Camila Martins Marchetti

Estudo da migração de células peritoneais na paracoccidioidomicose experimental murina: avaliação do efeito da estimulação de células peritoneais por lectina sobre a evolução da lesão paracoccidióidica

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Mestre em Doenças Tropicais.

Orientador: Prof. Titular Rinaldo Poncio Mendes
Coorientadora: Profa. Dra. Maria Sueli Parreira de Arruda

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Palavras-chave: Concanavalina A; Paracoccidioides brasiliensis; Paracoccidioidomicose.


PAI, O QUE É "UTOPIA"!
UTOPIA É COMO A LINHA DO HORIZONTE...
POR MAIS QUE A GENTE CAMINHE, NUNCA IREMOS ALCANÇAR!
E PARA ISSO SERVE A UTOPIA...
...PARA NOS FAZER CAMINHAR!
beckilustras@gmail.com
Fernando Birri, citado por Galeano

Aos meus pais

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Aos animais de experimentação

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LISTA DE ABREVIATURAS E SIGLAS

BCG: *Bacillus Calmette-Guérin*
BHI: Brain Heart Infusion
CD: Cluster of Differentiation
CEEPA: Comissão de Ética no Ensino e Pesquisa em Animal
CMX: Cotrimoxazole
CO_2: Carbon dioxide
COBEA: Brazilian College of Animal Experimentation
Con-A: Concanavalina A/ Concanavalin A
GPY: Glucose-peptone-yeast extract agar
H_2O_2: Hydrogen peroxide
HE: Hematoxylin-eosin
IFN-γ: Interferon-gamma
Ig : Imunoglobulina / immunoglobulin
IL: Interleucina/ Interleukin
MCP: Monocyte chemotactic protein
MHC: Complexo de histocompatibilidade principal/ major histocompatibility complex
MR: Mannose receptor
NK: Natural killer
NO: Nitric oxide
p.i.: post-infection
Pb: *Paracoccidioides brasiliensis*
PBS: Phosphate buffered saline
PCL: Phagocytic Cell Labeling
PCM: Paracoccidioidomicose/ Paracoccidioidomycosis
PL: Peritoneal lavage
SMZ-TMP: Sufametoxazol-trimethoprim
TCR: Receptor de linfócito/ T-lymphocyte receptor
Th: T helper
TNF: Fator de necrose tumoral/ Tumor necrosis factor

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1. INTRODUÇÃO

1.1. Paracoccidioidomicose

A paracoccidioidomicose (PCM) é micose sistêmica, granulomatosa, causada por fungos termo-dimórficos do gênero *Paracoccidioides.* [1] A infecção ocorre após a inalação de fragmentos de micélio ou de conídios (formas infectantes) que, ao atingirem os pulmões, transformam-se em leveduras (forma patogênica). [2] Os fungos se disseminam do parênquima pulmonar para linfonodos regionais, constituindo, em seu conjunto, o complexo primário; alguns fungos se disseminam pela corrente sanguínea e, ou, linfática, podendo atingir qualquer órgão, aparelho ou sistema. [2-3] O diagnóstico da PCM é baseado em sinais e sintomas e em exames laboratoriais, como o micológico direto a partir de amostras clínicas, isolamento do fungo em cultura e detecção de anticorpos específicos por técnicas sorológicas.[4]

As principais formas clínicas da PCM são a forma aguda/subaguda e a forma crônica. A forma aguda/subaguda, também chamada juvenil, geralmente acomete crianças, adolescentes e adultos jovens; com história clínica de curta duração (mediana de dois meses), esta forma exibe manifestações clínicas compatíveis com o comprometimento de órgãos do sistema fagocítico mononuclear. A forma crônica, em geral, compromete adultos com mais de 30 anos de idade; doença de longa duração – em geral acima de seis meses, acomete pulmões e mucosa das vias aerodigestivas superiores com grande frequência. Mesmo após tratamento antifúngico eficaz, muitos pacientes apresentam sequelas, como a fibrose e o enfisema pulmonar, e são classificados como apresentando formas residuais da doença. [5]

À semelhança do que ocorre em outras doenças causadas por microrganismos intracelulares, o controle da PCM depende de uma resposta imune celular efetiva por parte do hospedeiro. De modo geral, os pacientes apresentam uma depressão específica para antígenos do fungo; naqueles com formas graves da doença, essa depressão é mais acentuada e abrangente, não se limitando apenas aos antígenos do fungo. Foi observado, por exemplo, que pacientes com PCM ativa, quando comparados com pacientes curados, exibem diminuição na produção de citocinas de perfil Th1. [6]

1.2. Modelo experimental de PCM murina

Considerando que os estudos que envolvem os eventos iniciais da relação fungo-hospedeiro são difíceis de serem estudados em seres humanos, muitos pesquisadores têm utilizado modelos experimentais na busca da melhor compreensão dos mecanismos envolvidos na PCM [7] e no desenvolvimento de estratégias de intervenção terapêutica que resultem na cura ou na melhoria da qualidade de vida destes pacientes. [8-9]

Na busca de modelos experimentais, o camundongo tem se destacado por ser de fácil criação e manuseio. Diferente de outras espécies, a investigação imunológica em camundongos é privilegiada, ainda, por dispor de diversas ferramentas laboratoriais espécie-específicas, como proteínas recombinantes e anticorpos alvo.

Na paracoccidioidomicose experimental, os modelos mais utilizados são aqueles que apresentam características genéticas relacionadas à susceptibilidade e resistência ao fungo. A forma benigna da paracoccidioidomicose é representada pela linhagem de camundongos A/J; estes animais apresentam doença regressiva, com lesões bem delimitadas, de padrão granulomatoso, epitelióide e com poucos fungos, aspectos compatíveis com o padrão de resposta imune celular do tipo Th1. Os camundongos da linhagem B.10A, por outro lado, são susceptíveis à infecção; apresentam anergia ao teste de hipersensibilidade tardia e multiplas lesões caracterizadas por granulomas frouxos, ricos em fungos; exibem ainda um perfil de citocinas do tipo Th2, que é característico de pacientes com a forma grave da doença. [10-11] O padrão de resistência intermediária pode ser obtido em camundongos BALB/c. [12]

Em camundongos fungo-resistentes, o principal componente antigênico do *P. brasiliensis* (gp43) é apresentado preferencialmente por macrófagos e estimula a produção de uma resposta do tipo Th1. Em animais suscetiveis essa apresentação é realizada por células B, com ativação de linfócitos Th2. [10] Buscando entender o papel dessas células, Almeida et al, 1998, [10] verificaram que quando o antígeno é apresentado, *in vitro*, por macrófagos para linfócitos T de animais suscetiveis, a resposta muda para um perfil Th1. Da mesma forma, quando o antigeno é apresentado por células B para um linfócito T proveniente de um animal resistente, essa célula responde com um perfil Th2. Esses achados revelam que não há diferenças significativas no repertório de receptores de células T nas diferentes linhagens e que as células apresentadoras de antigenos podem determinar o resultado da infecção por *P. brasiliensis.*

O desenvolvimento da infecção no camundongo também é modulado pela via de inoculação e a fase morfológica do fungo. Entre as vias de inoculação mais utilizadas, destaca-se a via intratraqueal. Essa é uma via simples e segura que proporciona distribuição heterogênea da suspensão fúngica para os lóbulos pulmonares em até 90% dos camundongos BALB/c, com pouca disseminação para outros órgãos. [13-14] As lesões causadas nos pulmões são caracterizadas por possuírem células leveduriformes homogêneas, grandes e viáveis, por vezes em proliferação, com padrão histológico predominantemente granulomatoso. Assim, o uso dessa via permite avaliar o ambiente pulmonar e a interação entre o fungo e os macrófagos alveolares, possibilitando observações da progressão da doença e de possível migração de células para o local da infecção.

A opção pela utilização de um inóculo com fungos em fase micelial ou leveduriforme depende do objetivo do estudo. A inoculação de fungos na fase micelial foi pouco realizada e é de difícil reprodução, mas guarda a vantagem de possibilitar a investigação dos estágios iniciais da interação fungo-hospedeiro.[15] Em contrapartida, a inoculação da fase leveduriforme é mais segura, garantindo a infecção por parte do animal e proporcionando o estudo da interação parasita-hospedeiro que é semelhante à observada no paciente à admissão, para tratamento. A inoculação do fungo na sua fase leveduriforme tem sido utilizada para avaliar a progressão da infecção, desde a formação de granulomas[16] à eficácia de vacinas[17] e de novos tratamentos. [18]

1.3. Microambiente peritoneal e tráfego celular

O peritônio é um mesotélio composto por diferentes tipos celulares. Possui uma camada de tecido conjuntivo submesotelial, que contém vasos sanguíneos e linfáticos e é composta por feixes de fibras colágenas, fibroblastos e células livres, tais como macrófagos, linfócitos, granulócitos e mastócitos. [19] Estas células têm múltiplas funções biológicas, incluindo regulação da inflamação, angiogênese, fibrinólise e processos de remodelação tecidual. Em camundongos, estima-se que aproximadamente 30% delas sejam macrófagos e 55%, linfócitos do tipo B-1. Os 15% - 20% de células peritoneais restantes representam outros subtipos celulares, como linfócitos T, linfócitos B-2, células NK, eosinófilos e mastócitos. [20]

Os macrófagos são células que exibem heterogeneidade fenotípica e funcional, devida, pelo menos em parte, à sua capacidade de se adaptar às mudanças do meio ambiente tecidual. Esta plasticidade funcional é importante para que possa atuar no *clearance* de tecidos lesados

e de microrganismos invasores, no recrutamento de células do sistema imune adaptativo e, ainda, na resolução de lesões.

As células B-1 se diferenciam dos linfócitos B convencionais quanto a fenótipo, morfologia, ontogenia, distribuição tissular e função. Embora compartilhem marcadores de superfície típicos de células de linhagem B (CD19, IgM e CD45R/B220), não expressam o receptor CD23. [21-22-23] De modo geral, as células B-1, por produzirem grande quantidade de IgM [24], têm sido associadas à resposta imune inata exercida nas cavidades peritoneal e pleural. Além disso, essas células são altamente fagocíticas via receptor de IgG e de manose [25] e produzem grande quantidade de IL-10. [26]

Os estudos envolvendo a migração de células peritoneais para órgãos alterados têm utilizado especialmente modelos envolvendo tumores. Empregando a variedade subcutânea do tumor AK-5, um tumor histiocítico regressivo de rato, Bhaumik et al, 2001[27], verificaram que, na vigência do tumor, os animais exibiam um acúmulo de macrófagos hiperativos no peritônio e que, com sua regressão, a quantidade desses macrófagos diminuía drasticamente. Corando células do peritônio com o corante vital fluorescente PKH26, demonstrou-se que estas migravam para o tumor e, posteriormente, voltavam ao peritônio. Segundo os autores, esse tráfego não se limitaria apenas à condição tumoral, mas envolveria também outras alterações teciduais. [27]

Russo et al., 2010, [21] investigando este fenômeno, verificaram que as células B-1 migram para lesões inflamatórias crônicas. Em camundongos BALB.xid, deficientes de células B-1, demonstraram que a infecção pelo bacilo *Mycobacterium bovis* BCG levava à mortalidade de 90% deles; nesses animais, as lesões eram claramente acentuadas e difusas, caracterizadas por granulomas desorganizados. Em camundongos BALB/c, que não são deficientes em B-1, além da infecção não levar à morte, as lesões apresentavam infiltrado inflamatório focal e granulomas típicos. A transferência adotiva de células B-1 de BALB/c para camundongos BALB.xid confirmou que as células B-1 presentes nas lesões pulmonares induzidas pela infecção por BCG, migram da cavidade peritoneal para o pulmão infectado, modulam o padrão histológico da inflamação e favorecem a resistência à micobactéria. [21]

Fraga-Silva et al., 2013, [28] estudaram a migração de fagócitos peritoneais para órgãos infectados com candidíase disseminada. Empregando camundongos *Swiss* hipoinsulinêmicos-hiperglicêmicos e transferência adotiva de células, os autores observaram que animais livres de infecção apresentavam migração de fagócitos peritoneais para cérebro, fígado, rim e baço. Quando infectados com *C. albicans*, os animais exibiam células marcadas também no

pulmão. Considerando que os fagócitos peritoneais migram do peritônio para os demais órgãos e que, assim, podem auxiliar na resolução de infecções, os autores sugerem a utilização do peritônio como uma opção viável e promissora para estudos envolvendo imunoterapia. [28]

1.4. Concanavalina A e peritônio

A Concanavalina A (Con-A) é uma lectina mitogênica, obtida da planta *Canavalia ensiformis*, e é conhecida pelo seu potencial de ativação policlonal de linfócitos T. [29] Ao se ligar às moléculas de carboidratos (D-glucopiranosídeo e D-manopiranosídeo) presentes no complexo de histocompatibilidade principal (MHC) e ao receptor de linfócito T (TCR), a Con-A ativa a resposta celular [30] e a produção de citocinas tais como IL-2, IFN-γ e TNF-α. [31-32]

A utilização de Con-A como um estimulante do sistema imunológico, tem como suporte diversos estudos que demonstram a ativação de células peritoneais por essa lectina. [33] Assim, camundongos tratados com uma dose de Con-A por via intraperitoneal quatro dias antes da infecção por microorganismos patogênicos, tais como *Candida albicans* [35-36] e *Serratia marcescens* [37] demonstram maior capacidade de eliminação do patógeno e aumento de sua sobrevida. Além disso, Loyola et al., 2002, [33] utilizando camundongos *Swiss* inoculados intraperitonealmente com Con-A, observaram que após seis horas havia um aumento de neutrófilos ativados e, após três a quatro dias, um aumento na quantidade de macrófagos ativados na cavidade peritoneal. Demonstraram ainda, que a ativação de macrófagos por Con-A favoreceu a eliminação de *C. albicans* em camundongos infectados por via intravenosa, quatro dias após o tratamento. Os autores sugeriram que essa eliminação foi conseqüência do aumento da fagocitose por macrófagos peritoneais, via receptores de manose, uma vez que houve aumento de interiorização do ligante albumina-manose-FITC, além do aumento de sobrevida em relação aos animais apenas infectados. Interessante observar que a adição *in vitro* de Con-A sobre macrófagos de animais não tratados não alterou a fagocitose, sugerindo que a ativação de macrófagos pela Con-A é mediada por citocinas produzidas por linfócitos Th1. [33]

Nesta dissertação, apresentamos, pela primeira vez, resultados referentes à utilização da Con-A, como imunoestimulante, na paracoccidioidomicose experimental murina.

2. OBJETIVOS

2.1. Objetivo Geral.

Determinar a migração *in vivo* de fagócitos peritoneais durante a infecção via intratraqueal por *P. brasiliensis* em camundongos BALB/c e a influência da administração intraperitoneal de Concanavalina-A neste processo.

2.2. Objetivos Específicos

1. Avaliar a influência da Con-A na ativação e migração de fagócitos peritoneais.
2. Determinar a migração de fagócitos peritoneais para pulmões, fígado e baço de camundongos na infecção pulmonar por *P. brasiliensis*.
3. Avaliar o perfil de resposta de fagócitos peritoneais sob a influência de Con-A na infecção pulmonar por *P. brasiliensis.*
4. Avaliar o efeito da Con-A sobre a carga fúngica e a histopatologia pulmonar de animais infectados pelo *P. brasiliensis.*

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4. MANUSCRITO

Concanavalin A induces peritoneal phagocytes trafficking to the lungs in response to *Paracoccidoiodes brasiliensis* and stimulates pro-Th1 macrophage activity.

Camila Martins Marchetti [1,2], James Venturini [1], Thais Fernanda de Campos Fraga-Silva [1,3], Débora de Fátima Almeida [1], Marjorie de Assis Golim [2], Rinaldo Poncio Mendes [2], Maria Sueli Parreira de Arruda [1*].

[1] Laboratory of Experimental Immunology, Department of Biological Sciences, Faculty of Science, UNESP- Univ Estadual Paulista, Bauru, São Paulo, Brazil.
[2] Botucatu Medical School, UNESP- Univ Estadual Paulista, Botucatu, São Paulo, Brazil.
[3] Institute of Biosciences of Botucatu, UNESP – Univ Estadual Paulista, Botucatu, São Paulo, Brazil.

* Corresponding author
E-mail: camilamarchetti@fc.unesp.br

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Abstract

The paracoccidioidomycosis (PCM) is an infectious disease caused by *Paracoccicidioides brasiliensis*. The control of this disease depends on an effective and protective specific-host immune response, especially of the phagocytes. In this context, the peritoneal environment emerges as a possible site for immunotherapy, since peritoneal phagocytes exhibit plastic biological functions such as migration to inflamed and infected tissue. It was recently reported that peritoneal cells stimulated with Concanavalin A (Con-A) show an increased pro-inflammatory activation and high phagocytic activity. Thus, this study aimed to identify a possible action of Con-A in the peritoneal cavity, specifically the migration and the production of cytokines and reactive oxygen species by peritoneal phagocytes in a murine model of pulmonary PCM. Our findings showed that the treatment with three doses of Con-A in non-infected mice induces migration of peritoneal phagocytes to the lung and also a pro-Th1 profile by peritoneal phagocytes. In *Paracoccidioides brasiliensis* infected mice, the treatment with Con-A led to a protective immune response, characterized by higher production of H_2O_2 and IFN-γ and an increase in the percentage of peritoneal phagocytes in the lungs. Despite these findings, no changes were observed in the recovery of viable fungi as well as in the tissue damage. Our results showed that the intraperitoneal treatment with three doses of Con-A induces activation and migration of peritoneal phagocytes, but did not eliminate the fungus. Whereas PCM patients have a deficiency in antigen-specific immune response at admission, we suggest that Con-A immunotherapy, associated with antifungal therapy, could help the early elimination of the fungus.

Author Summary

Some individuals in Latin America, especially among males and rural workers, develop a fungal disease called paracoccidiomycosis (PCM). Although the mechanisms regarding why these individuals become ill are not well known, they exhibited a cellular-mediated antigen-specific immunodeficiency. The treatment is efficacious but long-term and almost all of the patients develop sequels such as lung fibrosis and emphysema. Furthermore, there is no treatment for these disabling conditions, which remain neglected. In the present study, we showed for the first time that Con-A, a lectin from jack-beans, improves the immune response

by peritoneal phagocytes and triggers the migration of these cells to the lungs in an experimental model of pulmonary PCM, but it did not eliminate the fungus. Thus, our study opened new perspectives for PCM treatment, i.e. conventional antifungal therapy associated with Con-A immunotherapy could improve the immune response and thus the early elimination of the fungi as well as avoid excessive fibrogenesis.

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the fungi of the genus *Paracoccidioides* [1]. The infection begins by inhalation of conidia or mycelial fragments (infective form), which are transformed into yeast, i.e., pathogenic form when they reach the lungs [2]. Afterwards, the fungi spread to regional lymph nodes, constituting the primary complex; some fungi also spread through the bloodstream or the lymphatic vessels and can affect any organ or system [2, 3].

Although PCM is highly prevalent in Brazil, Colombia and Venezuela and is the eighth most common cause of death among chronic infectious diseases in Brazil, the early events during the infection still remain unclear. Since these events cannot be studied in patients, experimental models have been used to better understand the immunological mechanisms [4] as well as to develop new approaches for therapeutic intervention [5-6]. Regarding the cellular physiology, the peritoneum has been the source for obtaining cells for *in vitro* studies, since the peritoneal mesothelium is composed of different types of cells, including B-1 cells and resident macrophages [7] that exhibit multiple biological functions, such as regulation of inflammation, angiogenesis, fibrinolysis and tissue remodeling processes.

When stimulated by Concanavalin A (Con-A) [8], a lectin originally extracted from the jack-bean, *Canavalia ensiformis*, influx of neutrophils is the first event in the peritoneal milieu, followed by macrophage activation after 3-4 days that is characterized by increased phagocytic activity, high production of TNF-α and INF-γ and expression of mannose receptor (MR) [8].

Several reports have shown that B-1 and macrophages could act at a distant site [9-10], since peritoneal cells are able to migrate to inflammatory [9] and infectious milieu [11]. Following these statements, we raised the following questions: could the Con-A trigger

phagocyte trafficking to the lungs? Could the Con-A elicits an effective response during *P. brasiliensis* infection?

In order to answer these questions, we evaluated the effect of Con-A treatment in a murine experimental model of pulmonary paracoccidioidomycosis.

Materials and Methods

Mice

Two-month-old male, weight-matched, BALB/c mice were maintained at the animal facilities of the Laboratory of Experimental Immunology (LIPE), School of Sciences, UNESP – Univ Estadual Paulista, Bauru, SP, Brazil. The mice were housed in groups of three to five and were provided with food and water *ad libitum*. The experimental protocol was performed in accordance with the ethical principles for animal research adopted by the Brazilian College of Animal Experimentation (COBEA) - CEEPA- # 041/2011.

Fungi

The *Paracoccidioides brasiliensis* (Pb), isolated 324, was newly isolated from a human lesion and obtained from the fungal collection of the Mycology Laboratory, Department of Tropical Diseases and Imaging Diagnosis, Botucatu Medical School, UNESP, and was maintained by frequent subculture in semi-solid medium containing 2% glucose, 1% peptone, 0.5% yeast extract and 2% agar (GPY) at 36º C in our laboratory.

Fungal inoculum

The isolated fungal was cultured on GPY agar for seven days at 36°C. The fungi were transferred with a platinum loop to tubes containing 10 ml of sterile phosphate buffered saline (PBS) and glass beads 4 mm in diameter, then mixed for 30 s on a vortex mixer and decanted for five min. After this time, the supernatant was collected and the viability of the suspension was determined by cotton blue staining [12]. The concentrations were adjusted to 2×10^7 viable *P. brasiliensis* yeasts per ml and 0.05 ml was inoculated by the intratracheal route. Before the fungal inoculation, the mice were anesthetized with an intraperitoneal administration of ketamine and xylazine at doses of 80 and 10 mg / kg body weight, respectively. The incision was sutured with surgical thread and the animals were kept in a

warm place and observed for their recovery process.

Con-A treatment

Mice were treated with 7.2 mg/kg body weight, by the peritoneal route, according to Loyola et al. [8].

Experimental design

The mice were randomly distributed into four experimental groups, and each group was composed of six animals: 1) PbConA group, composed of mice treated with Con-A and infected with *P. brasiliensis*; 2) Pb group, composed of mice treated with saline solution and infected; 3) Con-A group, composed of mice that were treated with Con-A and inoculated with saline solution; 4) CTL group, with mice only inoculated by saline solution in both routes of inoculation. Three different protocols were performed:

Protocol I: Mice of the Con-A group received three different treatment schedules: single dose, three doses, and eight doses. The doses were given each four days and the animals were evaluated after four days of the last inoculated dose, when the macrophage activity peak occurs, as previously evaluated (data not shown). The peritoneal phagocytes were labeled with fluorescent particles (PKH-PCL) three days after the first dose.

Protocol II: Mice of the PbConA group received three doses of Con-A. The first dose was given four days before the fungal inoculation; the second dose was given 24 hour after the fungal inoculation; and the last, four days after the fungal inoculation. The mice were evaluated four days after the last Con-A dose was administered. Twenty-four hours before the fungal inoculation, the peritoneal phagocytes were labeled with fluorescent particles (PKH-PCL).

Protocol III: Mice from the PbConA group received eight doses of Con-A. The first dose was given four days before the fungal inoculation; the second dose was given 24 hour after the fungal inoculation. The next six Con-A doses were inoculated each four days. The mice were evaluated four days after the last Con-A dose was administered.

Collection of the biological material

Mice were sacrificed via CO_2 asphyxiation and submitted to peritoneal lavage (PL) with cold and sterile PBS. The spleen, liver and lungs were dissected for microbiological and histopathological analyses.

Colony-forming unit (CFU) determination

Fragments of the collected organs were weighed and homogenized in 1 ml of PBS, and 0.1 ml of the homogenate was cultured on 15 x 90 mm plates containing BHI agar supplemented with 4% horse serum, plus 5% *P. brasiliensis* cell extract supplemented and 1%. gentamicin. The plates were incubated at 36°C for four weeks. Each sample was assessed in duplicate. Total colonies were counted, and the results were expressed as the number (log_{10}) of *P. brasiliensis* per gram of tissue [13].

Peritoneal adherent cells culture

The peritoneal cell suspensions were centrifuged, and the cells were re-suspended in 1.0 ml of RPMI-1640 (Nutricell, Campinas, SP, Brazil) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL, Grand Island, NY, USA), penicillin (100 UI ml^{-1}) and streptomycin (100 mg ml-1) (Gibco). The cell concentration was adjusted to 2.0×10^6 mononuclear phagocytes ml^{-1} as judged by the uptake of 0.02% neutral red. The cells were placed in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA, USA) and incubated for two hours at 37°C and 5% CO_2 in a humidified chamber to allow cells to adhere and spread. Non-adherent cells were removed by washing the wells three times with RPMI, and the remaining adherent cells (>95% mononuclear phagocytes as assessed by morphological examination) were used for the experiments. The adherent peritoneal cells were cultured at 37°C and 5% CO_2 in supplemented RPMI-1640 with or without *P. brasiliensis* antigen (exoantigen rich in gp43) – 10 μg ml^{-1}. As an internal control for macrophage activity, the cells were cultured with 10 μg ml^{-1} lipopolysaccharide (Sigma-Aldrich, St. Louis, MO, USA). After 24 hours, the cell-free supernatants were harvested and stored at -80°C pending the cytokine analysis.

Hydrogen peroxide release (H_2O_2)

The peritoneal adherent cells were obtained as described before and maintained in RPMI-1640 culture medium at 37ºC and 5% CO_2 for 24 hours. At the end of the cell culture period, the supernatant was removed and the macrophages were incubated with phenol red solution [dextrose (Sigma), phenol red (Sigma), horseradish peroxidase type II (Sigma)] and plated at 37ºC in 5% CO_2 for one hour according to the methods of Russo et al [14]. The reaction was stopped with the addition of 1 N NaOH and the H_2O_2 concentration was

determined using an ELISA microreader (ELx 800; Bio-tek Instruments Inc., Winooski, VE, USA).

Nitric oxide production (NO)

To determine the NO levels, the production of nitrite (a stable end product of NO) was measured in the cell-free supernatants of cultured peritoneal adherent cells according to the methods of Green et al [15]. Briefly, 0.1 ml of the cell-free supernatant was incubated with an equal volume of Griess reagent containing 1% sulfanilamide (Synth, Diadema, SP, Brazil), 0.1% naphthylene diamine dihydrochloride (Sigma) and 2.5% H_3PO_4, at room temperature for 10 min and the nitrite accumulation was quantified using an ELISA microreader. The concentration of nitrite was determined using sodium nitrite (Sigma) diluted in RPMI-1640 medium as a standard.

Determination of peritoneal cellular trafficking

Peritoneal phagocytes were labeled with the fluorescent vital dye PKH-26 PCL in accordance with the manufacturer's protocol (Sigma). Briefly, 2.0×10^{-6} M PKH-26 PCL was diluted in the diluents B and 24 hours before the fungal inoculation, 0.1 ml of the PKH-26 PCL use solution was injected into the peritoneal cavity of each mouse. After collection of the biological material, vertical sections were performed in each tissue using a scalpel. The pieces were pressed against glass slides and allowed to dry at room temperature. After washing them with PBS, the imprinted slides were mounted using Fluoroshield™ with DAPI (Sigma). The slides were analyzed with a fluorescence microscope (BX61, Olympus Optical, Tokyo, Japan) and images were captured using the Leica LAS-AF Software. After collecting the images, the cells of the lung were counted (100 cells per sample, in triplicate) and the percentage of PKH^+ cells was determined.

Cytokine production

IL-6, IL-10, TNF-α, MCP-1, IFN-γ and IL-12p70 levels in cell-free supernatants, serum and lung homogenate were determined using the Cytometric Bead Array (CBA) inflammation kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's

instructions. The levels of MIP-1α were measured using a cytokine Duo-Set Kit (R&D Systems, Minneapolis, MI, USA), according to the manufacturer's instruction.

Histopathological evaluation

Samples of the lung, spleen and liver were fixed in a solution of 10% buffered formalin for 24 hours and subjected to routine processing for paraffin embedding, Histological sections were cut in microtome with 4 µm thickness and stained with hematoxylin-eosin (HE).

Statistical analysis

The data was submitted to a normality test (Shapiro-Wilk) and the comparison of two independent samples was analyzed using *t* tests. Multiple comparisons of means were performed using the ANOVA with the Tukey`s post-test. All statistical tests were performed using the GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA), and the statistical significance level was set at 5% ($p \leq 0.05$) for all analyses [16].

Results

Treatment with Con-A induced the migration of peritoneal phagocytes to the lungs and enhanced the cytotoxicity response to *P. brasiliensis* antigens in non-infected mice.

Previous studies have showed that the treatment of one dose of Con-A triggers an intense influx of neutrophils in the first hours, and four days after, intense activation of macrophages characterized by increased phagocytosis, expression of MR, production of TNF-α and the maximum of the candidacidal activity by these cells [8]. In the present study, we extend these findings, showing the participation of other inflammatory mediators as well as one possible mechanism to explain the systemic effect of Con-A in a distant infectious site from the peritoneum.

The treatment with a single dose of Con-A in non-infected mice (Con-A group) induced a higher spontaneous production of H_2O_2 and INF-γ by LPS-stimulated peritoneal adherent cells than in the non-treated mice, and a lower spontaneous production of TNF-α and IL-6 (Figure 1).

The treatment with three doses of Con-A in the Con-A group induced high production of NO and IFN-γ by peritoneal adherent cells in all experimental conditions. We also observed low spontaneous levels of the chemokines MCP-1 and MIP-1α and the cytokines IL-6 and TNF-α (Figure 1).

The treatment with eight doses of Con-A resulted in a higher spontaneous production of NO, IFN-γ and IL-10 and *P. brasiliensis*-stimulated peritoneal adherent cells and lower levels of TNF-α in both conditions than the control group (Figure 1).

Comparing the groups who received three and eight doses, we observed a lower level of IFN-γ production and a higher level of IL-10 and MCP-1 by the eight doses group (Figure 1, $p < 0.05$).

Migration of PKH^+ cells to the spleen and liver was observed in the homeostasis condition, i.e., in non-infected and non Con-A-treated mice (Control group). In the Con-A groups, the injection of three doses of Con-A triggered PKH^+ cell migration to the spleen, liver and lungs (Figure 2).

Treatment with three doses of Con-A induced a protective immune response in mice infected with *P. brasiliensis*.

Peritoneal adherent cells of infected and treated mice with three doses of Con-A (PbConA group) showed: 1) higher production of H_2O_2 in the presence or absence of *P. brasiliensis* antigens, compared to the other groups; 2) increased spontaneous production of IFN-y and; 3) increased production of NO in the presence of fungal antigens, compared to the infected only group (Pb group) (Figure 3). We also observed an increase in the number of PKH^+ mononuclear phagocytes in the lungs of the PbConA group, an average of 24.6% versus 7.6% in the only infected group (Figure 4). Despite these results, no changes were observed in the recovery of viable fungi and tissue damage (Figure 5).

We also assessed whether eight doses of Con-A could enhance the fungicidal activity and result in fungal clearance in the infected mice. The continuous action of Con-A, with eight treatments, revealed no significant changes in the fungal burden, histopathological analyses and macrophage activity of peritoneal phagocytes (Figures 3 and 4).

Comparing the infected groups who received three and eight doses, we observed a lower level of H_2O_2 and IFN-γ production by the eight doses group (Figure 5).

Discussion

The drugs of choice for the treatment of paracoccidioidomycosis are itraconazole (ITC) and the trimethoprim-sufamethoxazole combination (cotrimoxazole - CMX). Amphotericin B is used for specific cases, due to its toxicity. The duration of treatment of PCM is usually time-consuming, with an average of 105 and 159 days for the itraconazole and CMX, respectively [17]. The use of immunotherapy has been rarely reported in PCM. Meira et al. [18] studied the effect of a treatment with the immunostimulant beta-glucan (beta-1, 3 polyglucose) in severe PCM. These patients were compared to those with moderate PCM that received only the antifungal drug. The immune stimulation resulted in a stronger and more favorable response to antifungal therapy, low rate of relapse (one out of ten patients) and significant reduction of serum levels of antibodies anti-*P. brasiliensis*. In an experimental study, Rezkallah-Iwasso et al. [19] showed that levamisole, an antihelmintic, enhances the cellular immune response in *P.brasileinsis* hamster without changed the fungal load. Whereas PCM patients have a deficiency in antigen-specific cellular-mediated immune response at the admission, conventional antifungal therapy combined with immunotherapy could help the host to eliminate the fungus early. In the present study, we showed at first that Con-A induces peritoneal phagocytes trafficking to the lungs in response to *P. brasiliensis* and stimulates pro-Th1 macrophage activity.

The mechanism involved in the activation of peritoneal phagocytes by Con-A remains unclear. It is well known that Con-A binds directly to the carbohydrate molecules (D-glucopyranoside and D-manopiranosídeo) present in major histocompatibility complex molecules (MHC) and T-lymphocyte receptor (TCR). The Con-A-TCRs oligomerization triggers cellular activation and high production of IFN-γ [20]. It has also been described that Con-Astimulation into the peritoneum increases phagocytosis mediated by MR [21]. Considering that peritoneal milieu comprises 15-20% of T lymphocytes [22], we suggest that Con-A acts in the macrophage activation directly by binding the MR and by INF-γ released from Con-A stimulated T lymphocytes.

The possibility of peritoneal cells becoming inflamed, neoplastic and infectious tissue has been achieved by several researches [9, 11, 23]. However, the mechanisms and their importance regarding immunotherapeutic approaches remain to be clarified. In the present study, we demonstrate for the first time that Con-A stimulated peritoneal cells are able to

enhance a pro-Th1 immune response by peritoneal cells during pulmonary paracoccicioidomycosis and increases the traffic of these cells to the lungs.

Previous studies have shown that single Con-A triggers high production of TNF-α by peritoneal cells of Swiss mice [24]. In the present study, although we observed low levels of this cytokine, phagocytes exhibited high levels of H_2O_2 and IFN-γ in the three dose treatment, consistent with the data regarding its activation. Considering that macrophages are the first line of defense against infection by *P. brasiliensis* [25] and these cells kill the yeast in an IFN-γ-dependent manner [26, 27, 28], we hypothesized whether Con-A treatment could improve the fungicidal response induced by migrant peritoneal cells to the lungs. We observed that in the Con-A treatment used protocol, it was not able to promote changes in fungal clearance as wells as in the histopathological architecture. Nevertheless, the fact that the treatment with Con-A stimulates the pro-Th1 response is promising because, in association with antifungal therapy, it could prevent, after the pathogen elimination, the granuloma switching to a M2-like profile, strongly associated to the development of fibrosis[29]. In addition, it is well-known that the Th1 response induces production of matrix metalloproteinase [30] responsible for collagen cleavage [31].

Three doses of Con-A enhanced the production of NO and IFN-γ by PC of infected-mice, including when the cells were challenged with *P. brasiliensis* antigens. Furthermore, we observed that three and eight doses of Con-A triggered low levels of TNF-α. Several studies have shown that unbalanced production of these cytokines is associated to tissue damage, such as thrombotic processes [32], increased pulmonary endothelial permeability *in vivo* [33] and pro-fibrotic effects by stimulating fibroblast maturation and collagen deposition [34].

Although in the present study we did not evaluate the subpopulation of peritoneal phagocytes separately, B-1 cells have been recognized to influence the outcome of pulmonary granulomatous infectious disease [11, 35]. Russo et al. [11] showed that the B1-cells from the peritoneum are crucial to granuloma development in the experimental pulmonary mycobacterial infection. Popi et al. [35] showed that BALB.Xid mice, deficient in B-1 cells, exhibit resistance to infection by *P. brasiliensis* and when the mice received B-1 cells by adoptive cell transfer, the fungal burden as well as the extent of lung lesions increased. These results were associated with a decrease of the phagocytic activity of *P. brasiliensis* via secretion of IL-10. In fact, Costa et al. [36] demonstrated that IL-10 plays a detrimental effect to pulmonary PCM, resulting in progressive infection and precocious mortality of infected hosts. In the present study, we also observed that eight Con-A doses in non-infected mice

resulted in increased production of IL-10; it is possible that long-term Con-A treatment could enhance the activation of B1-cells. Nevertheless, it is also possible that B1 cells and macrophages act in collaboration and further investigations are necessary to evaluate this interaction.

In summary, our results demonstrate that treatment with three doses of Con-A did not eliminate the fungus, but generates a favorable macrophage response to *P. brasiliensis* elimination by inducing a pro-Th1 response and enhances peritoneal phagocyte migration to the lungs. Despite these results, longer treatment with eight doses of Con-A does not demonstrate the same efficiency. Whereas PCM patients have a deficiency in antigen-specific cellular-mediated immune response at the admission, conventional antifungal therapy combined with immunotherapy with Con-A could help the host to eliminate the fungus early.

Acknowledgments

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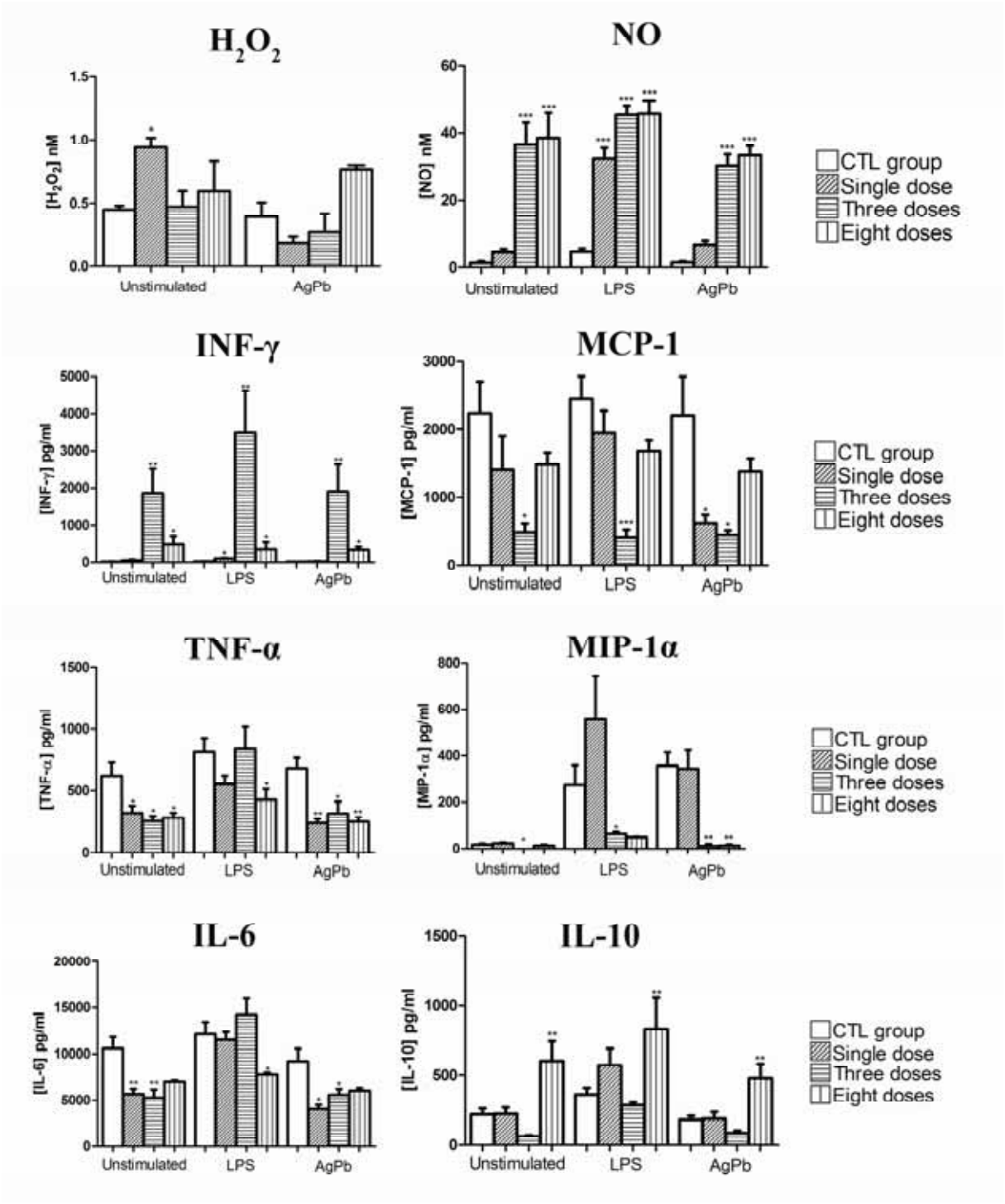

Figure 1. Production of H_2O_2, NO, IFN-γ, MCP-1, TNF-α, MIP-1α, IL-6 and IL-10 by adherent peritoneal phagocytes. BALB/c mice were inoculated with one, three or eight doses of Con-A i.p. and evaluated after four days of the last dose. The CTL group was composed of mice inoculated with sterile saline solution. Peritoneal phagocytes were stimulated or not *in vitro* with non-specific (LPS) or specific (AgPb) antigens. The symbols indicate statistical differences between the Con-A versus CTL groups. (* $p < 0.05$; ** $0.05 > p > 0.01$; *** $p < 0.01$; $n = 5$; *t* test).

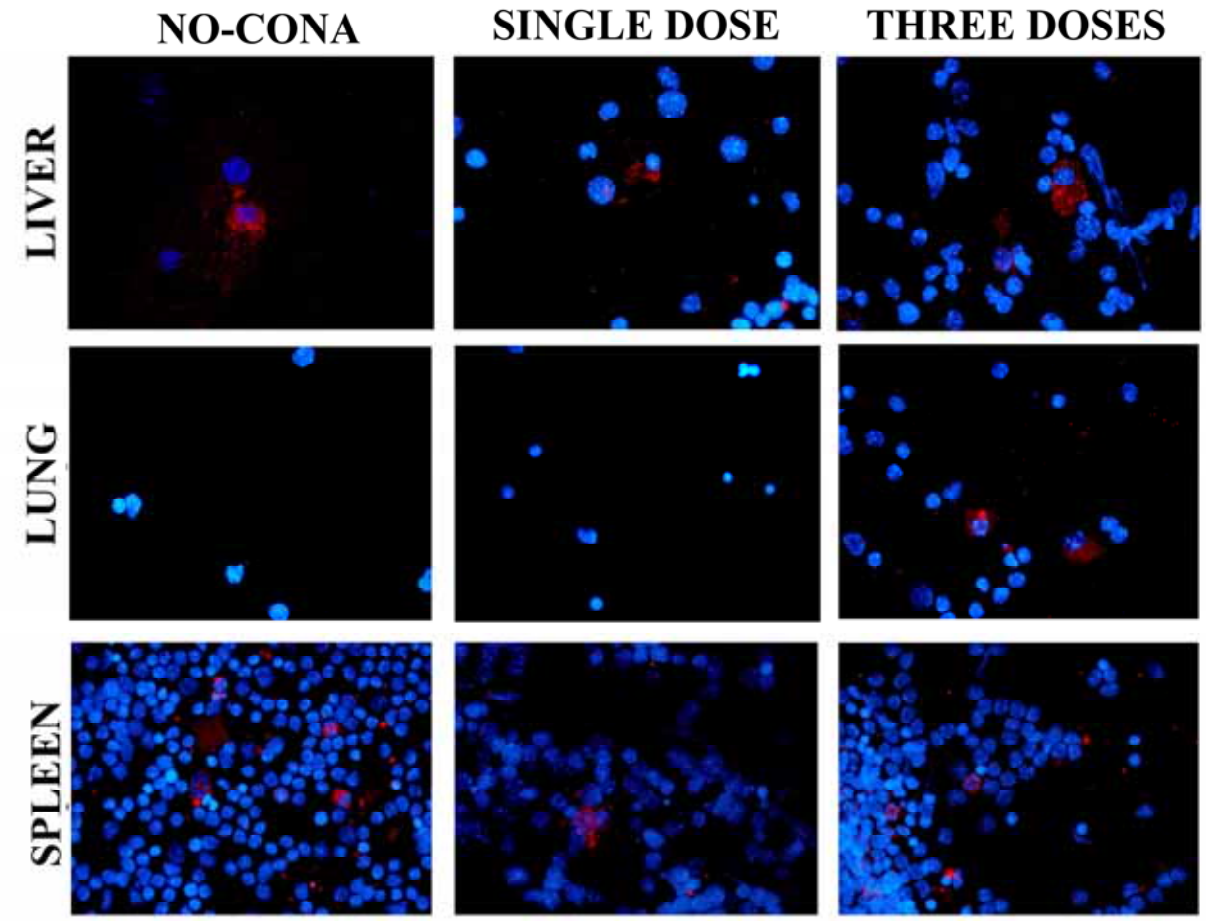

Figure 2. **Trafficking of peritoneal phagocytes in non-infected mice.** BALB/c mice were inoculated with Con-A, i.p., and after 3 days the mice were submitted to PKH-26 PCL by i.p. route. Four days after the last dose of Con-A the mice were euthanized and samples of the liver, lung and spleen were subjected to fluorescence analysis. PKH-26 PCL: red; DAPI: blue.

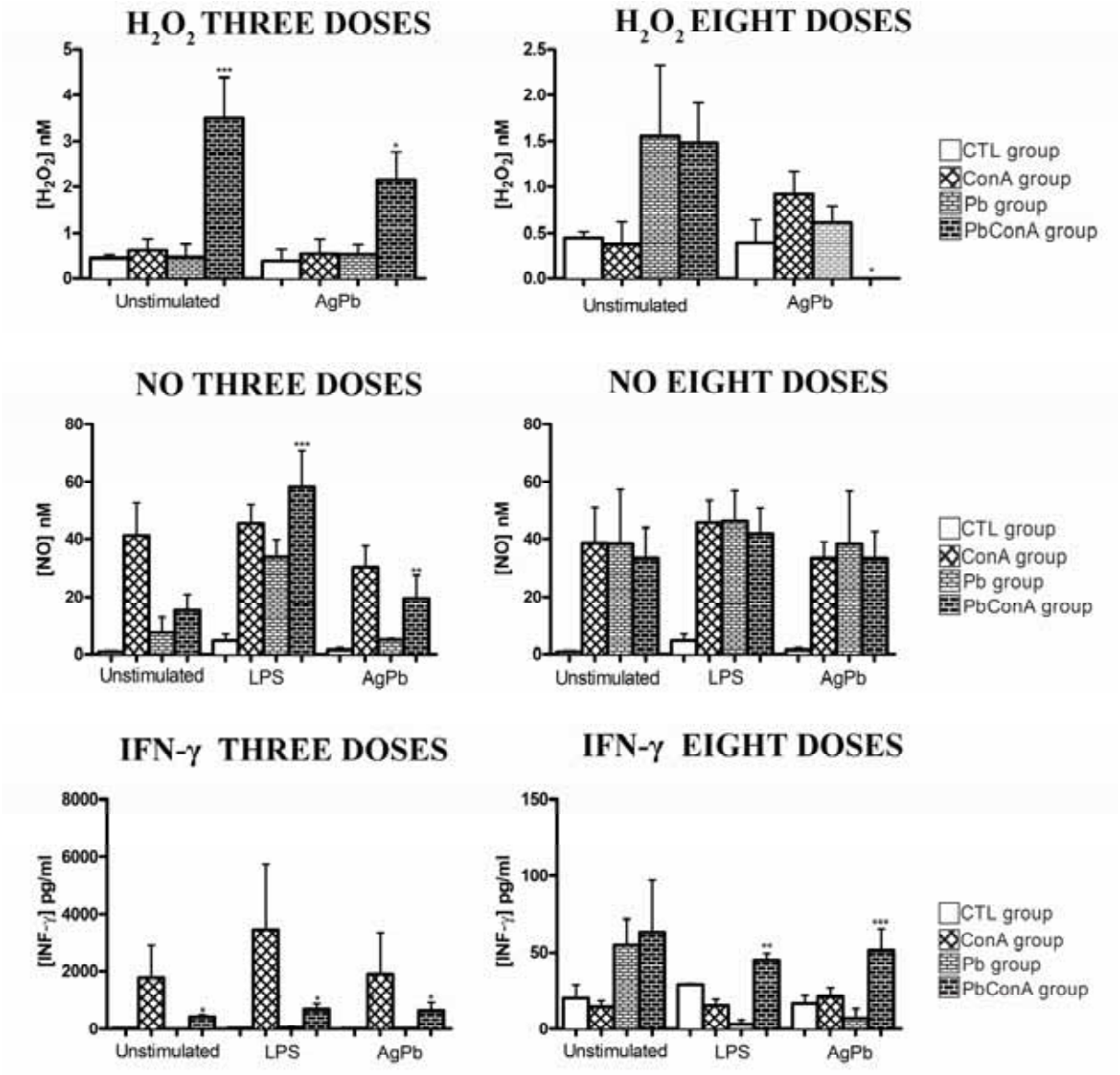

Figure 3. Production of H_2O_2, NO and IFN-γ by adherent peritoneal phagocytes of infected mice. BALB/c mice were inoculated with *P. brasiliensis* and received three or eight doses of Con-A i.p. The animals were evaluated after four days of the last dose. The Pb group was composed of infected mice inoculated with sterile saline solution i.p. Peritoneal phagocytes were stimulated or not *in vitro* with non-specific (LPS) or specific (AgPb) antigens. The symbols indicate statistical differences between the PbConA group versus the Pb group. (* $p < 0.05$; ** $0.05 > p > 0.01$; *** $p < 0.01$; $n = 5$; *t* test).

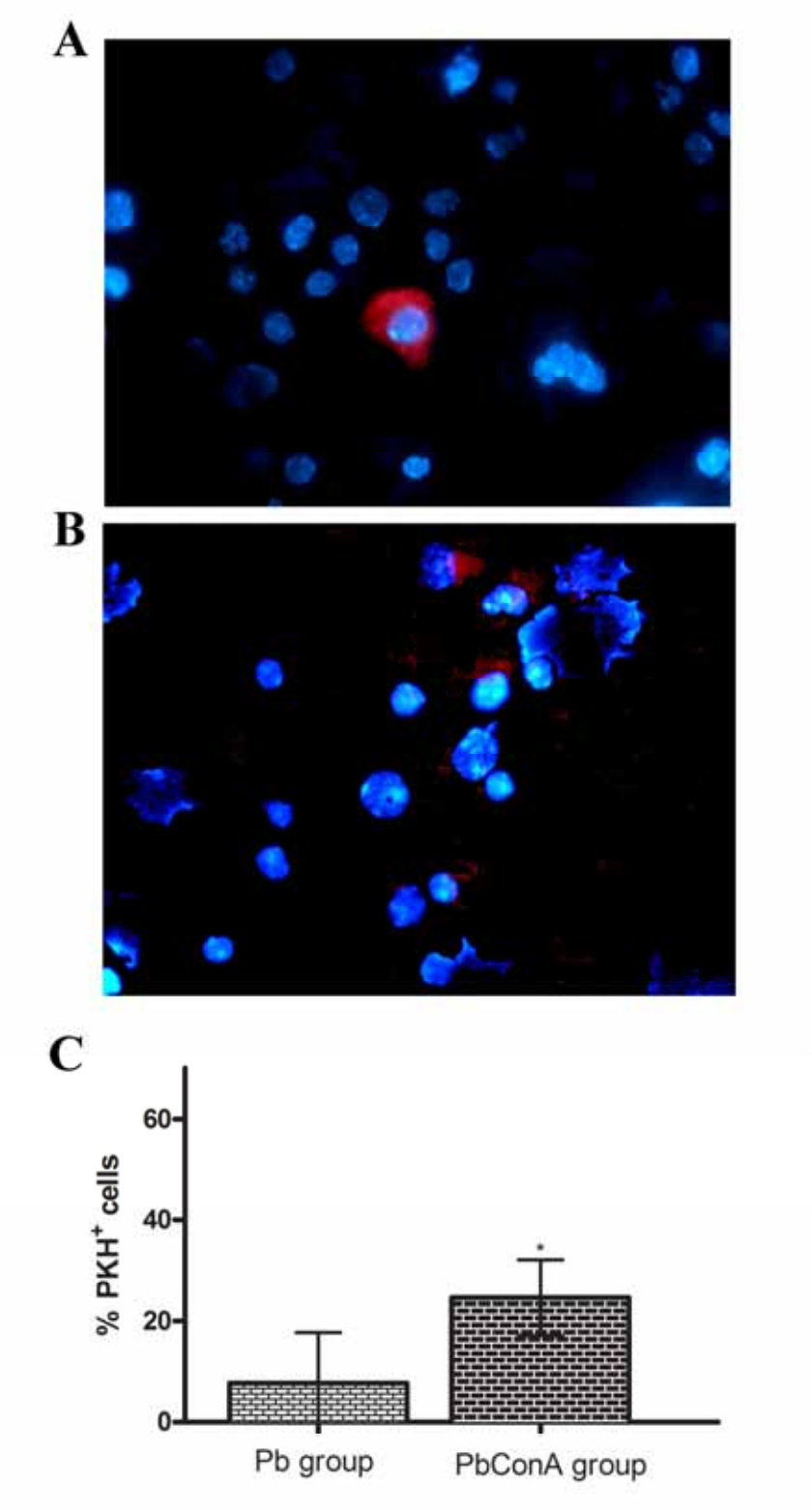

Figure 4. Trafficking of peritoneal phagocytes in infected mice. BALB/c mice were inoculated with *P. brasiliensis* and received three doses of Con-A i.p. One day before the fungal inoculation, the mice were submitted to PKH-26 PCL by i.p. route. (* $p < 0.05$; $n = 3$; *t* test).

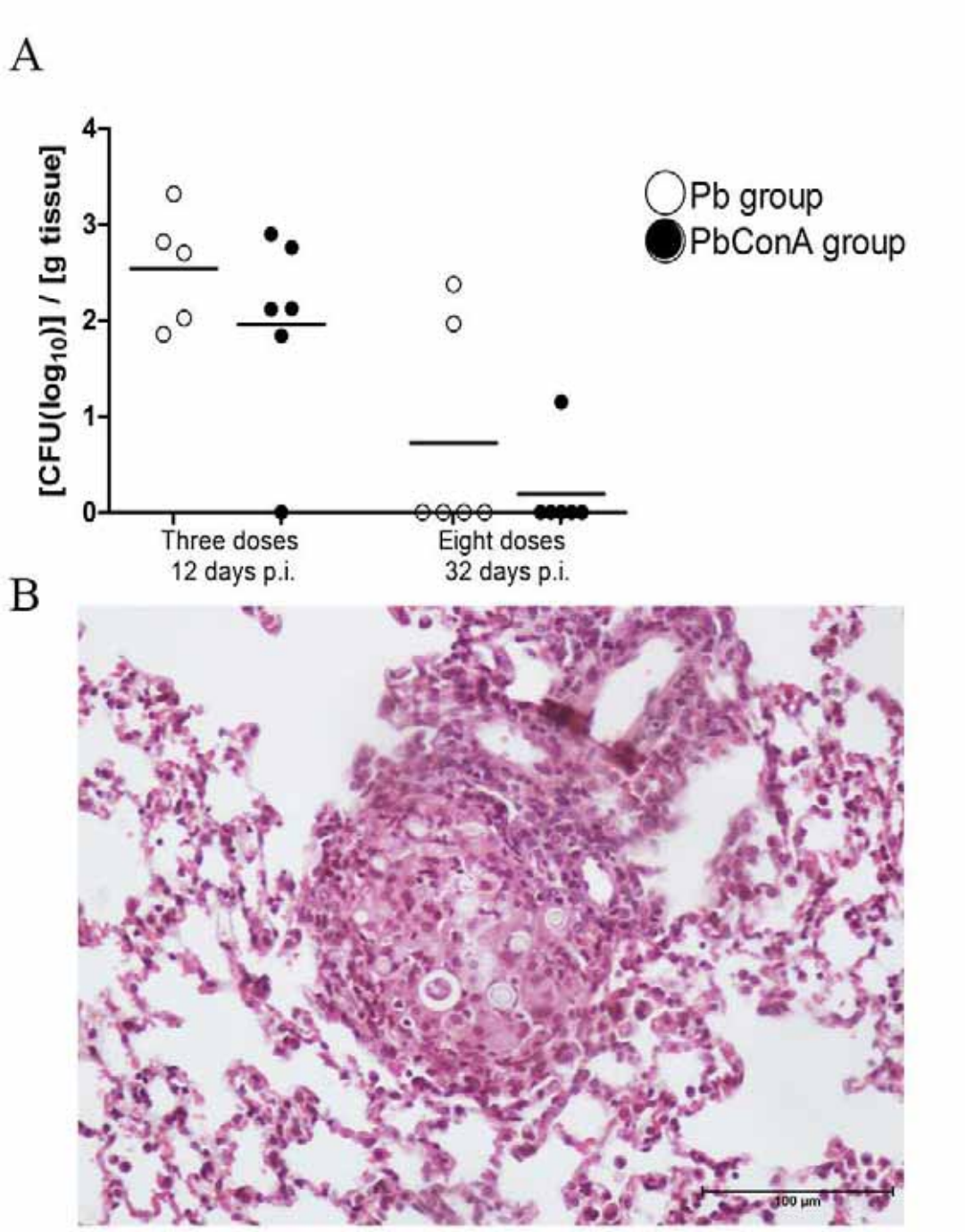

Figure 5. Microbiological and histopathological analysis of the lung. (A) Fragments of the lung were weighed and homogenized in PBS; homogenate was cultured on plates containing BHI agar supplemented and incubated at 36°C for four weeks. Total colonies were counted, and the results were expressed as the number (log_{10}) of *P. brasiliensis* per gram of tissue ($p > 0.05$, *t* test). (B) and (C) Typical early paracoccidioidal granulomatous lesion in the pulmonary parenchyma, containing viable fungal cells, modified macrophages and giant cell surrounded lymphohistiocytary infiltrate in Pb and PbConA groups, respectively (32 days, Hematoxylin-Eosine stain).

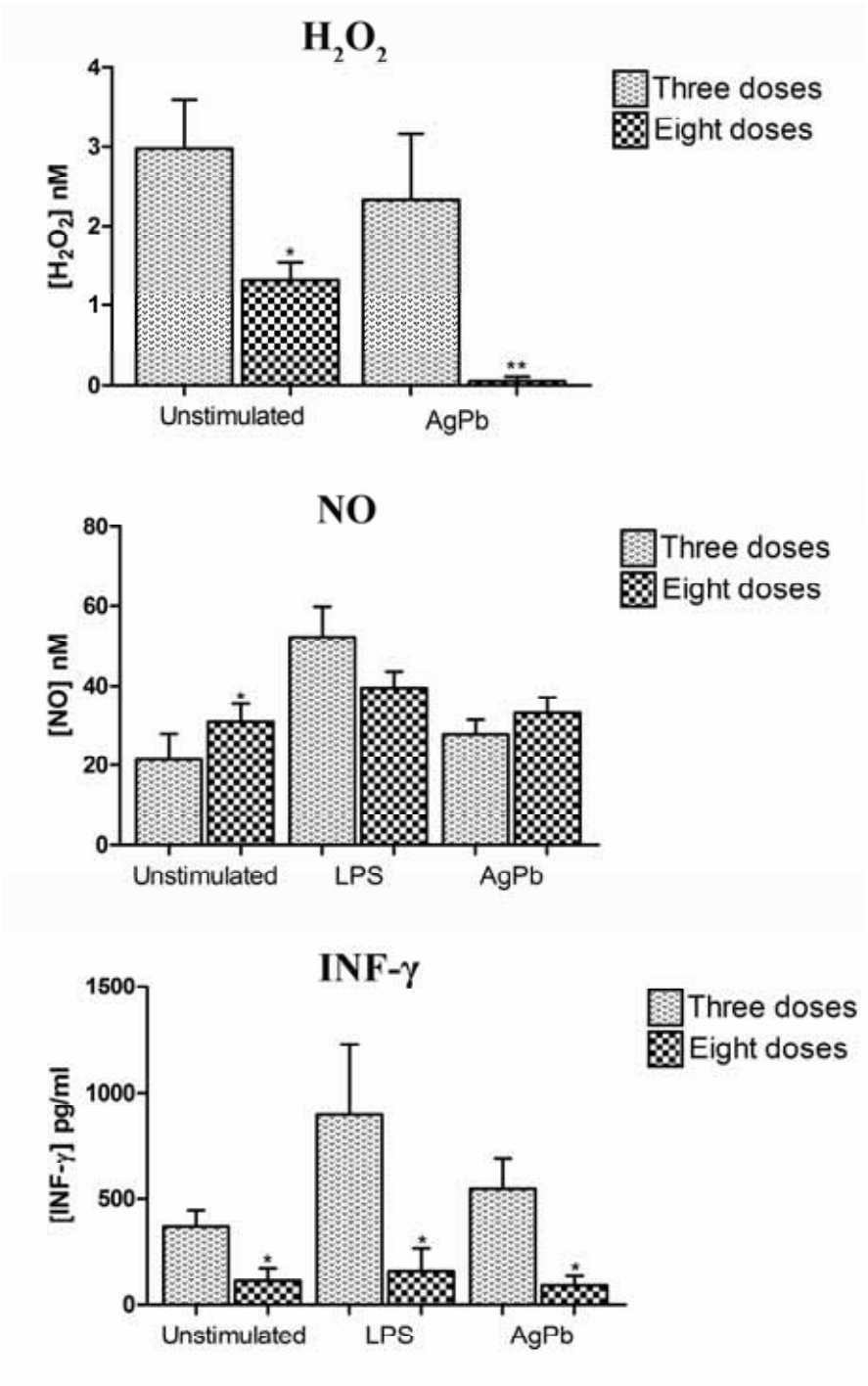

Figure 6. Production of H_2O_2, NO and IFN-γ by adherent peritoneal phagocytes by PbConA groups. BALB/c mice were inoculated with *P. brasiliensis* and received three or eight doses of Con-A i.p. The animals were evaluated after four days of the last dose. Peritoneal phagocytes were stimulated or not (CC) *in vitro* with non-specific (LPS) or specific (AgPb) antigens. The symbols indicate statistical differences between the groups. (* $p < 0.05$; ** $0.05 > p > 0.01$; *** $p < 0.01$; $n = 5$; *t* test).

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5. CONCLUSÕES

Objetivo 1: Avaliar a influência da Concanavalina-A na ativação e migração de fagócitos peritoneais.

A migração de fagócitos para baço e fígado é um processo natural, documentado por nosso grupo de pesquisa (Fraga-Silva et al, 2013), e também observada no presente estudo. Por outro lado, não foi detectada migração de fagócitos peritoneais para o pulmão em condições de homeostase.

Uma única dose de Con-A, via intraperitoneal, apesar de estimular os fagócitos no local, provocando aumento na produção de H_2O_2 e INF-γ, não leva a migração dessas células para pulmões.

O estímulo, pela mesma via, com três doses de Con-A, com um intervalo de 4 dias entre doses, provoca a migração de fagócitos peritoneais para os pulmões, além de um perfil de resposta Th1, com elevada produção de NO e INF-γ *in vitro.* Esse perfil de resposta se mantém em um estímulo mais prolongado, com oito doses de Con-A.

Conclui-se, portanto, que, nos protocolos utilizados, o uso de Con-A promove a migração para pulmões e a ativação de fagócitos peritoneais.

Objetivo 2: Determinar a migração de fagócitos peritoneais para pulmões, fígado e baço de camundongos na infecção pulmonar por *P. brasiliensis*.

No presente estudo, a infecção, realizada por via intratraqueal, não atingiu baço e fígado nos momentos avaliados. Esses órgãos recebem fagócitos peritoneais naturalmente, e não sofreram alterações na quantidade de células migrantes após a infecção. Por outro lado, o inoculo provoca uma infecção pulmonar e leva a migração de fagócitos peritoneais para esse órgão.

O estímulo com três doses de Con-A leva a um aumento no número de fagócitos migrantes encontrados no pulmão.

Conclui-se que a infecção por *P. brasiliensis* via intratraqueal promove a migração de fagócitos peritoneais para o pulmão, mas não para o fígado e baço. Além disso, o estímulo com três doses de Con-A provoca um aumento no número de células migrantes.

Objetivo 3: Avaliar o perfil de resposta de fagócitos peritoneais sob a influencia de Con-A na infecção pulmonar por *P. brasiliensis.*

Os perfis de resposta de fagócitos peritoneais de animais infectados, que receberam três doses de Con-A, de acordo com a produção de citocinas e reativos de oxigênio, é do tipo Th1, favorável à eliminação do fungo. Esse perfil de resposta é diferente daquele observado em animais apenas infectados, que não apresentam produção de INF-γ, e é semelhante ao observado em animais não-infectados que receberam três ou oito doses de Con-A.

Objetivo 4: Avaliar o efeito da Con-A sobre a carga fúngica e a histopatologia pulmonar de animais infectados pelo *P. brasiliensis.*

A inoculação de Con-A não altera a carga fúngica e a histopatologia pulmonar de animais infectados.

5.1. Conclusão final

Conclui-se que a Concanavalina-A estimula a migração de fagócitos peritoneais para o sítio da infecção. No entanto, esse estímulo não é suficiente para eliminação do fungo. Mais trabalhos se fazem necessários para avaliação do perfil de resposta desses fagócitos no local da infecção e da ação da Con-A associada ao tratamento antifúngico convencional.

6. RESUMO

A paracoccidioidomicose (PCM) é uma doença infecciosa causada por espécies do gênero *Paracoccidioides*, um patógeno intracelular cujo controle depende de uma resposta imune celular efetiva por parte do hospedeiro. Neste contexto, o ambiente peritoneal emerge como um possível local para a imunoterapia, uma vez que os fagócitos peritoneais apresentam plasticidade de funções biológicas que vão de indução/regulação da inflamação, angiogenese e processos de remodelação tecidual à migração para tecidos inflamados e infectados. Recentemente, foi relatado que células peritoneais estimuladas com Concanavalina-A (Con-A) exibem um aumento da ativação de células peritoneais, caracterizada por uma elevada atividade fagocítica, aumento da produção de TNF-α e expressão do receptor de manose. Assim, este estudo teve como objetivo identificar uma possível ação da Con-A sobre a migração de células e perfil de resposta imune por fagócitos peritoneais em modelo murino de paracoccidioidomicose pulmonar. Para tanto, foram utilizados camundongos BALB/c *naive* ou infectados por via intratraqueal com o fungo. Para avaliação da migração de fagócitos peritoneais foi utilizado o corante vital fluorescente PKH26 PCL. O tratamento com Con-A foi realizado por via intraperitoneal, as doses foram administradas com um intervalo de quatro dias (pico da ativivação macrofágica) e os fagócitos peritoneais foram analisados *in vitro* quanto à produção de IL-6, IL-10, TNF-α, MCP-1, IFN-γ e IL -12p70 e espécies reativas de oxigênio e nitrogênio. Os resultados demostraram que o tratamento com três doses de Con-A em animais não infectados induziu a migração de células peritoneais para o pulmão e, ainda, um perfil de resposta pró-Th1 por essas células. Além disso, o tratamento com três doses de Con-A induziu, o desenvolvimento de resposta imune protetora por fagócitos peritoneais de camundongos BALB/c infectados com *P. brasiliensis*. Apesar destes resultados, não foram observadas alterações na recuperação de fungos viáveis, bem como na arquitetura tecidual dos pulmões. Também foi avaliado se um tratamento prolongado com oito doses de Con-A poderia aumentar a atividade fungicida. No entanto, a ação contínua de Con-A não revelou alterações significativas na carga fúngica, arquitetura estrutural do pulmão e atividade de fagócitos peritoneais. Em conjunto, os nossos resultados mostraram que o tratamento intraperitoneal com três doses de Con-A induz a ativação dos fagócitos peritoneais, mas não elimina o fungo. Considerando que pacientes com PCM têm uma deficiência na resposta imune antígeno-específica no momento da internação, a terapia antifúngica convencional combinada com imunoterapia com Con-A poderia auxiliar na eliminação precoce do fungo pelo hospedeiro.

7. SUMMARY

The paracoccidioidomycosis (PCM) is an infectious disease caused by species of the genus *Paracoccidioides,* an intracellular pathogen that depends on an effective and protective specific-host cellular immune response. In this context, the peritoneal environment emerges as a possible site for immunotherapy, since phagocytes exhibit plastic biological functions related to induce/regulate inflammation, angiogenesis and tissue remodeling processes as well as to migrate to inflamed and infected tissue. Recently, it was reported that peritoneal cells stimulated with Concanavalin A (Con-A) show an increased cell activation characterized by high phagocytic activity, increased production of TNF-α and expression of mannose receptor. Thus, this study aimed to identify a possible action of Con-A in the peritoneal cavity, specifically the migration of peritoneal phagocytes and the profile of the response by peritoneal phagocytes in a murine model of pulmonary paracoccidioidomycosis. To this, BALB/c mice were used, infected intratracheally with the fungus or no. Assessment of migration of peritoneal phagocytes was by vital dye PKH26. The treatment with Con-A was by intraperitoneal route, the doses were given with an interval of four days (peak of macrophage activity) and the peritoneal phagocytes were analyzed *in vitro* for production of IL-6, IL-10, TNF-α, MCP-1, IFN-γ e IL -12p70 and reactive oxygen species by. Our findings showed that the treatment with three doses of Con-A in no-infected mice induces migration of peritoneal cells to the lung and also a pro-Th1 profile by peritoneal phagocytes. Furthermore, the treatment with three doses of Con-A lead a protective immune response in mice infected with *P. brasiliensis*, characterized by increased production of H_2O_2 in the presence or absence of *P. brasiliensis* antigens and in the production of IFN-γ and NO in the presence of fungal antigen. Despite of these findings, no changes were observed in the recovery of viable fungi as well as in the tissue damage. We also evaluated whether a longer treatment with eight doses of Con-A could enhances the microbicidal activity. The continuous action of Con-A revealed no significant changes in the fungal load, lung structural architecture and macrophage activity of peritoneal phagocytes. Together, our results showed that the intraperitoneal treatment with three doses of Con-A induces activation of peritoneal phagocytes, but did not eliminate the fungus. Whereas PCM patients have a deficiency in antigen-specific immune response at admission, conventional antifungal therapy combined with immunotherapy with Con-A could help the early elimination of the fungus by the host.