 7% Galactitol and 11% Mannitol (THOMAZ, 2012). When BALB/c mice were immunized 8 times with the Polysoluble extract, several IgM

immunoglobulins and only one IgG clone were generated. The IgG clone was named F1.4 and was used to produce an hybridoma cell line (mAb F1.4). Facing the possibility of a promising immunotherapy for the treatment of PCM, this work proposes the study of the mechanisms of action by which mAb F1.4, could be protective or not in the immunotherapy of the experimental PCM of C57Bl/6 mice.

Conclusions

- By western blot and dot blot we identified that mAb F1.4 recognizes glycosidic bonds β-1,3 e β-1,6 and also is unable to recognize α-(1,6), α-(1,2) and α-(1,3) - glucans.
- By direct immunofluorescence, confocal microscopy and by transmission electron microscopy it was possible to prove that mAb F1.4 binds to *P. brasiliensis* and *P. lutzii* yeasts.
- By phagocytic capacity, NO concentration and cell viability performed by murine J774.16 linage and C57Bl/6 bone marrow differentiated macrophages challenged with opsonized yeasts of *P. brasiliensis* and *P. lutzii* we confirmed that mAb F1.4 modulated the phagocytic capacity and had a fungicidal effect on the yeasts, associated with an increased NO concentration induced by the activated macrophages.
- By CFU it was possible to estimate in *vivo* the efficiency of mAb F1.4 to moderate the fungal burden in C57Bl/6 mice chronically with *P. brasiliensis*. We determined that therapy with TMP/SMX in association with mAb F1.4 significantly decreased the colony- forming units when compared with the TMP/SMX only treated group and with the infected-untreated group control.
- By analysis of the cytokine profile, we observed an increase in INF- γ, TNF- α, and IL-17 titers, as well as a decrease in IL-10 and IL-4 titers. These results suggest a Th1 and Th17 immune response modulated by the immunotherapy with TMP/SMX in association with mAb F1.4.
- By histopathological analysis we probed that the treatment with TMP/SMX associated with mAb F1.4 resulted in decrease of the granulomatous inflammatory response, less pulmonary fibrosis and lower viable CFUs

related to a better control of infection during the chronic PCM, suggesting that the mAb F1.4 exerted a protective effect and potentialized the efficiency of TMP/SMX treatment.

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