JULIÁN ESTEBAN MUÑOZ HENAO

Avaliação do Peptídeo Sintético (P10): associado ao tratamento quimioterápico em camundongos BALB/c anérgicos infectados com *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 Doutor em Ciências.

São Paulo
2012

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Área de concentração: Microbiologia

Orientador: Prof. Dr. Carlos Pelleschi Taborda

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Avaliação do peptídeo sintético (P10) associado ao tratamento quimioterápico em camundongos BALB/c anérgicos infectados com *Paracoccidioides brasiliensis* / Julián Esteban Muñoz-Henao. -- São Paulo, 2012.

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"Avaliação do peptídeo sintético (P10) derivado da glicoproteína gp43 de paracoccidioides brasiliensis. Atividade como adjuvante ao tratamento quimioterápico da paracoccidioidomicose e resposta pulmonar em camundongos Balb/c anérgicos infectados com P.brasiliensis"

Prezado Professor,

Informo que a sua licença para uso de animais em experimentação, constante no protocolo em epígrafe, **estará em vigência até 27.02.2013.**

Reitero que havendo alteração de metodologia e inserção de novos alunos ao projeto de pesquisa vinculado à referida licença a CEUA/ICB deverá ser informada.

Cordialmente,

Prof. Dr. WOTHAN TAVARES DE LIMA
Coordenador da Comissão de Ética
no Uso de Animal - ICB /USP

Ilmo. Sr.

Prof Dr. Carlos Pelleschi Taborda

Departamento de Microbiologia

Instituto de Ciências Biomédicas - USP

Parecer - Projeto Nº 98/12/CEUA-IPEN/SP

Com base nos pareceres apresentados pelos relatores, o protocolo de pesquisa **“AVALIAÇÃO DA BIODISTRIBUIÇÃO DO PEPTÍDEO SINTÉTICO P10 DA gp43 do *P. brasiliensis*, RADIOMARCADO COM TECNÉCIO-99M CAMUNDONGOS BALB/c”** de responsabilidade da pesquisadora **DR^a BLUMA L. FAINTUCH** foi considerado **APROVADO**.

Tendo em vista a legislação vigente, devem ser encaminhados, a este Comitê, relatórios anuais (parciais ou finais, dependendo da duração do projeto) referentes ao andamento da pesquisa. Após o término da pesquisa, uma cópia do trabalho deve ser encaminhada a este CEUA.

São Paulo, 27 de fevereiro de 2012.

Profa. Dra. Nanci do Nascimento
Coordenadora do CEUA-IPEN

Aos meus pais e familiares, que me apoiaram no dia a dia e sempre estiveram aí nos momentos difíceis da minha vida.

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Todos são muito especiais para mim, são a força de cada dia na minha vida, graças por existir...

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Obrigado por tudo!

E se estiver se arrastando na direção do cume de uma montanha nunca escalada e vir outro grupo de alpinistas subindo por um caminho paralelo? Quando se faz ciência, pode-se sugerir um trabalho conjunto – a cooperação parece tão mais criativa do que a concorrência.

Maurice Wilkins, The Third Man of the Double Helix.

Na longa história da humanidade (e também dos animais), aqueles que aprenderam a colaborar e a improvisar de forma mais eficaz, foram os que prevaleceram.

Charles Darwin

RESUMO

MUÑOZ, J. E. **Avaliação do Peptídeo Sintético (P10):** associado ao tratamento quimioterápico em camundongos BALB/c anérgicos infectados com *Paracoccidioides brasiliensis*. 2012. 231 f. Tese (Doutorado em Microbiologia) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2012.

A paracoccidioidomicose (PCM), doença sistêmica de caráter granulomatoso, é causada pelo fungo termodimórfico *Paracoccidioides brasiliensis*. A PCM é endêmica na América Latina em países como Argentina, Colômbia, Venezuela e Brasil e afeta principalmente pessoas das zonas rurais. Esta doença apresenta duas manifestações clínicas (aguda e crônica). Na maioria dos casos a PCM envolve primeiramente os pulmões, podendo se disseminar para outros órgãos e sistemas. Os pacientes com PCM são submetidos ao tratamento medicamentoso com Sulfametoxazol/trimetoprim, Anfotericina B, e derivados azólicos. Por ser uma doença com altos índices de recidivas, longos períodos de tratamento são necessários. A glicoproteína de 43kDa (gp43), possui 416 aminoácidos, onde um trecho específico de 15 aminoácidos (QTLIAIHTLAIRYAN) designado como (P10), é reconhecido por linfócitos T. No presente trabalho, avaliamos a ativação da resposta imune e o efeito aditivo da imunização com o peptídeo P10, em camundongos induzidos à imunossupressão com dexametasona. Reações de HTT e contagem de leucócitos foram realizadas periodicamente para verificar o estado do sistema imune dos animais. Os resultados indicam um efeito aditivo da imunização com P10 e o tratamento com as drogas em camundongos BALB/c imunossuprimidos e infectados; associado a redução significativa da carga fúngica no pulmão, baço e fígado desses animais, detectamos aumento de citocinas próinflamatórias como IFN- γ , TNF- α e IL-12, no homogenato de pulmão e no sobrenadante de cultura celular. Ensaio *in vitro* mostraram que a imunização com o P10 induziu uma efetiva resposta celular, com um aumento significativo na proliferação de linfócitos TCD4⁺ específicos provenientes de baço. Animais imunossuprimidos e imunizados com P10 apresentaram um aumento significativo na produção de Óxido Nítrico (NO) e foi observado a través de imunohistoquímica um aumento de células L3T4⁺, Ly-6G/Ly-6C⁺ e CD11b⁺ nos pulmões dos animais imunizados. A eficiência na resposta levou a um aumento na sobrevivência de 100%. Estes resultados sugerem que o peptídeo P10, pode atuar como adjuvante ao tratamento medicamentoso, representando uma alternativa promissória na geração de uma vacina anti-PCM e evitando possíveis recidivas no hospedeiro imunossuprimido.

Palavras-chave: P10. Imunossupressão. *P. brasiliensis*. Paracoccidioidomicose. Adjuvantes. Antifúngicos.

ABSTRACT

MUÑOZ, J. E. **Assessment of Synthetic peptide (P10):** associated with chemotherapy treatment in BALB/c mice infected with *Paracoccidioides brasiliensis*. 2012. 231 p. Ph. D. thesis (Microbiology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2012.

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease endemic of Latin America. It is caused by *Paracoccidioides brasiliensis* (Pb), a thermal dimorphic fungus. PCM is widespread mainly in Argentina, Colombia, Venezuela and Brazil affecting mainly rural workers. Inhalation of conidia is the provable route of infection. This sickness has multiple manifestations, and two progressive forms (acute and chronic) are recognized. In most cases, PCM involves primarily lungs and then disseminates to other organs and systems. PCM treatment is mandatory currently based on sulfamethoxazole/trimethoprim, amphotericin B and azolic derivatives. Because it is a disease with high recurrence rate, long periods of treatment are necessary. The gp43 has 416-mer and mice and human T lymphocytes are stimulated by a 15-mer peptide designated as P10 (QTLIAHTLAIRYAN). In the present work we evaluated the activation of immune responses and additive effect of P10 immunization in BALB/c mice induced for immunosuppression with Dexamethasone. HTT reactions and leucogram were performed periodically to check the status of the immune system of animals. The results indicate an additive effect of P10 immunization and treatment with drugs in BALB/c mice immunosuppressed and infected; associated with a significant reduction in fungal burden in the lung, spleen and liver of these animals, detected increase of proinflammatory cytokines such as IFN- γ , TNF- α e IL-12, in lung homogenates and cell culture supernatant. *In vitro* assays have shown that P10 peptide immunization led to an effective cellular immune response increasing the lymphoproliferation of CD4⁺ specific lymphocytes from the spleen. Immunosuppressed animals that were immunized with P10 showed increased in the NO production by macrophages and were observed through the immunohistochemistry augmented of cells as L3T4⁺, Ly-6G/Ly-6C⁺ and CD11b⁺ in the lungs of immunized mice. The efficiency of response led to a 100% survival in animals immunized with peptide and treated with antifungal drugs. The present work shows that P10 can act as an adjuvant to drug treatment, represents a promising alternative for the generation of anti-PCM vaccines and avoiding possible recurrences in host immunocompromised.

Keywords: P10. Immunosuppression. *P. brasiliensis*. Paracoccidioidomycosis. Adjuvant. Antifungal drugs.

LISTA DE ILUSTRAÇÕES

Figura 1 - Contagem do número de leucócitos no sangue periférico.....	55
Figura 2 - HTT realizado em camundongos BALB/c.....	56
Figura 3 - Sobrevida de camundongos BALB/c infectados i.t com 3×10^5 células leveduriformes de <i>P. brasiliensis</i> e tratados com dexametasona (0.15 mg/kg) na água de beber por 30 dias (●) grupo controle (○).....	57
Figura 4 - Baços de camundongos BALB/c submetidos ao tratamento com Dexametasona.....	58
Figura 5 - UFCs de pulmão (P), baço (B) e fígado (F) de camundongos BALB/C anérgicos, com 45 dias de infecção.....	60
Figura 6 - UFCs de pulmão (P), baço (B) e fígado (F) de camundongos BALB/C anérgicos, com 60 dias de infecção.....	61
Figura 7 - Dosagem da citocina IFN- γ , pelo método <i>Cytometric Bead Array</i> (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de <i>P. brasiliensis</i> e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.....	63
Figura 8 - Dosagem da citocina IL-12, pelo método <i>Cytometric Bead Array</i> (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de <i>P. brasiliensis</i> e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.....	64
Figure 9 - Dosagem da citocina TNF- α , pelo método <i>Cytometric Bead Array</i> (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de <i>P. brasiliensis</i> e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.	65
Figura 10 - Dosagem da citocina IL-10, pelo método <i>Cytometric Bead Array</i> (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de <i>P. brasiliensis</i> e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.....	66
Figura 11 - Dosagem da citocina IL-6, pelo método <i>Cytometric Bead Array</i> (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de <i>P. brasiliensis</i> e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.....	67

Figura 12 - Dosagem da citocina IL-8, pelo método <i>Cytometric Bead Array</i> (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de <i>P. brasiliensis</i> e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.....	68
Figura 13 - Dosagem da quimiocina MCP-1, pelo método <i>Cytometric Bead Array</i> (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de <i>P. brasiliensis</i> e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.....	69
Figura 14 – Dosagem da citocina IL-4, pelo método <i>Cytometric Bead Array</i> (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de <i>P. brasiliensis</i> e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.....	70
Figura 15 - Dosagem da citocina IL-2, pelo método <i>Cytometric Bead Array</i> (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de <i>P. brasiliensis</i> e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.....	71
Figura 16 - Relação do perfil Th1/Th2, pelo método <i>Cytometric Bead Array</i> (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos.....	72
Figura 17 - Detecção por ELISA, do isotipo IgG total presente no soro de camundongos BALB/c anérgicos.....	73
Figura 18 - Detecção por ELISA, do isotipo IgG2a total presente no soro de camundongos BALB/c anérgicos.....	74
Figura 19 - Detecção por ELISA, do isotipo IgG2b total presente no soro de camundongos BALB/c anérgicos.....	75
Figura 20 - Detecção por ELISA, do isotipo IgG1 total presente no soro de camundongos BALB/c anérgicos.....	76
Figura 21 - Determinação da produção de óxido nítrico no homogenato de pulmão de camundongos BALB/c anérgicos imunizados ou não com o P10 e tratados ou não com as drogas antifúngicas.....	77
Figura 22 - Determinação da produção de óxido nítrico no sobrenadante de cultura celular de esplenócitos de camundongos BALB/c anérgicos imunizados ou não com o P10 e tratados ou não com as drogas antifúngicas.....	78

Figura 23 - Identificação de células CD11b+ através de imunohistoquímica dos pulmões dos camundongos BALB/c anérgicos infectados com Pb18 e imunizados ou não com o P10.....	79
Figura 24 - Identificação de células Ly-6G/Ly-6C+ através de imunohistoquímica dos pulmões dos camundongos BALB/c anérgicos infectados com Pb18 e imunizados ou não com o P10.....	80
Figura 25 – Identificação de células L3T4+ através de imunohistoquímica dos pulmões dos camundongos BALB/c anérgicos infectados com Pb18 e imunizados ou não com o P10.....	81
Figura 26 - Quantificação de células do tecido pulmonar de camundongos BALB/c anérgicos infectados com Pb18 e imunizados ou não com o P10, marcadas pelo método de imunohistoquímica com os anticorpos Ly-6G/Ly-6C e L3T4.....	82
Figura 27 - Ensaio de proliferação celular utilizando linfócitos esplênicos de animais imunizados com o P10 ou não imunizados 60 dias após a infecção intratraqueal com 3×10^5 leveduras de <i>P. brasiliensis</i> e animais controle (sham)....	83
Figura 28 - Dosagem de IFN- γ , pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de <i>P. brasiliensis</i>	84
Figura 29 - Dosagem de IL-12, pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de <i>P. brasiliensis</i>	85
Figura 30 - Dosagem de IL-10, pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de <i>P. brasiliensis</i>	86
Figura 31 - Dosagem de IL-4, pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de <i>P. brasiliensis</i>	87
Figura 32 - Dosagem de IL-18, pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de <i>P. brasiliensis</i>	88
Figura 33 - Dosagem de IL-1 β , pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de <i>P. brasiliensis</i>	89
Figura 34 - Dosagem de IL-17A, pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de <i>P. brasiliensis</i>	90
Figura 35 - Percentual de linfócitos esplênicos com fenótipo de memória.....	91

Figura 36 - Percentual de linfócitos esplênicos com fenótipo de memória em camundongos BALB/c anérgicos.....	92
Figura 37 - Percentual de linfócitos esplênicos com fenótipo de reguladora em camundongos BALB/ c anérgicos.....	93
Figura 38 - Percentual de linfócitos esplênicos com fenótipo de reguladora em camundongos BALB/c anérgicos.	94
Figura 39 – Contagem global de leucócitos do sangue periférico de camundongos BALB/c anérgicos.....	95
Figura 40 - Curva de sobrevivência de camundongos BALB/c anérgicos infectados, por via intratraqueal, com 3×10^5 leveduras de <i>P. brasiliensis</i> isolado 18 e imunossuprimidos, com dexametasona.....	96
Figura 41 - Curva de sobrevivência de camundongos C57/BL6 <i>Knockout</i> iNOS ^{-/-} infectados por via intratraqueal com 3×10^5 leveduras de Pb18 e imunossuprimidos, com dexametasona.....	97
Figura 42 - Histopatologia dos pulmões dos camundongos BALB/c anérgicos infectados com Pb18 e imunizados, ou não, com P10.....	98
Figura 43 – Detecção da fibrose pulmonar através da histopatologia dos pulmões de camundongos BALB/c imunossuprimidos e infectados com Pb18, imunizados, ou não, com P10.....	100
Figura 44 - Comparação da formação de fibras de colágeno do tipo I.....	101
Figura 45 - Comparação da formação de fibras de colágeno do tipo III.....	102
Figura 46 – Representação esquemática do desenho de um radiofármaco	102
Figura 47 – Biodistribuição do ^{99m}Tc -HYNIC-P10 em camundongos BALB/c saudáveis.....	103
Figura 48 – Imagens cintilográficas em camundongos BALB/c ^{99m}Tc -HYNIC ligado no peptídeo P10.	104
Figura 49 - Citotoxicidade <i>in vivo</i> do peptídeo P10.	106
Figura 50 - Citotoxicidade <i>in vitro</i> do peptídeo P10.....	107

LISTA DE ABREVIATURAS E SIGLAS

Abs	Absorvância
Ac	Anticorpo
AcM	Anticorpo Monoclonal
B	Baço
BHI	Brain Heart Infusion (infusão de cérebro e coração)
BSA	Soro Albumina Bovina
°C	Graus celcius
CD	<i>cluster of differentiation</i>
CO ₂	Dióxido de carbono
DCs	Células Dendríticas
Dex.	Fosfato de Dexametasona
DMSO	dimetilsulfóxido
D.O	Densidade Ótica
DTH	Testes cutâneos de hipersensibilidade tardia
E ₂	Estradiol
ELISA	“Enzyme Linked Immuno Sorbent Assay”
EUA	Estados Unidos da América
F	Fígado
FcγR	Receptor da fração Fc das imunoglobulinas
g	Gramas
g	Relative centrifuge force-Gravities
gp	Glicoproteína

gp43	Glicoproteína de 43 kDa
HUVEC	<i>Human Umbilical Vein Endothelial Cells</i>
HYNIC	6-hidrazinonicotinamida
H ₂ O	Água
HTT	Hipersensibilidade Tipo Tardio
H ₂ O ₂	Peróxido de hidrogênio
H ₂ SO ₄	Ácido Sulfúrico
HCl	Ácido clorídrico
HLA	Antígeno Leucocitário Humano
HPLC	<i>High Performance Liquid Chromatography</i>
IFN	Inteferon gama
Ig	Imunoglobulina
IgA	Imunoglobulina A
IgG1	Imunoglobulina G isótipo 1
IgG3	Imunoglobulina G isótipo 3
IgG2a	Imunoglobulina G isótipo 2a
IgG2b	Imunoglobulina G isótipo 2b
IL-4	Interleucina 4
IL- 5	Interleucina 5
IL-10	Interleucina 10
IL-12	Interleucina 12
iNOS	Óxido Nítrico Sintase
i.t.	Intratraqueal
KDa	Quilodalton
Kg	Kilogramas

K ₂ HPO ₄	Fosfato de potássio
KO	<i>“Knockout”</i>
L-DOPA	L-3,4 dihidroxifenilalanina
M	Molar
MEFs	<i>Mouse Embryonic Fibroblasts</i>
mg	Mili gramos.
mM	Mili Molar
mg	Miligramas
mL	Mililitros
min	Minutos
MMcM	Meio líquido quimicamente definido
MS	Mass Spectrometry
MS	Ministério de Saúde
MTT	Thiazolyl Blue Tetrazolium Bromide
Mø	Macrófagos
µl	Microlitros
µg	Microgramas
µm	Micrometros
N	Normal
NaCl	Cloreto de sódio
NF-κB	<i>Nuclear Factor-KappaB</i>
NK	<i>natural killer</i>
nm	nanômetro
NO	Óxido Nítrico
P10	Peptídeo 10

Pb	<i>Pacoccidioides brasiliensis</i>
Pb 18	<i>Pacoccidioides brasiliensis</i> isolado 18
PBS	Fosfate buffer saline
PBMC	Células mononucleares de sangue periférico
PCM	Paracoccidioidomicose
pH	Potencial Hidrogeniônico
PMNs	polimorfonucleares
PRRs	Receptores de reconhecimento padrão
rpm	Rotações por minuto
SDS-PAGE	<i>Sodium Dodecyl Sulfate de PolyAcrylamide Gel</i>
SPF	<i>Specific Pathogen Free</i>
SZM-TMP	sulfametoxazol-trimetoprim
^{99m} Tc	Tecnécio-99 metaestável
TCD4+	Linfócito T CD4 positivo
TGF-β	Transforming Growth Factor-beta
Th1	Linfócito T “ <i>helper</i> ” 1
Th2	Linfócito T “ <i>helper</i> ” 2
TNF-α	Fator de necrose tumoral-alfa
Treg	Células T Reguladoras
UFC	Unidade formadora de colônia
VIH	Vírus da Imunodeficiência Humana

SUMÁRIO

1 INTRODUÇÃO	23
1.1 Paracoccidioidomicose.....	23
1.2 Agente etiológico.....	27
1.3 Fatores de virulência.....	29
1.4 Paracoccidioidomicose e o Sistema imune.....	32
1.5 Dexametasona e sistema imune.....	35
1.6 Tratamento na Paracoccidioidomicose.....	36
1.6.1 Derivados azólicos.....	36
1.6.2 Derivados sulfonamídicos.....	37
1.6.3 Polienos.....	37
2 JUSTIFICATIVA.....	39
3 OBJETIVOS	40
3.1 Objetivo geral.....	40
3.2 Objetivos específicos.....	40
4 MATERIAL E MÉTODOS	41
4.1 Animais.....	41
4.2 Imunossupressão dos camundongos BALB/c.....	41
4.3 Avaliação do estado de imunossupressão.....	41
4.3.1 Leucograma.....	41
4.3.2 Reação de HTT.....	42
4.4 Isolado e Cultivo do <i>Paracoccidioides brasiliensis</i>.....	42
4.5 Infecção e Terapia.....	42
4.5.1 Preparo do inóculo.....	42
4.5.2 Determinação da viabilidade das leveduras.....	42
4.5.3 Infecção intratraqueal (i.t).....	43
4.5.4 Terapia com agentes antimicrobianos.....	43
4.5.4.1 <u>Primeiro protocolo</u>	43
4.5.4.2 <u>Segundo protocolo</u>	44
4.6 Síntese e purificação do peptídeo (P10).....	44
4.7 Imunização dos camundongos.....	44
4.8 Grupos utilizados.....	45

4.9	Análise da carga fúngica através das unidades formadoras de colônias....	45
4.10	Dosagem de citocinas do homogenato de pulmão pelo método de CBA..	46
4.11	Detecção por ELISA de anticorpos (IgG Total, IgG2a, IgG2b, IgG1 e IgG3) no soro de camundongos BALB/c anérgicos infectados intratraquealmente com <i>P. brasiliensis</i> e imunizados ou não com o peptídeo P10.....	46
4.12	Dosagens de óxido nítrico.....	47
4.13	Análise imunohistoquímico através da reação de estreptavidina-biotina-peroxidase.....	47
4.14	Ensaio de proliferação celular.....	48
4.15	Dosagem de citocinas do sobrenadante de cultura celular.....	49
4.16	Imunofenotipagem por citometria de fluxo.....	50
4.17	Contagem global de leucócitos.....	50
4.18	Curva de sobrevida.....	51
4.18.1	Curva de sobrevida de camundongos Knockout iNOS.....	51
4.19	Histopatologia.....	51
4.20	Determinação da fibrose pulmonar.....	52
4.21	Marcação com radiofármaco.....	52
4.22	Estudios de imagem por biomarcação.....	53
4.23	Determinação da atividade citotóxica <i>in vivo</i> do peptídeo P10.....	53
4.24	Determinação da atividade citotóxica <i>in vitro</i> do peptídeo P10.....	54
4.25	Análise estatística.....	54
5	RESULTADOS.....	55
5.1	Padronizações do modelo de anergia com o glicocorticóide sintético “fosfato de dexametasona” em camundongos BALB/c.....	55
5.2	Análise da carga fúngica através das unidades formadoras de colônias 1º protocolo: (Os animais foram sacrificados com 45 dias de infecção).....	58
5.3	Análise da carga fúngica através das unidades formadoras de colônias 2º protocolo: (Os animais foram sacrificados com 60 dias de infecção).....	60
5.4	Análise das citocinas IFN- γ , IL-12, TNF- α , IL-10, IL-6, IL-8, MCP-1, IL-4 e IL-2 nos camundongos anérgicos infectados e tratados com drogas antifúngicas, associadas, ou não, à imunização com P10 pelo método de CBA.....	62
5.4.1	Padrão Th1/Th2 determinado pela análise do perfil de citocinas.....	71

5.5 Detecção por ELISA de anticorpos (IgG Total, IgG2a, IgG2b e IgG1) no soro de camundongos BALB/c anérgicos infectados intratraquealmente com <i>P. brasiliensis</i> e imunizados ou não com o peptídeo P10.....	72
5.5.1 Dosagem de IgG total.....	72
5.5.2 Dosagem de IgG2a.....	73
5.5.3 Dosagem de IgG2b e IgG1.....	74
5.6 Dosagem de óxido nítrico (NO) do homogeneizado de pulmão.....	76
5.7 Dosagem de óxido nítrico (NO) do sobrenadante de cultura celular.....	77
5.8 Análise imunohistoquímica através da reação de estreptavidina-biotina-peroxidase.....	78
5.9 Ensaio de proliferação celular.....	82
5.10 Dosagem de citocinas do sobrenadante de cultura celular.....	83
5.11 Caracterização de células T com fenótipo de memória.....	90
5.12 Caracterização de células T com fenótipo de reguladoras (Treg).....	92
5.13 Contagem global de leucócitos.....	94
5.14 Curva de sobrevivência de camundongos BALB/c anérgicos.....	95
5.14.1 Curva de sobrevivência de camundongos Knockout iNOS.....	96
5.15 Histopatologia do pulmão de camundongos anérgicos infectados e imunizados, ou não, com o peptídeo 10.....	97
5.16 Determinação da fibrose pulmonar.....	98
5.17 Biodistribuição do conjugado radiomarcado.....	102
5.18 Aquisições de imagens cintilográficas.....	103
5.19 Determinação da atividade citotóxica in vivo do peptídeo P10.....	104
5.20 Determinação da atividade citotóxica in vitro do peptídeo P10.....	106
6 DISCUSSÃO.....	108
7 CONCLUSÕES.....	115
REFERÊNCIAS.....	117
APÊNDICES.....	131
APÊNDICE A - Artigos publicados em periódicos indexados.....	131
APÊNDICE B - Artigos no prelo.....	132
APÊNDICE C - Patente.....	132

1 INTRODUÇÃO

1.1 Paracoccidioidomicose

A paracoccidioidomicose (PCM), micose sistêmica com manifestações clínicas de uma doença granulomatosa, causada pelo fungo termodimórfico *Paracoccidioides brasiliensis*, foi descrita pela primeira vez, por Adolpho Lutz, ao verificar lesões bucais em dois pacientes, em 1908. A micose descrita por Lutz foi inicialmente nomeada de Blastomicose Sul-Americana ou Doença de Lutz-Splendore e Almeida (LACAZ et al., 1991), porém, a oficialização do termo Paracoccidioidomicose foi estabelecida em 1971, em Medellín, Colômbia, durante reunião de micologistas do continente Americano, sendo mundialmente aceita, desde então (LACAZ et al., 1982).

A PCM é endêmica e considerada a micose sistêmica de maior prevalência na América Latina, reportada desde o México até a Argentina e afetando principalmente Brasil com 80% dos casos seguido pela Colômbia e Venezuela (BRUMMER et al., 1993; WANKE et al., 1994). Casos atípicos ou não autóctones foram notificados no Chile, Suriname, Guiana, Nicarágua e Belize segundo Restrepo (2003¹ apud RESTREPO et al., 2008). Um aspecto interessante na distribuição da doença é que países onde a PCM é endêmica, não se apresenta uma distribuição homogênea da doença e sim em certas regiões que oferecem condições aparentemente favoráveis para o fungo (RESTREPO et al., 2001).

No Brasil, os índices de mortalidade mais elevados dessa micose, foram encontrados nas regiões Sudeste, especialmente nos estados de São Paulo, Minas Gerais e Rio de Janeiro e na região sul nos estados de Paraná e Rio Grande do Sul (PRADO et al., 2009); sendo considerado um grave problema de Saúde Pública, devido à existência de extensas áreas endêmicas, ao alto potencial incapacitante e à quantidade de mortes prematuras que provoca, principalmente para segmentos sociais específicos, como os trabalhadores rurais, trazendo importantes repercussões econômico-produtivas dos indivíduos acometidos (BLOTTA et al., 1999; SHIKANAI-YATSUDA et al., 2006).

No Brasil, a investigação epidemiológica sobre a mortalidade é realizada pelo Sistema de Informações sobre Mortalidade (SIM), onde a taxa anual de mortalidade

¹RESTREPO A. Paracoccidioidomycosis. In: DISMUKES, W. E.; PAPPAS, P. G.; SOBEL, J. (Ed.) **Clinical mycology**. New York: Oxford University Press, 2003. p. 328-345.

detectada foi de 1,45/milhão de habitantes, mostrado que 94,6% das mortes ocorreram no Sul, 90,9% no Sudeste, 87,3% no Centro-Oeste e 74,6% no Norte e Nordeste do Brasil, onde o sub-registro é mais comum o sub-registro tem sido essencialmente atribuído à falta de atestado de óbito padronizado, que geralmente ocorre em áreas pobres e rurais (PRADO et al., 2009).

Epidemiologicamente apresenta incidência maior em pacientes do sexo masculino, com idades entre 30 e 50 anos, e uma proporção de homem para mulher de (13:1), esta proporção não se aplica na infância, onde a micose é distribuída uniformemente entre ambos os sexos, com ligeiro predomínio do masculino em adultos jovens (BRUMMER et al., 1993; RESTREPO; TOBON, 2005; RESTREPO et al., 2008).

A doença no homem pode-se desenvolver desde o momento em que o fungo entra em contato com o hospedeiro, ou passar por um período de latência que pode ser de meses a vários anos, dificultando assim, a determinação precisa do local onde foi adquirida a infecção, o *P. brasiliensis* também tem a capacidade de permanecer latente no pulmão por um período de tempo indefinido (BRUMMER et al., 1993; RESTREPO, 1994).

Deve-se salientar que a PCM apresenta maior incidência em populações com baixos recursos econômicos, que não têm acesso aos serviços de saúde, principalmente aqueles que desempenham atividades na lavoura (MARQUES et al., 1983). Relatos de pacientes com PCM residentes em grandes centros urbanos ou países não endêmicos como EUA, África, Europa e Ásia são conhecidos como *doença de importação*, devido ao fato dos pacientes terem residido em regiões endêmicas em época anterior à manifestação clínica da micose, e nestes grandes centros a doença é diagnosticada, um estudo realizado por Forjaz (1989), indicou o fluxo migratório de zonas endêmicas do Brasil à cidade de São Paulo, e foi observado que 80,8% dos pacientes tinham desempenhado atividades na lavoura e 67,7% provinham de outros estados.

Diversas opções têm sido publicadas para clasificar as formas clínicas da PCM, baseadas em diferentes critérios tais como topografia das lesões, gravidade da doença, resultados de reações sorológicas e história natural, entre outras. Algumas adaptações da classificação apresentada no *International Colloquium on Paracoccidioidomycosis*, clasificam a doença em: PCM infecção, PCM doença,

Forma aguda/subaguda, Forma crônica, Forma unifocal, Forma multifocal e a forma residual ou sequelar. A classificação mais citada atualmente da doença baseia-se em duas formas clínicas principais, a aguda e subaguda (tipo juvenil) e a crônica (tipo adulto) é determinada por uma série de sinais e sintomas (FRANCO et al., 1987), dependentes da evolução, da localização das lesões e da ativação do sistema imune do hospedeiro (MONTENEGRO; FRANCO, 1994).

Pacientes com a forma crônica da PCM, que compreendem aproximadamente 90% dos casos, apresenta uma duração prolongada com uma instalação lenta e gradual, tendendo a se localizar em órgãos e tecidos de modo mais focal, comprometendo principalmente o pulmão e a mucosa das vias respiratórias, com manutenção da resposta imune celular e baixos títulos de anticorpos específicos, o que corresponde à forma hiperérgica ou localizada (MENDES; RAPHAEL, 1971; MONTENEGRO; FRANCO, 1994). A forma crônica progride lentamente, de forma silenciosa e é denominada unifocal quando é restrita a um órgão, geralmente a doença envolve vários órgãos (forma multifocal), sendo os pulmões (90% dos pacientes), mucosa e pele os sítios mais acometidos pela infecção (SHIKANAI-YATSUDA et al., 2006).

Enquanto que as formas agudas e subagudas apresentam altos níveis de anticorpos específicos e o sistema imune celular comprometido. A resposta imune desenvolvida por pacientes com a forma aguda e subaguda leva à formação de granulomas frouxos, que estão associados ao alto número de células fúngicas viáveis (FRANCO et al., 1987).

A forma aguda apresenta evolução rápida, e é responsável por 15 a 20% de todos os casos da doença, com frequente linfadenomegalia, hepatoesplenomegalia, envolvimento ósteo-articular, mediastinal, intra-abdominal e lesões cutâneas (SHIKANAI-YATSUDA et al., 2006), apresentando lesões progressivas e afetando o sistema fagocítico mononuclear de jovens de ambos os sexos e pessoas imunocomprometidas, apresentando ativação policlonal de linfócitos B por antigenemia, que corresponde à forma anérgica ou disseminada (CAMARGO; FRANCO, 2000; MONTENEGRO; FRANCO, 1994).

O estabelecimento da infecção parece estar intimamente relacionado ao dimorfismo do *P. brasiliensis*, conforme observado em outros fungos patogênicos (*Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Sporothrix schenckii* e

Coccidioides immitis). Essa conversão considera-se fundamental para o estabelecimento da infecção e de interesse particular, pois se acredita que seja importante na patogenicidade do fungo (CAMARGO; FRANCO, 2000; FRANCO, 1987; SAN-BLAS et al., 2000). A primeira interação fungo-hospedeiro ocorre nos alvéolos pulmonares, onde dependendo da capacidade invasiva do fungo e do estado imunológico do hospedeiro, começa o processo de dimorfismo e colonização. Estudos realizados no modelo experimental de infecção com conídios de *P. brasiliensis*, descrevem que o processo de dimorfismo ocorre entre 12 e 18 horas após infecção (MCEWEN et al., 1987).

A mulher está mais protegida da doença, devido à presença de estrógenos endógenos que atuam a través de proteínas ligantes no citosol do fungo, inibindo a transformação de micélio em levedura (STOVER et al., 1986). Assim, sem a interferência hormonal, a infecção pode aparecer antes da puberdade ou depois da menopausa, uma vez que o fungo *P. brasiliensis* tem receptores para 17- β -estradiol (E2) no citoplasma.

Aristizabal et al (2002), avaliaram o curso clínico da doença no modelo experimental de camundongos machos e fêmeas, eles empregaram diferentes condições hormonais e observaram que os machos que receberam o E2 conseguiram restringir a progressão da doença, enquanto que as fêmeas que receberam testosterona não foram capazes de controlar a doença.

No envolvimento pulmonar é importante resaltar as lesões fibróticas residuais, observadas em ~60% dos pacientes com PCM, esta é uma severa e progressiva sequela que altera as funções respiratórias e incapacita o paciente nas suas atividades diárias tornando-se um problema econômico-productivo (NARANJO et al., 2011; TOBÓN et al., 2003).

Interpretações de estudos de função pulmonar têm sido dificultadas pela alta frequência de uso do tabaco entre os doentes de PCM, qual excede o 93% dos pacientes, apresentando defeito predominante obstrutivo, principalmente das pequenas vias respiratórias, com hipoxemia de grau variado. Além disso, o fumo parece ser um factor de risco para o desenvolvimento da micose (LEMLE et al., 1983).

Casos de PCM em pacientes imunossuprimidos não são maioria, já que o *P. brasiliensis* não é um fungo “oportunista”, mas alguns casos de infecção associados

a imunossupressão tem sido relatados, como por exemplo em pacientes submetidos a tratamento citotóxico ou terapia imunossupressora contra o câncer (MARQUES; LASTÓRIA; MARQUES, 2011), pacientes submetidos a transplantes (SHIKANAI-YASUDA et al., 1995; SUGAR et al., 1984), desordens imunológicas como Lúpus eritematoso (LONDERO; SANTOS; RAMOS, 1987), artrite reumatoide (WOYCIECHOWSKY et al., 2011) e doença de Hodgkin (ALTERIO; NEGRO, 1960). Presume-se que fármacos citotóxicos pode reativar uma lesão latente, causando uma infecção pulmonar aguda, provavelmente pelo comprometimento do sistema imunológico devido ao tratamento quimio e radioterápico.

1.2 Agente etiológico

P. brasiliensis, agente etiológico da PCM, apresenta dimorfismo térmico, assim, à temperatura ambiente, entre 18-25 °C, assume forma de micélio, composto por hifas septadas que produzem propágulos asexuais, conhecidos como microconídeos (< 5 µm). Já no hospedeiro ou em cultura a uma temperatura entre 35-37 °C, apresenta a forma de levedura unicelular, com múltiplos brotamentos (blastoconídeos) e tamanho variável entre 6 a 40 µm (RESTREPO et al., 2008). Na fase de levedura, as colônias são enrugadas e com coloração creme; o seu crescimento começa aparentemente entre 10 a 15 dias após a incubação (LACAS et al., 1991). Durante vários anos, o *P. brasiliensis* tem sido conhecido apenas na sua forma assexuada, atualmente sabe-se que, pelo menos, uma quinta parte dos fungos descritos não é conhecida a sua fase sexual (GEISER; PITT; TAYLOR, 1998). No entanto, a incapacidade para demonstrar a fase sexual, não significa a sua inexistência. Recentemente, genomas sequenciados e alguns dados mostram evidências de recombinação, corroborando a existência de uma fase sexual do *P. brasiliensis* (MATUTE et al., 2006).

A infecção produzida nos humanos por *P. brasiliensis* acontece principalmente pela inalação dos conídios ou propágulos na forma miceliana do fungo; com o aumento da temperatura, os conídios recebem um estímulo e se transformam em leveduras nos alvéolos pulmonares, constituindo a forma parasitária nos tecidos do hospedeiro, como foi comprovado experimentalmente em camundongos (McEWEN et al., 1987). Até recentemente, os humanos eram tidos

como os únicos hospedeiros naturalmente infectados por este fungo, mas é importante resaltar que alguns animais foram encontrados portadores da infecção, como tatus especialmente *Dasypus novemcinctus* (BAGAGLI et al., 1998., CORREDOR et al., 1999; NAIFF et al., 1986; SILVA-VERGARA et al., 2000), cachorros (FARIAS et al., 2005, 2011; RICCI et al., 2004), morcegos frugívoros *Artibeus lituratus* (GROSE; TAMSITT, 1965), bicho-preguiça *Choloepus didactylus* (TREJO-CHAVES et al., 2011), espécies de macacos (JOHNSON; LANG 1984; NAIFF et al., 1996) e o fungo também foi encontrado em fezes de pinguins da antártida (GARCIA et al., 1993; GEZUELE, 1989) provavelmente, estes últimos animais foram infectados a partir de solos contaminados com o fungo (FRANCO et al., 2000).

Após a descrição do fungo *P. brasiliensis* por Adolpho Lutz, em 1908, iniciou-se uma fase de estudos das principais características do agente infectante, como diferenças morfológicas e temperatura de cultivo. Splendore, em 1912, sugeriu a classificação do agente no gênero *Zymonema*, recebendo, assim, a denominação de *Zymonema brasiliense*. A doença passou, então, a ser denominada de "blastomicose brasileira" e, logo a seguir, "blastomicose sul-americana", já que foram relatados casos isolados em outros países da América do Sul. Após estudos sistematizados, cria-se um novo gênero dentro do reino *Fungi* - o *Paracoccidioides*, revalidando o nome da espécie criada por Splendore, em 1912. Em 1930, Floriano Paulo de Almeida oficializou o nome *Paracoccidioides brasiliensis* (ALMEIDA, 1930). Taxonomicamente, o fungo *P. brasiliensis* foi, inicialmente, classificado por Ajello (1977) da seguinte forma: Reino *Fungi*, Filo *Eumycota*, Subdivisão *Deuteromycotina*, Classe *Hyphomycetes*, Ordem *Moniales*, Família *Moniliaceae*, Gênero *Paracoccidioides* e Espécie *brasiliensis*. Contudo, outra classificação foi proposta, pois estudos filogenéticos empregando ferramentas moleculares posicionaram o agente etiológico da PCM junto aos demais fungos dimórficos (*Coccidioides posadasii*, *Coccidioides immitis*, *Blastomyces dermatitidis* e *Histoplasma capsulatum*) como pertencentes à seguinte categoria taxonômica: Reino *Fungi*, Filo *Ascomycota*, Classe *Pleomycetes*, Ordem *Onigenales*, Família *Onygenaceae*, Gênero *Ajellomyces* e Espécie *brasiliensis*, sendo denominado, então, de *Paracoccidioides brasiliensis* (SAN-BLAS; NINO-VEGA; ITURRIAGA, 2002).

O habitat natural do fungo não tem sido precisamente definido, o solo é considerado o habitat do *P. brasiliensis* apesar de seu isolamento ser raro (FRANCO et al., 2000). Na intenção de identificar o micronicho de fungo tem sido prejudicado por várias circunstâncias, incluindo a falta de informações sobre focos que poderiam ajudar a determinar uma fonte comum de infecção, além disso, o fungo tem a capacidade de entrar em prolongados estágios de repouso, como tem sido observado em casos relatados em indivíduos que vivem em países não endêmicos que muitos anos antes viveram em uma área endêmica.

Há poucos dados a respeito da forma em que o fungo *P. brasiliensis* encontra-se na natureza (RESTREPO, 1985); entretanto, Negroni (1966) e Albornoz (1971) isolaram o fungo *P. brasiliensis* do solo. Fatores ecológicos também foram demonstrados em municípios da Colômbia onde pacientes tinham vivido durante muitos anos, encontrando uma correlação com residência entre 1000 a 1499 m acima do nível do mar, com uma alta taxa de precipitação anual (2000 a 2999 mm) e com culturas agrícolas como café e tabaco (CALLE et al., 2001; TORRADO et al., 2000). A partir de então, estudos a respeito do habitat do fungo começaram a ser realizados. Silva-Vergara et al. (1998) isolaram o fungo a partir de solo de regiões com plantação de café no Estado de Minas Gerais, sugerindo que esse é um dos habitats do fungo *P. brasiliensis*, reafirmando, assim, a hipótese da PCM ser comum nas áreas agrícolas.

1.3 Fatores de virulência

Os fatores de virulência auxiliam na aderência, colonização, disseminação e habilidade do fungo para sobreviver a ambientes hostis e escapar dos mecanismos da resposta imune do hospedeiro. Tanto os fatores de virulência apresentados por diferentes fungos, como os mecanismos de defesa do hospedeiro requerem ação e interação de processos complexos, cujo conhecimento permitirá a melhor compreensão da patogenia das micoses sistêmicas (KUROKAWA, 1998).

Pouco se conhece sobre os mecanismos de virulência que permitem ao fungo adaptar-se às condições presentes no tecido do hospedeiro, para iniciar a colonização e proceder à invasão (LACAZ et al., 2002; RESTREPO; BENARD 2004; SAN-BLAS et al., 2002). Não obstante, podemos assumir que o dimorfismo é um

fator de virulência importante, já que em sua ausência não se daria a conversão dos conídios em leveduras no pulmão, interrompendo-se assim a infecção. A conversão dimórfica é a regra em modelos experimentais, por isso, seu significado na patogênese da PCM é claro (MENDES-GIANNINI et al., 2000; RESTREPO-MORENO, 2003).

A parede celular dos microrganismos desperta grande interesse e concentra muitos estudos acerca de sua biossíntese e composição, pois representa uma barreira de proteção contra processos físico-químicos, aos quais, a célula fúngica está sujeita, e por ser, também, o ponto de contato para adesão e subsequente invasão do tecido hospedeiro (REISS et al., 1992).

A composição de polissacarídeos da parede celular de *P. brasiliensis* pode estar em parte, relacionada à sua virulência. Estudos bioquímicos sobre os polissacarídeos da parede celular do fungo na forma filamentosa e de levedura mostraram que os principais constituintes são: glucanas, quitina, proteínas e lipídios (KANETSUNA et al., 1969, 1972; KANETSUNA, CARBONELL, 1970).

A virulência do *P. brasiliensis* tem sido associada à α -1,3 glucana contida na parede da célula leveduriforme do fungo (MORAES; SCHAFFER, 2002). Estudos têm mostrado que α -glucana é o principal polissacarídeo da parede de leveduras, formas estas que apresentam apenas traços de β -glucanas. Já na parede do micelio, a β -glucana é a única glucana presente. Isso tem suscitado a hipótese de que a transformação dimórfica de *P. brasiliensis* exija controle rigoroso da síntese de glucana (BRUMMER; CASTANEDA; RESTREPO, 1993).

San-Blas et al. (1984) demonstraram alteração na síntese de α -1,3 glucana quando amostras passaram por repiques sucessivos (*in-vitro*), ocasionando a perda ou a atenuação da sua virulência. Os mesmos autores verificaram, entretanto, que a adição de soro fetal bovino à cultura induz a produção desse polissacarídeo, com conseqüente reativação da virulência. Entretanto, um estudo que compara três isolados distintos de *P. brasiliensis* (Pb192, Pb18 e Pb265) mostrou uma contradição à observação e demonstrou que a virulência das células leveduriformes do fungo *P. brasiliensis* não esteve correlacionado com os níveis de α -1,3 glucana na parede celular (ZACHARIAS et al., 1986).

Estudos realizados *in-vitro* com isolados de *P. brasiliensis* sugerem que α -1,3 glucana protege o fungo contra enzimas digestivas do hospedeiro como também

leucócitos e macrófagos (SAN-BLAS, G., SAN-BLAS, F, 1977). Brummer et al. (1990) observaram, também, que a virulência de isolados de *P. brasiliensis* era atenuada ou perdida quando este era mantido por muito tempo *in-vitro*.

Componentes da parede celular de *P. brasiliensis*, como β -glucana, estimulam a resposta imune em uma intensidade mais alta ou mais reduzida. Uma resposta imune intensa permitiria uma menor sobrevivência das células fúngicas, prevenindo a instalação e o crescimento do patógeno. Assim, a β -glucana presente na parede celular do fungo *P. brasiliensis* é capaz de induzir uma resposta inflamatória mais vigorosa e de produzir o fator de necrose tumoral (TNF), uma citocina importante que estimula a atividade fungicida dos macrófagos (FIGUEIREDO et al., 1993; SILVA; ALVES; FIGUEIREDO, 1994).

Gómez et al. (2001) demonstraram que o *P. brasiliensis* é capaz de sintetizar melanina *in vitro* e durante a infecção. Conídios e células leveduriformes cultivadas em meio contendo L-DOPA foram reativas com anticorpos monoclonais que reconhecem a melanina. Através de camundongos infectados com conídios de *P. brasiliensis*, foram recuperados resíduos escuros reativos com anticorpos monoclonais contra melanina (NOSANCHUK et al., 1999).

Por métodos físico-químicos e imunológicos mostrou-se que os conídios e leveduras podem produzir ou sintetizar, compostos melânicos, tanto *in-vivo* como *in vitro* (GÓMEZ et al., 2001). Com base no reconhecimento da melanina como fator de virulência, tem sido demonstrado que esse pigmento pode ter um papel importante na patogênese da PCM (GÓMEZ et al., 2001). A produção de melanina pelo fungo *P. brasiliensis* parece contribuir para a virulência do fungo, pela redução da fagocitose das células leveduriformes por linhagens de macrófagos peritoneais, alveolares e primários (J774.16 e MH-S), aumentando a resistência do patógeno contra o ataque dessas células efectoras. As células melanizadas do fungo são também menos susceptíveis a drogas antifúngicas, particularmente à anfotericina B (DA SILVA et al., 2006).

A análise por SDS-PAGE do material eluído do sobrenadante de cultura do *P. brasiliensis* revelou quatro componentes de 43, 55, 70 kDa e um de alto peso molecular, com migração difusa. Em muitos casos estes componentes glicoproteicos induzem uma resposta imune baseada na secreção de anticorpos a qual não é a mais eficiente, embora tenha sido demonstrado a importância de alguns anticorpos

monoclonais na resposta a algumas doenças fúngicas como a PCM (BUISSA-FILHO et al., 2008) (Apêndice 3). Além de ser fundamental no diagnóstico da PCM, a gp43 tem sido assinalada como fator de virulência, demonstrando se ligar especificamente na laminina, proteína da matriz extracelular. A ligação à laminina induz um aumento na adesão do fungo às células epiteliais (VICENTINI et al., 1994). Além disso, foi observado que células mononucleares de sangue periférico (PBMC) de pacientes com PCM quando estimuladas com gp43, aumentam a secreção de IL-10, uma importante citocina imunossupressora capaz inibir a expressão de citocinas próinflamatórias. Nesse mesmo estudo, foi observado um aumento na taxa de apoptose nos PBMC estimuladas com esta glicoproteína (CACERE et al., 2002).

1.4 Paracoccidioidomicose e o sistema imune

O estabelecimento da doença, sua disseminação e gravidade além de estarem relacionado ao próprio fungo, como sua virulência, composição antigênica e até condições ambientais, dependem muito dos fatores ligados ao próprio hospedeiro na sua capacidade de desenvolver ou não uma resposta imune eficaz.

Células da resposta imune inata como células *natural killer* (NK), neutrófilos, monócitos, macrófagos e células dendríticas, desempenham um papel central na resistência, já que constituem a primeira linha de defesa a entrar em contato com o *P. brasiliensis* assim que ele se instala nos pulmões. A participação destas células na reação inflamatória e na atividade fungicida é induzida pelo fungo e por citocinas produzidas pelas células durante sua interação com fagócitos (CALVI et al., 2003).

As células NK foram estudadas no sangue periférico de pacientes com a PCM e no modelo experimental em hamster. Estas células apresentam atividade citotóxica diminuída quando se tem a doença, sugerindo distúrbio imunológico associado à depressão da imunidade celular tanto em pacientes quanto no modelo experimental (PERAÇOLI et al., 1995).

Os neutrófilos exercem um importante papel imunoprotetor na fase inicial da defesa, mas deve-se resaltar que dependendo dos padrões genéticos do hospedeiro podem apresentar um papel imunoprotetor ou imunoregulador. Em camundongos suscetíveis à PCM (B10.A), a depleção dos neutrófilos, resultou em um aumento nos níveis de anticorpos IgG1, IgA e IgG3 relacionados com a secreção de IL-4, TGF- β e

IFN- γ , respectivamente. Já em animais resistentes (A/J), houve a produção de altos títulos de IgG2a, IgG3 e IgG2b os quais são isotipos Th1 ou IFN-reguladores, desta forma os neutrófilos são fundamentais em situações onde a imunidade mediada por células esta debilitada (PINA et al., 2006).

Outras células que desempenham um papel importante na resposta à infecção por *P. brasiliensis* são os monócitos e macrófagos (M ϕ), sendo a ativação destas células um dos primeiros eventos da resposta imune inata para combater o fungo. Esta ativação é induzida pelo reconhecimento de componentes extracelulares do fungo por alguns dos receptores de reconhecimento padrão (PRRs) (NAKAIRA et al., 2011). Também é possível ativar os M ϕ por ação do IFN- γ , o qual induz um aumento na produção de óxido nítrico (NO) e outros óxidos nitrogenados induzidos pela NO sintase induzível (iNOS), potencializando assim a atividade microbicida dos M ϕ . O papel do NO no controle da infecção por *P. brasiliensis* é importante tanto na fase micelial (GONZALEZ et al., 2000) quanto na fase leveduriforme no modelo *in vivo* (BOCCA et al., 1998). No entanto o papel do NO na PCM permanece controverso, já que a produção excessiva de NO pode estar relacionada com susceptibilidade no processo de infecção (NASCIMENTO et al., 2002).

As células dendríticas (DCs) são as principais células apresentadoras de antígeno e recentes estudos experimentais têm demonstrado que a infecção com o *P. brasiliensis* ativa estas células a migrar para a região dos nódulos linfáticos, providenciando o primeiro contato para a posterior ativação de uma resposta do tipo Th1 (SILVANA DOS SANTOS; FERREIRA; ALMEIDA, 2011).

Dados clínicos e experimentais indicam que a imunidade mediada por células desempenha um papel essencial na defesa do hospedeiro contra a infecção por *P. brasiliensis*, ao passo que níveis elevados de anticorpos específicos e a ativação policlonal das células B estão associados com as formas mais graves da doença (CANO et al., 1998).

Subpopulações de células T auxiliares são conhecidos por estimular diferentes vias de resposta imune (CHERWINSKI et al., 1987). A imunidade contra o *P. brasiliensis* pode ser regulada por subconjuntos Th1 ou Th2, o que determina o resultado da infecção. Linfocinas produzidas pelos linfócitos Th1 estão correlacionadas com a resistência contra certas doenças entre elas a PCM (NEGRONI, 1993).

A persistência a longo prazo de patógenos em um hospedeiro são características de certas doenças infecciosas, entre elas a esquistossomose, leishmaniose e paracoccidioidomicose (PCM). Algumas células do sistema imune podem ser utilizadas pelos microrganismos para permanecer no hospedeiro, como é o caso das células Treg conhecidas por suprimir a ativação e funções efectoras das células T (BELKAID; ROUSE, 2005).

Estudos de imunoprecipitação com soros de pacientes com PCM demonstraram que a glicoproteína de 43 kDa (gp43) era constantemente imunoprecipitada no soro dos pacientes com PCM e não era reativa com soro de indivíduos normais (PUCCIA; TAKAOKA; TRAVASSOS, 1991). Assim, a gp43 tem sido vista como o principal marcador sorológico da PCM, aumentando a especificidade e a sensibilidade dos testes sorológicos (CAMARGO et al., 1994; TABORDA; CAMARGO, 1993, 1994). A gp43 contém epítomos com a capacidade de produzir uma resposta imune celular (DTH) em cobaias (RODRIGUES; TRAVASSOS, 1994) e em pacientes humanos (SARAIVA et al., 1996). A sensibilidade da resposta imune em camundongos pela gp43 ocorre pela proliferação de CD4⁺ (TRAVASSOS et al., 1995). Estes epítomos estimulam os linfócitos CD4⁺ Th1, os quais produzem interferon (IFN- γ), que tem a função de estimular à formação de granulomas que podem conter as leveduras (BRUMMER et al., 1988). Entretanto, a contribuição de cada subtipo de células T na resposta imune depende dos padrões genéticos do hospedeiro, e uma imunidade com um balanço das células T CD4/CD8 regula a secreção de citocinas do tipo Th1 e Th2, o que se correlaciona com a resistência do hospedeiro à infecção por *P. brasiliensis* (CHIARELLA et al., 2007).

A gp43 é expressa de forma constitutiva na fase micelial e leveduriforme do fungo (GOLDANI et al., 1994), sendo constituída por uma única cadeia de oligossacarídeos (ALMEIDA et al., 1996) e caracterizada bioquimicamente por Puccia et al. (1986).

A glicoproteína de 43 kDa (gp43) possui 416 aminoácidos, onde um trecho específico de 15 aminoácidos designado como P10, é reconhecido pelos linfócitos T de camundongos e humanos. O efeito protetor do P10 está relacionado em induzir uma resposta imune do tipo Th1 dependente de IFN- γ - dependente em camundongos isogênicos (TABORDA et al., 1998).

Camundongos de linhagens isogênicas, ao serem imunizados com P10, desenvolveram infecção pulmonar 200 vezes menos intensa do que os animais não imunizados (TABORDA et al., 1998). Iwai et al. (2003) e a análise através do programa TEPITOPE, que verifica a probabilidade de HLA-DR de caucasianos reconhecerem diferentes peptídeos, demonstrou que o P10 é um peptídeo promíscuo, sendo um importante candidato vacinal para ser utilizado em humanos.

Este peptídeo, quando associado às drogas comumente utilizadas no tratamento da paracoccidioidomicose, apresenta um efeito aditivo no modelo experimental utilizando camundongos BALB/c, o que demonstra a capacidade do peptídeo P10 em auxiliar na diminuição do tempo de tratamento desta micose (MARQUES et al., 2006).

1.5 Dexametasona e resposta imune

A dexametasona (Dex) é um potente corticosteróide sintético que possui baixo custo e é amplamente utilizada com propriedades imunossupressoras e antiinflamatórias (RHODUS et al., 2006). Os corticóides são hormônios esteróides que atuam nos processos alérgicos, inflamatórios, imunológicos e no metabolismo de carboidratos, proteínas e gorduras (DIASIO; LOBUGLIO, 1996). A Dex possui mecanismos de ação idênticos ao do cortisol (ou hidrocortisona), que é o mais importante corticosteróide natural produzido pelo córtex da glândula supra-renal. A Dex, quando comparada ao cortisol, apresenta as seguintes propriedades: maior potência anti-inflamatória, meia-vida e tempo de ação prolongado, redução dos efeitos de retenção de sal, atividade tópica e oral (KATZUNG; TREVOR, 1995). Um dos mecanismos de supressão da dexametasona é o bloqueio da expressão do *Nuclear Factor-KappaB* (NF- κ B) e a diminuição nos níveis de citocinas proinflamatórias como IL-8, IL-6 e IL-1 β . Estes corticoides são conhecidos por diminuir a inflamação através da diminuição do influxo de células inflamatórias.

Seus efeitos na inibição da inflamação são profundos e rápidos, entre eles, a diminuição da fase vascular da inflamação, através de vasoconstrição; diminuição da permeabilidade capilar; menor liberação de cininas vasoativas e queda da histamina tecidual. Promove também a indução de um menor afluxo de eosinófilos e inibição

da função dos macrófagos, um dos efeitos mais importantes e mais citados (BAXTER; FORSHAM, 1972; CLAMAN, 1975).

1.6 Tratamento da Paracoccidioidomicose

A micose quando não é diagnosticada e tratada oportunamente, pode levar as formas disseminadas graves e letais, com rápido e progressivo envolvimento dos pulmões, tegumento, gânglios, baço, fígado e órgãos linfóides do tubo digestivo (SHIKANAI-YASUDA et al., 2006).

O tratamento da PCM é muito difícil, exigindo uma terapia prolongada para obter um resultado bem sucedido (RESTREPO, 1994). Apesar da limitação das informações disponíveis em estudos comparativos com diferentes esquemas terapêuticos, sugere-se o itraconazol como a opção terapêutica. Entretanto, considerando que o medicamento não está disponível na rede pública da maioria dos estados, a combinação sulfametoxazol-trimetoprim é a alternativa mais utilizada na terapêutica ambulatorial dos pacientes com PCM. Pacientes com formas graves, necessitando internação hospitalar, devem receber anfotericina B ou associação sulfametoxazol/trimetoprim por via intravenosa. A duração do tratamento relaciona-se à gravidade da doença e ao tipo de droga utilizada. Usualmente, o tratamento é de longa duração, para permitir o controle das manifestações clínicas da micose e evitar as recaídas. O paciente deve permanecer em tratamento e acompanhamento até a obtenção dos critérios de cura, com base nos parâmetros clínicos, radiológicos e sorológicos (SHIKANAI-YASUDA et al., 2006).

1.6.1 Derivados azólicos

Os antifúngicos azólicos foram descobertos na década de 1960 e são totalmente sintéticos, sendo dividido em duas classes segundo sua estrutura: imidazóis (duas moléculas de nitrogênio) e triazóis (três moléculas de nitrogênio). Entre os imidazóis encontramos o miconazol e o cetoconazol, este último com atividade sistêmica. Contudo todos os triazóis possuem atividade sistêmica, incluindo o fluconazol, itraconazol e voriconazol. Tanto os imidazóis como os triazóis inibem a biossíntese do ergosterol pela inibição da enzima 14- α -desmetilase do

citocromo P-450 que é responsável pela demetilação do lanosterol. No entanto, os triazóis apresentam uma maior seletividade pelo sítio de ação em relação aos imidazóis, devido ao acréscimo de nitrogênio em seu anel azóico (GEORGOPAPADAKOU, 1998). Geralmente o mais utilizado destes azóis no tratamento da PCM é o itraconazol o qual é sugerido como uma opção terapêutica que permitiria o controle das formas leves e moderadas da doença em menor período de tempo, sendo utilizado na concentração de 200 mg/dia para adultos durante um período de 6 a 9 meses nas formas leves da doença e de 12 a 18 meses nas formas moderadas (SHIKANAI-YASUDA et al., 2006). O itraconazol é considerado um dos antifúngicos mais eficientes para combater a PCM, com uma eficiência de 95% dos casos e associado com baixas porcentagens de recidivas.

1.6.2 Derivados sulfonamídicos

Estes derivados sulfonamídicos foram as primeiras drogas empregadas no tratamento da paracoccidioidomicose (SHIKANAI-YATSUDA, 2005). Entre os derivados sulfonamídicos, a sulfadiazina é um dos principais compostos ativos (DEL NEGRO, 1982, 1985). Ocasionalmente o *P. brasiliensis* pode desenvolver resistência a esta droga durante o tratamento (RESTREPO; ARANGO, 1980). A combinação sulfametoxazol-trimetoprim é uma opção útil e tem sido utilizada com melhores resultados. A dose recomendada é de 1200 mg de sulfametoxazol e de 160 a 240 mg por dia de trimetoprim, durante 12 meses para formas leves da doença e de 18 a 24 meses em infecções clínicas moderadas (SHIKANAI-YASUDA et al., 2002). Esta associação sulfametoxazol-trimetoprim (5:1), também conhecida como cotrimoxazol, potencializa a atividade de cada um destes antibióticos por separado, inibindo a síntese do tetraidrofolato e impedindo que, os microrganismos não possam sintetizar purinas (BRUMFITT; HAMILTON-MILLER, 1993).

1.6.3 Polienos

Nos casos mais graves, a anfotericina B, um polieno, é a droga de escolha, administrada numa dose de 0,75 mg/kg por dia, a dose cumulativa total deve ser equivalente a 30 mg/kg. Dependendo da dose, a anfotericina B pode ter uma ação

fungistática ou fungicida. Esta droga atua através da permeabilização da membrana plasmática, mas sua atividade, inicialmente, depende de sua ligação ao esterol da membrana. Essa ligação pode resultar na perda de íons intracelulares, o que caracteriza a natureza fungistática da droga. A anfotericina B em concentrações fungicidas pode levar a formação de poros na membrana, com conseqüente perda de constituintes celulares de baixo peso molecular. Essa condição é irreversível (BRAJTBURG et al., 1990).

2 JUSTIFICATIVA

Com o propósito de diminuir os efeitos colaterais e ativar uma resposta imune prolongada o uso de vacinas preventivas ou terapêuticas em doenças infecciosas tem se expandido e exige a seleção de componentes imunogênicos que possam levar a proteção do hospedeiro. No caso de doenças fúngicas, antígenos de *H. capsulatum*, *P. brasiliensis*, *B. dermatitidis*, *C. neoformans*, *C. immitis* e *C. albicans* estão sendo estudadas com resultados promissores (DIXON et al., 1998; JIANG et al., 1999). O conhecimento recente de aspectos estruturais de proteínas e peptídeos permitiu a definição de seqüências reativas e a importância de determinados aminoácidos. De modo particular peptídeos são apresentados por moléculas de histocompatibilidade participando em complexos que determinam o reconhecimento de seqüências “self” e “nonself” para efeito de respostas imunes ativas ou de tolerância imunológica. Tais peptídeos têm sido explorados largamente na constituição de vacinas juntamente aos adjuvantes apropriados.

Nossos dados prévios são promissores, portanto um melhor entendimento em relação ao processo inflamatório pulmonar local em animais imunizados com P10 se torna necessário, pois este peptídeo “promíscuo” é um forte candidato vacinal. Além disso, nossa proposta é também avaliar a utilização do P10 num modelo experimental que mimetize um processo de anergia, comum em pacientes com as formas agudas e subagudas, e como se comportam os animais imunossuprimidos com dexametasona, infectados e tratados com sulfametoxazol/trimetoprim ou itraconazol, associados ou não à imunização com P10.

3 OBJETIVOS

3.1 Objetivo geral

Avaliar o papel do peptídeo 10 (P10) associado ao tratamento quimioterápico em camundongos BALB/c anérgicos infectados com o *Paracoccidioides brasiliensis*.

3.2 Objetivos específicos

- ❖ Padronizar um modelo experimental de anergia em camundongos BALB/c infectados intratraquealmente com isolado Pb18;
- ❖ verificar se o tratamento com antifúngicos associado à imunização com P10, em animais imunossuprimidos, reduzirá a quantidade de fungos em diferentes órgãos e/ou impedirá a disseminação para outros sítios anatômicos;
- ❖ verificar a produção de citocinas pró-inflamatórias nos pulmões dos animais infectados e imunossuprimidos, submetidos ou não ao tratamento com antifúngicos associado à imunização com P10;
- ❖ verificar a produção de óxido nítrico no homogeneizado de pulmão e no sobrenadante de cultura celular dos animais que foram imunizados com o peptídeo P10;
- ❖ identificar a geração de células T de memória durante o processo de imunização dos camundongos;
- ❖ identificar a geração de células T regulatórias durante o processo de imunização dos camundongos.

4 MATERIAIS E MÉTODOS

4.1 Animais

Foram utilizados camundongos machos da linhagem BALB/c, com a média de idade entre 6 e 8 semanas e com um peso entre 25 e 30 gramas. Os animais foram criados em condições SPF (*Specific Pathogen Free*), no biotério de camundongos isogênicos do Departamento de Imunologia do Instituto de Ciências Biomédicas, e mantidos no biotério de animais de experimentação do Departamento de Microbiologia do Instituto de Ciências Biomédicas II da Universidade de São Paulo.

4.2 Imunossupressão dos Camundongos BALB/c

Os animais foram submetidos à imunossupressão com dosagens diárias de 0,15 mg/kg de fosfato de dexametasona, por um período de 20 dias. Os animais foram mantidos em gaiolas fechadas com filtros, tentando reproduzir um ambiente totalmente estéril, autoclavando-se a maravalha, a alimentação e a água distribuída para os animais. As trocas das gaiolas foram realizadas duas vezes por semana em fluxo laminar. Os animais foram considerados anérgicos quando não mais responderam ao teste de hipersensibilidade tardia (HTT), realizado no coxim plantar da pata traseira, utilizando-se uma solução de exoantígenos.

4.3 Avaliação do estado de imunossupressão

4.3.1 Leucograma

Com o propósito de examinar o número de leucócitos, foram realizados leucogramas, nos quais foram coletados 20 µl de sangue (diluição 1:20) e posteriormente adicionados a 380 µl do reagente de Turk (15 mL (ácido acético glacial); 0,002 g (violeta de genciana); 500 mL (H₂O deionizada q.s.p)), após misturar levemente a contagem global de células foi feita em câmara de Neubauer.

4.3.2 Reação de HTT

Para verificar a integridade da resposta imune-celular, reações de HTT contra o antígeno bruto de Pb 18 foram avaliadas durante a administração da dexametasona. No coxim plantar da pata esquerda dos camundongos, foram injetados 25 µl de solução do antígeno bruto, pela via subcutânea. A espessura da pata dos animais foi medida 24 h após a injeção utilizando-se calibrador visual (precisão de 0,01 mm, Mitutoyo, Japan). As medições foram diárias e as alterações na espessura das patas dos animais foram avaliadas.

4.4 Isolado e cultivo de *Paracoccidioides brasiliensis*

Paracoccidioides brasiliensis (Pb) isolado virulento Pb18 foi mantido e adaptado para a fase leveduriforme no meio Sabouraud-Dextrose (Sanofi, França) e mantido a 37 °C. 5 a 7 dias antes da inoculação, foi transferido para o meio líquido Mc Veigh & Morton (McVM), quimicamente definido, a 35 °C (CASTAÑEDA et al., 1988; HANDAM et al., 1998; RESTREPO; ARANGO 1980; SINGER-VERMES et al., 1992).

4.5 Infecção e Terapia

4.5.1 Preparo do inóculo

O fungo foi coletado e lavado 3 vezes com tampão fosfato (PBS pH 7,2). Após a última lavagem, a solução permaneceu imóvel, por alguns minutos, para que ocorra a sedimentação dos grumos celulares. A suspensão, contendo células individuais ou com poucos brotamentos, foi coletada e a contagem realizada em câmara de Neubauer.

4.5.2 Determinação da viabilidade das leveduras

A viabilidade das leveduras foi determinada através do corante azul de Tripan (Sigma, St. Louis, EUA). Todos os procedimentos cirúrgicos executados no

experimento foram realizados com suspensão fúngica com viabilidade entre 90 e 95%.

4.5.3 Infecção Intratraqueal (i.t.)

Os camundongos foram infectados por via intratraqueal conforme descrito previamente por Taborda et al. (1998). Foi utilizado inóculo contendo 3×10^5 leveduras em 50 μ l para cada camundongo. O procedimento foi realizado com os animais sob anestesia (80 mg/kg de ketamina e 10 mg/kg de xilazina).

Quando os animais se apresentaram insensíveis à dor (após dez minutos), foi feita uma pequena incisão longitudinal na pele do pescoço e a traquéia ficou exposta. Após a inoculação do fungo, a incisão foi suturada e os animais colocados sob uma fonte moderada de calor para controlar a hipotermia transitória produzida pela anestesia, até acordarem.

4.5.4 Terapia com agentes antimicrobianos

As doses foram definidas com as concentrações de: 15 – 3 mg kg⁻¹ de peso para sulfametoxazol-trimetoprim (Bac-Sulfitrin® Ducto, Brasil) e 10 mg kg⁻¹ de peso para o Itraconazol (Janssen-Cilag®, Brasil). Os antimicrobianos foram administrados pela via intraperitoneal, todas as quantidades administradas das drogas foram baseadas nos tratamentos seguidos em humanos. As drogas foram conservadas e mantidas em temperatura ambiente e preparadas com uma prévia dissolução em 2% de DMSO. Para o sulfametoxazol/trimetoprim (Bac-sulfitrin, Ducto, Brasil), a diluição foi feita em PBS, com 2% de DMSO.

4.5.4.1 Primeiro protocolo

A terapia foi iniciada 15 dias após a infecção intratraqueal e realizada por um período de 30 dias consecutivos, com administração, a cada 24 h, para Itraconazol e para sulfametoxazol/Trimetoprim, com as concentrações acima citadas, após 15 dias de infecção todos os antimicrobianos foram administrados por via intraperitoneal. O

peptídeo P10 foi aplicado em 4 doses 1 por semana. Os animais foram sacrificados após 45 dias de infecção.

4.5.4.2 Segundo protocolo

A terapia foi iniciada 30 dias após a infecção intratraqueal e realizada por um período de 30 dias consecutivos, com administração, a cada 24 h, para Itraconazol e para sulfametoxazol/Trimetoprim, com as concentrações acima citadas, após 30 dias de infecção todos os antimicrobianos foram administrados por via intraperitoneal. O peptídeo P10 foi aplicado em 4 doses 1 por semana. Os animais foram sacrificados com 60 dias de infecção.

4.6 Síntese e Purificação do Peptídeo (P10)

O peptídeo foi sintetizado e purificado no departamento de Biofísica, da UNIFESP, assim como descrito por Taborda et al. (1998). Os estudos de biodistribuição foram realizados com o Peptídeo P10 fabricado pela Peptide 2.0 (Peptide 2.0., Inc. Chantilly, Virginia, VA., USA), com uma pureza de 98%, determinada por análise de HPLC e MS.

4.7 Imunização dos camundongos

Camundongos BALB/c machos pesando aproximadamente 25 g foram imunizados subcutaneamente com 20 µg do peptídeo 10 (P10) em adjuvante completo de Freund na primeira dose; as subseqüentes (3 doses; 1 por semana) foram administradas com adjuvante incompleto. A emulsão (50 µL) foi injetada em cada animal em uma das patas traseiras na primeira dose e as últimas três foram por via intraperitoneal. Os camundongos controles receberam somente o adjuvante sem o peptídeo.

4.8 Grupos utilizados

Foram utilizados 10 grupos de camundongos machos BALB/c, contendo 6 animais cada (os mesmos grupos de animais foram utilizados no desenvolvimento dos dois protocolos), sendo:

1) Sham NI: não infectado, não imunossuprimido e não tratado; 2) infectado NI: infectado com Pb18, não imunossuprimido e tratado com PBS; 3) Sham: Imunossuprimido, não infectado; 4) infectado: infectado com Pb18, imunossuprimido e tratado só com PBS; 5) Adj: infectado, imunossuprimido e imunizado somente com adjuvante completo e/ou incompleto de Freud; 6) ITRA: infectados, imunossuprimidos e tratados somente com itraconazol, 7) SULFA: infectados, imunossuprimidos e tratados somente com sulfametoxazol/trimetoprim; 8) P10: infectado, imunossuprimido e imunizado somente com P10; 9) Itra+P10: infectado, imunossuprimido tratado com sulfametoxazol/trimetoprim e imunizado com P10; 10) Sulfa+P10: infectado, imunossuprimido tratado com sulfametoxazol/trimetoprim e imunizado com P10.

4.9 Análise da carga fúngica através das unidades formadoras de colônias

O grau de infecção foi caracterizado em animais tratados com drogas em associação, ou não, com a imunização com P10 e seus respectivos controles, através da recuperação de fungos viáveis do pulmão, fígado e baço dos camundongos, após 45 e 60 dias de infecção, em meio Brain Heart Infusion (BHI) (Difco, laboratories, Detroit, MI, USA), suplementado com 4% (vol/vol) de soro bovino (Gibco, NY, USA) e 5 % de filtrado de cultura do isolado 192 do *P. brasiliensis*, streptomomicina/penicillina 10 IU ml⁻¹ (Cultilab, Brasil) e cicloheximida 500 mg ml⁻¹ (Sigma, St Louis, Mo). Após a morte dos animais com punção cardíaca, os órgãos (pulmão, baço e fígado) foram extirpados, divididos ao meio e pesados, imediatamente. Com o auxílio de um homogeneizador manual, as células foram rompidas em 1 ml de PBS; dessa solução, 100 µL foram plaqueados e incubados a 37 °C, e as colônias foram contadas, com 10 dias de incubação.

4.10 Dosagem de citocinas do homogeneizado de pulmão pelo método de CBA

A quantificação das citocinas foi feita pelo método de Cytometric Bead Array (CBA) (FACSCalibur, EUA) no software BD FACSCComp™ & BD CaliBRITE™ Beads. Antes de analisar as amostras, foi realizada a curva padrão com as diluições (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256) definindo a concentração do padrão entre 20 - 5000 pg/ml. Após a realização do padrão, foi feita a reconstituição do Mix, no qual são adicionados 10µl do anticorpo específico para cada citocina em cada amostra. Todos os procedimentos foram realizados a temperatura de 4 °C. Após adicionar o mix foram acrescentados 50µl do anticorpo de detecção fluorocromo, PE, por amostra e estas foram protegidas da luz por 2 horas. Foram preparadas as Setap Beads, marcando três tubos (A, B e C): nos tubos B e C foram adicionados 50µl dos reagentes de marcação, FITC e PE respectivamente, e foram adicionados 400µl da solução de lavagem. No tubo A foram adicionados 450 µl desta solução. Os Setap Beads foram incubados a temperatura ambiente por 30 minutos, protegidos da luz. Após este procedimento, as amostras foram analisadas, calibrando-se o citômetro com o CaliBRITE Beads e os resultados foram obtidos em pg/g de tecido.

4.11 Detecção por ELISA de anticorpos (IgG total, IgG2a, IgG2b e IgG1) no soro de camundongos BALB/c anérgicos infectados i.t com *P. brasiliensis* e imunizados ou não com o peptídeo P10. (2º protocolo)

Foram utilizadas placas de microtitulação de 96 poços, que foram sensibilizadas com 200 mg de gp43, durante 12 h, a 4-8 °C. As placas foram, então, lavadas com PBS Tween 20 0,05% (PBS-T) e bloqueadas com BSA 1%, a 37 °C. Após nova lavagem, foram adicionados 50 µl de soro de camundongos, diluídos, inicialmente, a 1/200, seguindo-se titulações com diluições subseqüentes na razão 2, e incubação, por 1 hora, a 37 °C. Um anticorpo específico para cada isotipo marcado com biotina (IgG1, IgG2a, IgG2b, IgG3, IgM e IgG total – BD PharMingen, San Diego) foi adicionado após as lavagens, seguindo-se incubação, por 1 hora, a 37 °C. As placas foram novamente lavadas com PBS-T e os conjugados enzimáticos adicionados (streptoavidina-fosfatase alcalina). A reação foi desenvolvida pela

adição de substrato para fosfatase (Sigma, St. Louis). A leitura foi realizada em microleitor de ELISA (Labsystems iEMS Analyser, Helsinki), com filtro de 405 nm.

As amostras foram consideradas positivas para anticorpos anti-gp43, quando a D.O. (405 nm) fosse superior a 0,200, segundo o “cut off” obtido pela média das absorbâncias obtidas para os animais-controle, durante a realização dos testes.

4.12 Dosagem de óxido nítrico (NO)

O sobrenadante de cultura celular e o homogeneizado do tecido pulmonar foram utilizados para determinar a produção de óxido nítrico, esta dosagem foi realizada no analisador de quimioluminescência (NOATM280, Sievers Inc., USA). A curva de calibração foi determinada usando padrões de nitrato de sódio. Usando o analisador NOATM280, o nitrato foi reduzido a óxido nítrico (NO) com Vanadium (III) a 90 °C, e o NO formado foi detectado por fase gasosa de quimioluminescência após reação com o ozônio.

4.13 Análise imunohistoquímica através da reação de estreptovidina-biotina-peroxidase

Os fragmentos de tecido das amostras foram gradativamente congelados em nitrogênio líquido e armazenados no freezer -80°C até análise. As secções congeladas foram processadas no criostato (Leica CM1850) e os cortes de 5 micrômetros foram colocados em lâminas de poli-L-lisina (Sigma-Aldrich) e fixadas com acetona para a realização da análise imunohistoquímica (CAVASSANI et al., 2006).

No momento da análise por imunohistoquímica, as lâminas foram incubadas com tampão de lavagem PBS (pH 7,6) por 3 minutos, após este período foi feito o bloqueio de peroxidase endógena com solução 3% de peróxido de hidrogênio (30% - Merck, Germany) em Metanol (Synth, Brasil) sob agitação, por 5 minutos, após o bloqueio os cortes foram lavados com tampão de lavagem (2 vezes de 3 minutos cada). Em seguida, foi aplicado sobre os cortes BSA 2% para o bloqueio de reações inespecíficas, por 2 minutos. A solução bloqueadora foi removida com tampão de lavagem (3 vezes de 3 minutos cada). Além disso, foi empregada uma solução

tampão TBS (Tris-buffered salina – salina tamponada com Tris) (Pierce Inc.) sobre os cortes de tecido para o bloqueio da biotina endógena. Às lâminas foram adicionados os anticorpos primários específicos para células CD11b⁺, Ly-6G/Ly-6C⁺ e L3T4⁺ nas diluições adequadas em PBS/BSA 1%, posteriormente incubadas “overnight” a 4 °C. No caso do anticorpo anti-L3T4, a incubação foi de 30 minutos a 4 °C. O anticorpo primário foi lavado com tampão de lavagem (3 vezes de 3 minutos cada). Posteriormente, as lâminas foram incubadas com o anticorpo secundário biotilado (Vector Laboratories.), diluído 1:500 em PBS, por 30 minutos à temperatura ambiente. O anticorpo secundário foi lavado com tampão de lavagem (3 vezes de 3 minutos cada). A seguir, as lâminas foram incubadas com estreptovidina-peroxidase (1 mg mL⁻¹, vector laboratories) na diluição 1:100 em PBS por 30 minutos à temperatura ambiente e a reação foi revelada com tetra-hidrocloreto de 3,3 diaminobenzidina (Sigma Biochemical Co.). As reações foram interrompidas após a visualização de cor marrom nos cortes de tecidos e a seguir, as lâminas foram contracoradas com hematoxilina de Mayer, desidratadas e montadas em solução Permount e observadas em microscópio óptico (Nikon) com aumentos de 40x, 100x, 250x e 400x.

Lâminas com cortes de tecido de camundongos BALB/c imunossuprimidos e infectados foram utilizadas como controles, lâminas com estes cortes e com ausência do anticorpo primário foram empregadas para avaliar a especificidade da reação.

4.14 Ensaio de proliferação celular

Após uma semana da última imunização ou 60 dias após a infecção, os animais foram sacrificados, retirando-se o baço para pesquisa de células T reguladoras e células T de memória. As células deste tecido foram desagregadas através de maceração em meio RPMI estéril e as hemácias foram lisadas. As células foram contadas em Câmara de Neubauer e a viabilidade destas foi determinada utilizando-se o teste de exclusão com azul de Trypan, com a viabilidade em torno de 90%. As células do baço foram plaqueadas em placas de 96 poços, fundo chato, na concentração de 4×10^5 células/poço, sendo que em cada poço o volume final foi de 200 µl. O meio utilizado foi RPMI 1640 suplementado com 20 mM NaHCO₃, 10 mM Hepes, 100 U/ml de penicilina, 100 mg/ml de estreptomicina, 2 mM de L-glutamina,

50 μ M de β -mercaptoetanol, 5 mM de piruvato de sódio, 100 mM de aminoácidos não essenciais e 10% de soro fetal bovino. Às células dos poços controle acrescentou-se Concanavalina A (1000 ng/poço) e o peptídeo P10 (20 μ g/ml) a todos os outros poços. As placas foram mantidas por 144 horas em estufa com 5% de CO₂ a 37 °C, retirando-se a Concanavalina A após 48 horas. Para análise da proliferação celular foi utilizado o sal de tetrazolium MTT (Thiazolyl Blue Tetrazolium Bromide – Sigma, St. Louis) conforme protocolo adaptado de Mosmann (1983). Após as 144 horas, foi acrescentado 50 μ l/poço de MTT (1 mg/ml) e as placas retornaram a estufa com 5% de CO₂ a 37 °C por quatro horas. Para parar a reação e dissolver os cristais de formazana formados, adicionou-se 100 μ l/poço de Isopropanol – HCl 0,04N. Procedeu-se à leitura em leitor de ELISA no comprimento de onda de 590 nm.

4.15 Dosagem de citocinas do sobrenadante de cultura celular pelo método de ELISA

Procedeu-se a dosagem das seguintes citocinas quantitativamente pelo método de ELISA de captura (BD Pharmingen, San Diego): IFN- γ , IL-12, IL-4 e IL-10. Placas para ELISA foram sensibilizadas com 100 μ l dos anticorpos de captura e mantidas a 4 °C *overnight*. Após, procedeu-se a lavagem dos poços com PBS-Tween 20 0,05% e bloqueadas com 200 μ l de Assay Diluent (PBS-10% Soro Fetal Bovino) por uma hora, a temperatura ambiente. Após serem novamente lavadas, acrescentou-se aos poços 100 μ l das amostras (sobrenadante de cultura celular) ou das citocinas recombinantes de camundongos (padrão), realizando-se as diluições necessárias e as placas foram incubadas a temperatura ambiente por duas horas. Após esse período, as placas foram novamente lavadas com PBS – Tween 20 0,05% e foram adicionados 100 μ l do conjugado, composto dos anticorpos de detecção e a enzima. Assim, as placas foram novamente incubadas por uma hora, à temperatura ambiente. Após, procedeu-se a mais uma etapa de lavagem dos poços e acrescentou-se 100 μ l do substrato da enzima (TMB substrate reagent – BD Pharmingen, San Diego). As placas foram incubadas no escuro, a temperatura ambiente por trinta minutos e a reação foi parada com 50 μ l/poço de H₂SO₄ 2N. A

leitura foi realizada em leitor de ELISA no comprimento de onda de 450 nm (Multiskan EX, USA).

4.16 Imunofenotipagem por citometria de fluxo

Após o estímulo com o peptídeo P10 por 144 horas, as células do baço foram retiradas das placas e centrifugadas a 450 x g por cinco minutos a 4 °C. O sobrenadante foi coletado e armazenado a -70 °C para posterior análise de citocinas. Às células foi adicionado 80µl de sobrenadante de cultura do hibridoma 24G2 contendo anticorpos anti-CD16/CD32 (bloqueador de fração Fc) durante trinta minutos a 4 °C. Posteriormente, as células foram marcadas para as moléculas de superfície, utilizando-se os seguintes anticorpos, anti-CD4-FITC (clone RM4-5, 0,5 µg/10⁶ células), anti-CD8-PerCP (clone 53-6.7, 0,5 µg/10⁶ células), anti-CD44-PE (clone IM7, 0,5 µg/10⁶ células) e anti-CD25-PE (clone PC61, 0,5 µg/10⁶ células) e incubadas por trinta minutos a 4 °C. Após este período, as células foram lavadas com PBS acrescido de 3% de Soro Fetal Bovino e, a seguir, realizou-se a fixação e permeabilização das células para então proceder a marcação para Foxp3 utilizando o anticorpo anti-Foxp3-PE (clone FJK-16s, 0,5 µg/10⁶ células). A fixação e permeabilização foi realizada utilizando o kit “Foxp3 Staining Buffer Set” (e-Bioscience), seguindo as instruções do fabricante. Como controle foram utilizadas células não marcadas e tubos FMO (*fluorescence minus one*), os quais apresentam marcação para todas as moléculas de interesse menos uma delas. As células foram incubadas novamente por trinta minutos a 4°C e lavadas com PBS acrescido de 3% de Soro Fetal Bovino. Após a lavagem, foram adicionados 300 µl de PBS acrescido de 3% de Soro Fetal Bovino e procedeu-se a aquisição dos dados em citômetro de fluxo (FACScalibur). A análise das amostras adquiridas foi realizada utilizando o software FlowJo 7.2.4 (TreeStar).

4.17 Contagem diferencial dos Leucócitos

Lâminas de vidro foram utilizadas para realizar os esfregaços de uma gota de sangue, extraída do plexo ocular dos animais imunizados ou não com o peptídeo P10. Estes esfregaços foram tratados com o kit Panótico Rápido (Laborclin Brasil

Ltda). Para evitar erros de contagem, esta foi feita percorrendo a lâmina toda porém, descrevendo um zigue-zague. Aconselha-se iniciar a contagem da região média do esfregaço (corpo) para cauda. Foi realizada a contagem de 100 leucócitos para obter o resultado em porcentagem.

4.18 Curva de sobrevida

Cada grupo com 5 animais foram infectados com 3×10^5 leveduras de Pb18. Como controles foram utilizados animais anérgicos infectados e tratados com PBS. A mortalidade foi registrada diariamente por um período de 200 dias e os camundongos foram mantidos em condições livre de patógenos no biotério de experimentação animal do Departamento de Microbiologia do Instituto de Ciências Biomédicas da Universidade de São Paulo.

4.18.1 Curva de sobrevida de camundongos *knockout* iNOS^{-/-}

Com o propósito de determinar o papel do óxido nítrico, no processo de imunização com o peptídeo P10, camundongos imunossuprimidos, *knockout* para iNOS, foram infectados e imunizados ou não com o peptídeo P10. A sobrevida destes animais foi observada a por um período de 200 dias, as condições de manutenção foram idênticas à curva de sobrevida realizada nos camundongos BALB/c anérgicos.

4.19 Histopatologia

Uma fração do pulmão foi acondicionada em um tubo contendo formalina (Merck, Alemanha) 10% e enviada para realização da análise histopatológica no laboratório de Histologia do Departamento de Imunologia da Universidade de São Paulo. Os cortes foram submetidos à coloração HE e Gomori-Grocott.

4.20 Determinação da fibrose pulmonar

Pulmões de camundongos BALB/c imunossuprimidos e infectados com Pb18, foram retirados, fixados em formalina tamponada 10 % e enviada para realização da análise histopatológica no laboratório de Histologia do Departamento de Imunologia da Universidade de São Paulo. Os cortes foram submetidos à coloração Gomori Silver-reticulina, para confirmar as mudanças encontradas na organização das fibras de reticulina (colágeno tipo III), e a coloração tricrômico de Mason foi usada para identificar as fibras de colágeno do tipo I nos pulmões.

4.21 Marcação com radiofármaco

Uma molécula modificada do peptídeo sintético P10, na qual a cadeia de 15 aminoácidos (QTLIAHTLAIRYAN) associada ao 6-hidrazino nicotinamida (HYNIC), foi utilizada para avaliar a biodistribuição do peptídeo no modelo murino. O HYNIC-P10 foi sintetizado pela PEPTIDE 2.0 (Chantilly, VA, United States). A conjugação do HYNIC-P10 foi realizada com 95% de grau de pureza, analisada por cromatografia líquida ligada à espectrometria de massa.

O conjugado HYNIC-P10 foi marcado com o radioisótopo tecnécio-99m. A uma alíquota de 10 µg do HYNIC-P10 foram adicionados a 40 mg de tricina e 2 mg de ácido nicotínico, dissolvidos em 0,5 mL de tampão fosfato 0,1 M (pH 7,2) previamente nitrogenado. Em seguida, 10 µg de cloreto de estanho bihidratado dissolvido em ácido clorídrico (HCl) 0,1 N, também nitrogenado, foi adicionado, seguida da solução (0,5 mL) de pertecnetato de sódio (Na^{99m}TcO₄). A reação ocorreu por aquecimento a 100 °C por 20 min (FAINTUCH et al., 2005).

O produto HYNIC-P10-99mTc (0,1 mL) com atividade aproximada de 74 MBq (2 mCi) foi administrado aos animais pela via intravenosa (veia caudal). Os animais foram sacrificados por deslocamento cervical aos 5, 30, 60, 120, 240 e 360 minutos após a injeção do radioproduto.

Para os estudos de biodistribuição do P10 foram utilizados camundongos da linhagem BALB/c com 6 a 8 semanas de vida e pesando de 25 a 30 gramas, os quais foram fornecidos pelo biotério do Instituto de Pesquisas Energéticas e Nucleares (IPEN/CNEN, SP).

Os órgãos (rins, baço, fígado, pulmões, cérebro, coração, intestino grosso e delgado, pâncreas, estômago, músculos, ossos, cauda) foram dissecados, pesados e transferidos para tubos de contagem radioativa. A contagem foi realizada em contador de radiação gama tipo poço de NaI(Tl), utilizando-se como padrão a mesma dose injetada nos animais. Para o cálculo de captação por órgãos e tecidos, descontou-se a radioatividade da cauda (por ser o local de administração do material radioativo), do padrão.

O cálculo da captação do radioproduto por órgão foi realizado pela equação:

$$\% \text{ DI} = (\text{CPM órgão} / \text{CPM padrão}) \times 100$$

Em que %DI = Porcentagem da dose injetada e CPM = Contagem por minuto.

4.22 Estudos de imagem por biomarcação

A aquisição de imagem cintilográfica planar de animais foi efetuada em gama-câmara equipada com um colimador de baixa energia e alta resolução, ângulo de 90° e uma matriz de 256 x 256 X 16, durante 180 segundos. O estudo foi realizado em animais previamente anestesiados aos 30, 60 e 120 min após administração dos radiotraçadores. A captação em regiões de interesse (ROI) foi quantificada considerando-se uma atividade do animal equivalente a 100% (FAINTUCH et al., 2008).

4.23 Determinação da atividade citotóxica *in vivo* do peptídeo P10

O ensaio de toxicidade foi realizado de duas formas, em tecidos animais, onde camundongos BALB/c com idade entre 6 – 8 semanas foram imunizados com 100 ug do peptídeo P10 pela via i.p, 3 vezes a cada 24 h, na ausência de adjuvante. No quinto dia após o começo do tratamento, os animais foram sacrificados por deslocamento cervical e os órgãos (pulmão, baço, rins e fígado) foram dissecados. A histologia dos órgãos foi realizada por coloração H&E.

4.24 Determinação da atividade citotóxica *in vitro* do peptídeo P10

Para os ensaios de citotoxicidade do peptídeo P10, células das linhagens, endotelial (*Human Umbilical Vein Endothelial Cells*) HUVEC (5×10^3) e fibroblasto murino MEFs (*mouse embryonic fibroblasts*), (1×10^4) foram distribuídas em placas de 96 poços. Após 24 h, o peptídeo foi acrescentado às células em diferentes concentrações (100, 80, 60, 40 e 20 $\mu\text{g/mL}$) e as mesmas foram incubadas a 37 °C e 5% CO₂ por 20 horas. Para determinação da viabilidade celular após este período, as células foram coletadas com Tripsina e contadas em câmara de Neubauer, utilizando o teste de exclusão de Trypan Blue (ARRUDA et al., 2012). Estes experimentos de viabilidade celular foram realizados em triplicata e os resultados expressos em número total de células por poço.

4.25 Análise estatística

Análise Estatística. Os resultados foram analisados utilizando-se o software GraphPad Prism 5.0 (GraphPad Inc., San Diego, CA) e a análise de variância (ANOVA) foi realizada seguida do pós-teste de Tukey. O resultado foi considerado significativo quando o $p < 0,05$. Para os dados obtidos do experimento de sobrevivência foi utilizado o teste Long-Rank (Matel-Cox).

5 RESULTADOS

5.1 Padronizações do modelo de anergia com o corticóide sintético “fosfato de dexametasona” em camundongos BALB/c

Ao examinar o efeito da imunossupressão com dexametasona foi observada uma nítida redução no número global de leucócitos no sangue periférico, de camundongos tratados com dexametasona, a contagem dos leucócitos apresentou redução progressiva no decorrer do tempo no experimento (Figura 1). O grupo controle não recebeu o fosfato de dexametasona na água de beber, e manteve no décimo, vigésimo e trigésimo dia a média na contagem de leucócitos desde o início dos experimentos.

Figura 1 - Contagem do número de leucócitos no sangue periférico.

A contagem foi realizada (leucócitos/mm³) no 10º, 20º e 30º dia de protocolo, nos camundongos tratados com dexametasona em dose imunossupressora 0,15 mg/kg⁻¹. ** p<0,01 *** p<0,0001 diferença entre animais controle (não tratados com dexametasona) e animais tratados com dexametasona.

Outro método para observar o efeito imunossupressor da dexametasona foi realizado, com o propósito de identificar o tempo necessário para completar a anergia dos animais. A reação de HTT ou teste de hipersensibilidade tardia foi feito

em camundongos BALB/c, infectados intratraquealmente com 3×10^5 leveduras de Pb18, na qual foi observada uma diminuição significativa na inflamação apresentada na extremidade posterior dos animais a partir do décimo quinto dia, mas a partir do vigésimo dia podemos considerar os camundongos anérgicos, já que não respondem mais ao teste de HTT, conforme o observado na figura 2 (APÊNDICE C).

Figura 2 - HTT realizado em camundongos BALB/c.

O HTT foi realizado em camundongos BALB/c infectados i.t (○) e camundongos BALB/c infectados i.t e imunossuprimidos com 0,15 mg/kg-1 de dexametasona (●). As barras de erro indicam o desvio padrão. * $p < 0,05$.
Fonte: Marques et al., 2008.

O estado de anergia foi observado depois do dia vigésimo dia de tratamento com dexametasona na água de beber, uma condição que causa a morte de 100% dos camundongos infectados entre os dias 70 e 80 de infecção experimental (Figura 3) (APÊNDICE A, Artigo 2).

É importante resaltar que o fosfato de dexametasona provocou uma drástica redução de peso (relativo e absoluto) nos animais que receberam o tratamento com este corticoide. Já ao observar os órgãos destes animais se destacou a diminuição do baço (Figura 4).

Figura 3 - Sobrevida de camundongos BALB/c infectados i.t com 3×10^5 células leveduriformes de *P. brasiliensis* e tratados com dexametasona.

Animais tratados com dexametasona (0.15 mg/kg) na água de beber (●) grupo controle que não recebeu tratamento com Dexametasona (○).
Fonte: Marques et al., 2008.

Figura 4 - Baços de camundongos BALB/c submetidos ao tratamento com Dexametasona.

Baços de animais submetidos ao tratamento com fosfato de dexametasona (0,15 mg/kg) por 20 dias (A) e de animais controle não tratados com o fosfato de dexametasona (B). Estes baços são de animais da mesma idade e não infectados.

Com a padronização do modelo de anergia em camundongos BALB/c infectados intratraquealmente com *P. brasiliensis* estabelecido, conforme apresentado acima; nos experimentos seguintes avaliamos o efeito aditivo do peptídeo 10 (P10) no modelo experimental, com objetivo de mimetizar o estado anérgico, comum em pacientes com as formas aguda e subaguda da doença.

5.2 Análise da carga Fúngica através das Unidades Formadoras de Colônias

1º protocolo: (Os animais foram sacrificados com 45 dias de infecção)

Neste protocolo, os animais foram infectados intratraquealmente com 3×10^5 células leveduriformes de *P. brasiliensis*, e a terapia iniciou-se após 15 dias da infecção, com doses administradas, intraperitonealmente, de 10 mg/kg^{-1} de peso para o itraconazol e $15 - 3/\text{mg kg}^{-1}$ para sulfametoxazol-trimetoprim, durante 30 dias, diariamente. O sacrifício foi após 45 dias de infecção.

Ao sacrificar os animais, a simples vista, observamos que os pulmões dos animais controle infectados e tratados com o fosfato de Dexametasona apresentaram um aumento no tamanho. O estado destes órgãos estava bastante comprometido pelo fungo e nos outros órgãos, também foi observada uma

agressividade maior da infecção. Estes animais apresentaram um aumento nas UFCs quando comparados aos animais que não receberam a Dexametasona. Já nos animais infectados que receberam o tratamento quimioterápico com os antifúngicos, houve uma redução significativa da carga fúngica, igual aos que foram somente imunizados com o peptídeo P10.

Os melhores resultados foram obtidos ao associar o tratamento quimioterápico à imunização com o P10, onde foi observada uma queda significativa da carga fúngica. A diminuição da carga fúngica foi similar tanto no grupo tratado com Itraconazol, associado ao P10, como no grupo de animais tratado com Sulfametoxazol/Trimetoprim, associado ao P10. Estes grupos de animais também mostraram uma diminuição significativa da disseminação das leveduras para outros órgãos como fígado e baço (Figura 5).

É importante ressaltar que animais imunossuprimidos e infectados com *P. brasiliensis* com 30 dias o mais de infecção apresentam perda de peso e alterações na pelagem. Nos grupos de animais que receberam o tratamento quimioterápico, associado à imunização com o P10, não foram observadas estas alterações fenotípicas, o que evidencia a eficácia do tratamento (dados não mostrados).

Figura 5 - UFCs de pulmão (P), baço (B) e fígado (F) de camundongos BALB/c anérgicos, com 45 dias de infecção.

Os animais foram infectados intratraquealmente e tratados (15 dias após i.t.) com itraconazol e sulfametoxazol-trimetoprim, por 30 dias, associadas, ou não, à imunização com P10, e sacrificados após 45 dias de infecção. Foram utilizados, como controles, animais somente infectados e não tratados/imunizados (controle), * $p < 0,05$ ** $p < 0,01$ *** $p < 0,0001$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.3 Análise da carga Fúngica através das Unidades Formadoras de Colônias

2º protocolo: (Os animais foram sacrificados com 60 dias de infecção)

Neste protocolo, os animais foram infectados intratraquealmente com 3×10^5 células leveduriformes de *P. brasiliensis*, e a terapia iniciou-se após 30 dias da infecção, com doses administradas, intraperitonealmente, de 10 mg/kg^{-1} de peso para o itraconazol e $15 - 3/\text{mg kg}^{-1}$ para sulfametoxazol-trimetoprim, durante 30 dias, diariamente. O sacrifício foi, portanto, após 60 dias de infecção.

Os grupos de animais tratados apenas com itraconazol e sulfametoxazol-trimetoprim, não apresentaram diminuição significativa das UFCs e a disseminação

da doença foi maior do que nos animais tratados com itraconazol e sulfametoxazol-trimetoprim, associado à imunização com P10, onde observamos uma queda significativa na carga fungica nos pulmões, quando comparados com os pulmões do grupo controle e também não houve disseminação da doença a outros órgãos, como o baço e o fígado (Figura 6). Mostrando assim, uma eficácia no controle da paracoccidioidomicose experimental.

Figura 6 - UFCs de pulmão (P), baço (B) e fígado (F) de camundongos BALB/c anérgicos, com 60 dias de infecção.

Os animais foram infectados intratraquealmente e tratados (30 dias após i.t.) com itraconazol e sulfametoxazol-trimetoprim, por 30 dias, associado, ou não, à imunização com P10, e sacrificados após 60 dias de infecção. Foram utilizados, como controles, animais somente infectados e não tratados/imunizados (controle). * $p < 0,05$ ** $p < 0,01$ *** $p < 0,0001$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

Com a intenção de definir o papel do P10 como um adjuvante imunológico no tratamento da PCM experimental, associado às drogas, animais infectados foram analisados em dois protocolos diferentes. Nos dois procedimentos, realizados de quimioterapia, observamos melhora significativa nos animais infectados com *P. brasiliensis* e tratados com antifúngicos e imunizados com P10.

5.4 Análise das citocinas IFN- γ , IL-12, TNF- α , IL-10, IL-6, IL-8, MCP-1, IL-4 e IL-2 nos camundongos BALB/c anérgicos infectados e tratados com drogas antifúngicas, associadas, ou não, à imunização com P10

Uma fração dos pulmões dos camundongos BALB/c anérgicos, infectados e tratados com as drogas em associação, ou não, com o P10, foi pesado e homogeneizado, na presença de inibidores de protease. O sobrenadante foi conservado a -80 °C, até o momento do ensaio. A quantificação das citocinas foi feita através do programa Cytometric Bead Array (CBA) (FACSCalibur, EUA).

Ao observar e comparar os resultados obtidos nos dois protocolos (após 45 e 60 dias de infecção) não foi possível identificar diferença significativa entre os protocolos, na secreção de citocinas dos perfis tanto Th1 como Th2.

É importante ressaltar que ocorreu um aumento significativo nos níveis de citocinas pró-inflamatórias, como IFN- γ , IL-12, TNF- α e IL-2 e diminuição das citocinas IL-4 e IL-10 (citocinas imunoreguladoras), nos grupos que receberam o tratamento com itraconazol e sulfametoxazol-trimetoprim associados ao P10. Estas análises foram comparadas ao grupo controle (animais tratados com dexametasona e infectados), os quais apresentavam uma fisiologia aproxima do perfil da resposta da PCM aguda humana; foco do trabalho em questão.

A figura 7 indica os valores de IFN- γ , onde podemos observar que os imunizados com P10 tiveram um aumento significativo desta citocina, tanto após 45 como 60 dias de infecção, enquanto que os tratados somente com as drogas apresentaram níveis mais baixos, em relação ao grupo infectado e tratado com dexametasona.

Os animais que receberam somente o adjuvante não apresentaram diferença significativa. Entretanto, nos animais que receberam dexametasona foi observada uma diminuição desta citocina quando comparado com os animais infectados e sham que não receberam o tratamento com o corticóide.

Figura 7 - Dosagem da citocina IFN- γ , pelo método *Cytometric Bead Array* (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de *P. brasiliensis* e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.

* $p < 0,05$ ** $p < 0,01$ *** $p < 0,0001$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

Outra citocina pro-inflamatória que desempenha um papel importante direcionando a uma resposta imune do tipo Th1 é a IL-12. Como podemos observar na figura 8 houve um aumento significativo nos níveis de IL-12, no grupo de animais imunizados com P10 e também nos camundongos tratados com itraconazol e sulfametoxazol-trimetoprim em associação ao P10, quando comparados ao grupo de animais infectados e tratados com dexametasona. Esse resultado pode indicar um perfil de resposta polarizado para o tipo Th1, fundamental como resposta protetora à infecção pelo fungo.

Os grupos de animais que receberam somente as drogas e o adjuvante de Freund não apresentaram aumento significativo na secreção de IL-12.

Figura 8 - Dosagem da citocina IL-12 pelo método *Cytometric Bead Array* (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de *P. brasiliensis* e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.

* $p < 0,05$ ** $p < 0,01$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

Na figura 9, a qual indica os valores de $\text{TNF-}\alpha$, é possível observar que o grupo de animais imunizados somente com P10 e, os grupos imunizados com o P10, associado ao tratamento com itraconazol e sulfametoxazol-trimetoprim apresentaram um aumento significativo desta citocina no homogeneizado dos pulmões, após 45 e 60 dias de infecção. Enquanto que os animais tratados somente com as drogas apresentaram níveis mais baixos e similares ao grupo que recebeu o adjuvante de Freund, sem presença de P10. Estes grupos não apresentaram um aumento significativo na secreção de $\text{TNF-}\alpha$ em relação ao grupo somente infectado e tratado com dexametasona.

Os grupos de animais que foram tratados com dexametasona e que foram infectados ou não (Sham), apresentaram uma queda significativa desta citocina, quando comparados aos animais que não receberam dexametasona.

Ao observar os animais que não receberam o tratamento com o corticoide, o grupo de animais infectado apresentou um aumento na secreção de TNF- α provavelmente, devido ao processo de infecção pelo *P. brasiliensis*.

Figura 9 - Dosagem da citocina TNF- α pelo método *Cytometric Bead Array* (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de *P. brasiliensis* e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.

* $p < 0,05$ ** $p < 0,01$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

Na figura 10 podemos observar os níveis de secreção de uma das principais citocinas imunoreguladoras, a IL-10. O homogeneizado dos pulmões apresentou uma diminuição significativa da IL-10 nos grupos de animais imunizados com P10, tanto após 45 como 60 dias de infecção. O grupo de animais que recebeu o adjuvante de Freund, sem peptídeo, mostrou também diminuição na secreção desta citocina igualmente ao grupo de animais que foi sacrificado 60 dias após a infecção e que foi tratado somente com itraconazole. Os demais grupos apresentaram níveis similares ao controle.

Figura 10 - Dosagem da citocina IL-10 pelo método *Cytometric Bead Array* (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de *P. brasiliensis* e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.

* p<0,05 ** p<0,01 *** p<0,0001 quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

Com relação à secreção da interleucina 6 (IL-6) nos pulmões dos camundongos, o grupo de animais anérgicos imunizado com o P10 e também os animais que receberam o adjuvante de Freund sem P10, apresentaram diminuição significativa na secreção desta interleucina, após 60 dias de infecção.

Os animais que foram imunizados com o P10, associado ao tratamento quimioterápico, não apresentaram diferenças significativas na secreção de IL-6, e a secreção nestes grupos, foi similar à observada nos animais que receberam somente tratamento com os antifúngicos. Deve-se destacar o aumento na secreção de IL-6 nos animais (controle) tratados com dexametasona, infectados e sacrificados com 45 e 60 dias após infecção com Pb18.

Também na figura 11 podemos observar, que na maioria dos grupos, imunizados ou não com o P10, a secreção desta citocina foi menor aos 45 dias de infecção. No grupo que não foi infectado e que recebeu o tratamento com o corticóide (Sham), foi observado uma secreção maior aos 45 dias de sacrificado.

Figura 11 - Dosagem da citocina IL-6 pelo método *Cytometric Bead Array* (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de *P. brasiliensis* e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.

** $p < 0,01$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

Outra citocina que foi quantificada é a interleucina 8, e nos protocolos abordados neste trabalho, não foi possível ver diferença significativa na secreção de IL-8, nos diferentes grupos de animais imunizados com o peptídeo, como também nos grupos tratados com as drogas, tanto com 45 como 60 dias após infecção em relação ao grupo controle infectado e tratado com dexametasona (Figura 12).

O perfil de secreção desta citocina também se mostrou menor na maioria dos grupos aos 45 dias de infecção, o que demonstra, que no decorrer do tempo de infecção, o nível de secreção desta citocina aumenta, podendo cumprir um importante papel na PCM crônica.

No processo de infecção, esta citocina pode atuar na atração de neutrófilos e basófilos para o foco da infecção. Porém, se secretada em excesso, pode estar relacionada com a gravidade da doença.

Figura 12 - Dosagem da citocina IL-8 pelo método *Cytometric Bead Array* (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de *P. brasiliensis* e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.

Amostras comparadas com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

A secreção da quimiocina MCP-1 (*monocyte chemoattractant protein 1*) pelos pulmões dos camundongos anérgicos também foi dosada.

A figura 13 indica os valores de MCP-1, onde podemos visualizar uma diminuição significativa na secreção desta quimiocina, em todos os grupos de animais, tratados com dexametasona e sacrificados com 60 dias de infecção. Os camundongos que foram sacrificados com 45 dias de infecção, embora tenham apresentado diminuição na secreção de MCP-1, esta diminuição não foi significativa quando comparado ao grupo controle imunossuprimido e infectado.

Os animais imunossuprimidos, mas não infectados (sham), mostraram um aumento na secreção desta quimiocina, quando comparados aos animais sham que não foram imunossuprimidos.

Os animais anérgicos e infectados apresentaram uma secreção maior desta quimiocina quando comparados aos animais infectados, que não receberam o corticóide.

Figura 13 - Dosagem da quimiocina MCP-1 pelo método *Cytometric Bead Array* (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de *P. brasiliensis* e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.

** $p < 0,01$ *** $p < 0,0001$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

Na figura 14 podemos observar que os animais imunizados com P10 e sacrificados após 60 dias de infecção, apresentaram diminuição significativa na secreção de IL-4. Já os animais imunizados com o P10 e sacrificados 45 dias após a infecção, mostraram diminuição na secreção desta citocina.

Nesta figura pode-se observar que, os grupos de animais imunossuprimidos apresentaram níveis mais elevados de IL-4, em relação aos grupos de animais não imunossuprimidos.

Figura 14 - Dosagem da citocina IL-4 pelo método *Cytometric Bead Array* (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de *P. brasiliensis* e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.

* $p < 0,05$ ** $p < 0,01$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

A Figura 15 indica os valores de IL-2, onde pode-se observar que os animais imunizados somente com o P10 apresentaram um aumento significativo na secreção desta citocina pro-inflamatória. Este aumento foi observado aos 60 e 45 dias após infecção. Os animais que receberam o tratamento com as drogas, associado à imunização com o P10, demonstraram um aumento significativo na secreção de IL-2, após 60 dias de infecção. Com 45 dias de infecção, este aumento não foi significativo, quando comparado ao grupo imunossuprimido e infectado.

Podemos ressaltar também, que o grupo sham imunossuprimido não apresentou uma queda representativa na secreção desta citocina, ao comparalo com o grupo de animais sham não imunossuprimido (Figura 15).

Figura 15 - Dosagem da citocina IL-2 pelo método *Cytometric Bead Array* (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos, infectados por via intratraqueal com 3×10^5 células de *P. brasiliensis* e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.

* $p < 0,05$ *** $p < 0,0001$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.4.1 Padrão Th1/Th2 determinado pela análise do perfil de citocinas

A relação de citocinas Th1/Th2 nos pulmões dos animais imunossuprimidos é um dado importante, que nos mostra o padrão de resposta imune que está sendo polarizada após à imunização com o P10. Em conjunto, estes dados sugerem que a predominância de citocinas do tipo Th1 (IFN- γ e IL-12) pode ser suficiente para controlar o desenvolvimento da doença, conforme verificamos nos grupos de animais imunizados somente com P10 ou quando este foi associado ao tratamento quimioterápico (Figura 16).

Esta relação de citocinas (IFN- γ /IL-10, IFN- γ /IL-4, IL-12/IL-10, IL-12/IL-4), também nos mostra que, em vários grupos, a secreção de citocinas pro-inflamatórias

foi maior nos animais sacrificados com 45 dias de infecção, quando comparados aos animais sacrificados com 60 dias de infecção.

Figura 16 - Relação do perfil Th1/Th2, pelo método *Cytometric Bead Array* (CBA) (FACSCalibur, EUA), de homogeneizado dos pulmões de camundongos BALB/c anérgicos.

Estes animais foram infectados por via intratraqueal com 3×10^5 células de *P. brasiliensis* e tratados com as drogas antifúngicas, associadas, ou não, à imunização com P10, e sacrificados com 45 e 60 dias de infecção.

5.5 Detecção por ELISA de anticorpos (IgG Total, IgG2a, IgG2b e IgG1) no soro de camundongos BALB/c anérgicos infectados intratraquealmente com *P. brasiliensis* e imunizados ou não com o Peptídeo P10

5.5.1 Dosagem de IgG total

A alta secreção do anticorpo IgG pode regular a resposta imunológica mediada por células e pode suprimir ou inibir diretamente a ativação dos linfócitos B

(por meio da ligação a receptores para FC nos linfócitos B). O impacto da IgG neste caso é altamente dependente da concentração do anticorpo e da sua afinidade pelo antígeno, como podemos observar na figura 17, os animais imunizados com o peptídeo apresentaram uma secreção significativamente maior desta imunoglobulina quando comparados com os animais controle. Isto está provavelmente relacionado á regulação da resposta imune no hospedeiro.

Figura 17 – Detecção por ELISA, do isotipo IgG total presente no soro de camundongos BALB/c anérgicos.

Análise, por ELISA, do isotipo IgG total presente no soro de camundongos BALB/c anérgicos, infectados intratraquealmente com Pb18 e imunizados ou não com o P10, após 60 dias de infecção. * significância ($p \leq 0,05$) relativa aos camundongos somente infectados. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.5.2 Dosagem de IgG2a.

Nós animais imunizados com o peptídeo foi observado um aumento significativo da imunoglobulina do isotipo IgG2a. Isto devido ao aumento de IFN- γ , citocina que regula esse isotipo, assim, demonstrando, uma resposta do tipo Th1 nestes grupos de animais.

Figura 18 – Detecção por ELISA, do isotipo IgG2a presente no soro de camundongos BALB/c anérgicos.

Análise, por ELISA, do isotipo IgG2a presente no soro de camundongos BALB/c anérgicos, infectados intratraquealmente com Pb18 e imunizados ou não com o P10, após 60 dias de infecção. * significância ($p \leq 0,05$) relativa aos camundongos somente infectados. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.5.3 Dosagem de IgG2b e IgG1

A secreção das imunoglobulinas IgG2b e IgG1, (Figuras 19 e 20) nos camundongos BALB/c anérgicos imunizados com o peptídeo, não apresentou diferença significativa ao ser comparada com os grupos controle. Isto, provavelmente, foi devido a resposta imune do tipo Th1, desencadeada nos animais imunizados com o P10, observada através do aumento na secreção de IFN- γ e IL-12.

Figura 19 - Detecção por ELISA, do isotipo IgG2b presente no soro de camundongos BALB/c anérgicos.

Análise, por ELISA, do isotipo IgG2b total presente no soro de camundongos BALB/c anérgicos, infectados intratraquealmente com Pb18 e imunizados ou não com o P10, após 60 dias de infecção. Análise por One-way ANOVA seguida de pós-teste Tukey.

Figura 20 - Detecção por ELISA, do isotipo IgG1 presente no soro de camundongos BALB/c anérgicos.

Análise, por ELISA, do isotipo IgG1 total presente no soro de camundongos BALB/c anérgicos, infectados intratraquealmente com Pb18 e imunizados ou não com o P10, após 60 dias de infecção. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.6 Dosagens de óxido nítrico do homogeneizado de pulmão

A geração de óxido nítrico foi observada no sobrenadante de pulmão, de camundongos anérgicos, infectados intratraquealmente, e imunizados ou não com P10, associado ao tratamento com as drogas. Foram detectadas concentrações significativas para todos os grupos imunizados com P10, conforme apresentado na figura 21. Embora o grupo imunizado com P10, associado ao tratamento com sulfametoxazol/trimetoprim, tenha apresentado níveis similares ao grupo somente infectado, este aumento foi significativo, isto devido à alta sensibilidade do aparelho na detecção do óxido nítrico.

Nos animais imunizados com o peptídeo, que obtiveram o menor número de UFCs, as concentrações de óxido nítrico mostraram-se elevadas em relação aos demais grupos. Destacam-se os animais imunossuprimidos e infectados que apresentaram níveis baixos de óxido nítrico, igual ao grupo sham não infectado.

Pode-se observar nesta figura, que a imunização com P10 promoveu uma recuperação do sistema gerador de nitrito, o qual é fundamental para que os macrófagos possam executar sua função na resposta imunológica do hospedeiro.

Figura 21 - Determinação da produção de óxido nítrico no homogenato de pulmão de camundongos BALB/c anérgicos imunizados ou não com o P10 e tratados ou não com as drogas antifúngicas.

A dosagem do óxido nítrico no homogenato foi realizada através do analisador de quimioluminescência (NOATM280, Sievers Inc., USA). As barras indicam a média de três leituras. *** $p < 0,0001$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.7 Dosagem de óxido nítrico (NO) do sobrenadante de cultura celular

A geração de óxido nítrico foi observada no sobrenadante de cultura celular de esplenócitos de camundongos anérgicos, infectados intratraquealmente, imunizados ou não com P10, e tratados ou não com as drogas. Todas as amostras foram submetidas ao analisador de quimioluminescência (NOATM280, Sievers Inc., USA) para mensuração desse mediador citotóxico. Foram detectadas concentrações significativas para todos os grupos imunizados com P10, conforme apresentado na figura 22. Este mesmo resultado não observado nos animais controle infectados e sham os quais apresentaram níveis de óxido nítrico baixos.

Os grupos de animais tratados somente com itraconazol e sulfametoxazol-trimetoprim, também apresentaram níveis elevados de óxido nítrico quando comparados ao grupo somente infectado.

Os animais infectados e não infectados (sham) submetidos ao tratamento com o corticóide, apresentaram uma queda significativa na produção de óxido nítrico o que pode demonstrar, neste modelo, um dos efeitos imunossupressores da dexametasona.

Figura 22 - Determinação da produção de óxido nítrico no sobrenadante de cultura celular de esplenócitos de camundongos BALB/c anérgicos imunizados ou não com o P10 e tratados ou não com as drogas antifúngicas.

A dosagem do óxido nítrico no homogenato foi realizada através do analisador de quimioluminescência (NOATM280, Sievers Inc., USA). As barras indicam a média de três leituras. * $p < 0,05$ *** $p < 0,0001$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.8 Análise imunohistoquímico através da reação de estreptavidina-biotina-peroxidase

No tecido pulmonar dos animais infectados com Pb18, podem ser observadas lesões com granulomas bem definidos, constituídos por agregados de macrófagos,

células epitelióides, células gigantes multinucleadas, linfócitos e células polimorfonucleares, ao redor de células fúngicas leveduriformes. Em nosso modelo experimental, estas populações celulares se encontram diminuídas pelo efeito da dexametasona.

No ensaio de imunohistoquímica utilizou-se tecido pulmonar dos camundongos BALB/c anérgicos, para se observar a quantidade e a distribuição de diversas células, através da ligação de anticorpos a receptores específicos para identificação destas células.

Como podemos observar na figura 23, os animais imunizados com o P10 (Figura 23B) apresentaram uma marcação mais intensa, específica ao anticorpo anti-mouse CD11b, o qual é expresso principalmente por macrófagos. Com o aumento destas células, também é possível observar um número menor de células leveduriformes nos animais que receberam o P10. No grupo controle, só infectado e anérgico, podemos observar mais leveduras e um número menor de células marcadas (Figura 23A).

Figura 23 – Identificação de células CD11b+ através de imunohistoquímica dos pulmões dos camundongos BALB/c anérgicos infectados com Pb18 e imunizados ou não com o P10.

(A) Controle positivo (somente infectado) e tratado com PBS, 60 dias após a infecção. (B) Animais infectados e imunizados com P10. Cortes submetidos à marcação pelo anticorpo CD11b e contracoloração com Hematoxilina de Mayer, 10X. As setas pretas indicam os macrófagos, as vermelhas leveduras.

Na figura 24, podemos observar a marcação de células Ly-6G/Ly-6C+, receptor geralmente mais expresso em neutrófilos, com uma coloração marrom intensa, onde os animais imunizados com o P10 apresentaram um número maior destas células (Figura 24 B1 e 24 B2). Também pode ser observado, que as células Ly-6G/Ly-6C+ ficam aglomerados ao lado das leveduras como indicam as setas vermelhas. Os animais imunizados com o P10 quando apresentavam leveduras, estas estavam contidas em granulomas compactos.

O grupo controle, composto de animais imunossuprimidos e infectados (Figura 24 A1 e 24 A2), mostraram um número menor destas células, assim como foram observadas muitas células leveduriformes disseminadas pelo tecido e formação de granulomas frouxos.

O aumento no número de células Ly-6G/Ly-6C+ nos animais imunizados com o P10, foi significativo quando comparado ao grupo de animais não imunizado (Figura 26).

Figura 24 - Identificação de células Ly-6G/Ly-6C+ através de imunohistoquímica dos pulmões dos camundongos BALB/c anérgicos infectados com Pb18 e imunizados ou não com o P10.

(A1 e A2) Controle, animais somente infectados e imunossuprimidos e sacrificados 60 dias após a infecção. (B1 e B2) Animais infectados, imunossuprimidos e imunizados com P10. Cortes submetidos à marcação pelo anticorpo Ly-6G/Ly-6C e contracoloração com Hematoxilina de Mayer, 10X. As setas vermelhas indicam as células Ly-6G/Ly-6C+ e as amarelas indicam as leveduras.

Como podemos observar na figura 25 os animais imunossuprimidos e imunizados com o P10 apresentaram um aumento no número de células L3T4+, dentro destas células encontramos a subpopulação de linfócitos T maduros (incluindo células T “herper”, células T MHC-II restritas e um subtipo de células NKT), este aumento observado nos pulmões dos animais imunizados com o peptídeo demonstra que o P10 incrementa a resposta específica contra o fungo, tornando-se um fator importante no controle da doença.

Ao quantificar o número de células marcadas no tecido pulmonar, observamos que este aumento de células L3T4+ foi significativo, quando comparado ao grupo de animais somente infectado (Figura 25).

Figura 25 - Identificação de células L3T4+ através de imunohistoquímica dos pulmões dos camundongos BALB/c anérgicos infectados com Pb18 e imunizados ou não com o P10.

(A) Controle, animais somente infectados e imunossuprimidos e sacrificados 60 dias após a infecção. (B) Animais infectados, imunossuprimidos e imunizados com P10. Cortes submetidos à marcação pelo anticorpo L3T4 e contracoloração com Hematoxilina de Mayer, 10X. As setas vermelhas indicam as células L3T4+ e as amarelas indicam as leveduras.

Figura 26 – Quantificação de células do tecido pulmonar de camundongos BALB/c anérgicos infectados com Pb18 e imunizados ou não com o P10, marcadas pelo método de imunohistoquímica com os anticorpos Ly-6G/Ly-6C e L3T4.

Grupo infectado, animais somente infectados e imunossuprimidos. Grupo P10 animais infectados, imunossuprimidos e imunizados com P10, os dois grupos foram sacrificados 60 dias após a infecção. * $p < 0,05$ ** $p < 0,01$ quando comparado com os animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.9 Ensaio de proliferação celular

Os linfócitos dos camundongos imunossuprimidos e somente infectados (Controle) não mostraram proliferação significativa quando comparada com o grupo não infectado (sham). Entretanto, mesmo após 60 dias da infecção, os linfócitos esplênicos dos animais desafiados que haviam sido imunizados somente com P10 e o peptídeo associado as drogas sulfametoxazol/trimetoprim e itraconazol, apresentaram intensa proliferação, quando estimulados com o peptídeo P10 *in vitro*, comparando-se com o grupo controle (sham). Além disso, as células provenientes de animais anérgicos, que foram imunizados com adjuvante de Freund foram significativos, proliferando em resposta ao peptídeo P10 de forma significativa quando comparado com o grupo controle (sham), conforme é observado na figura 27.

Figura 27 - Ensaio de proliferação celular utilizando linfócitos esplênicos de animais imunizados com o P10 ou não imunizados 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis* e animais controle (sham).

As células permaneceram sob estímulo do peptídeo P10 ou Concanavalina A por 144 horas. ** $p < 0,01$ *** $p < 0,0001$ quando comparado ao grupo controle (sham).

5.10 Dosagem de citocinas do sobrenadante de cultura celular

Antes da marcação das células observadas no ensaio de proliferação celular foi coletado o sobrenadante de cultura das células provenientes do baço de animais imunizados com o peptídeo ou animais infectados, não imunizados; as quais foram estimuladas com o peptídeo P10 por 144 horas. No sobrenadante de cultura celular dos animais imunizados com o peptídeo P10, podemos observar um aumento significativo de IFN- γ e IL-12, dois importantes citocinas pró-inflamatórias que atuam na efetiva resposta do hospedeiro contra fungo o que demonstra a atividade do peptídeo em induzir resposta imune do tipo CD4⁺ Th1.

Os animais que receberam o tratamento quimioterápico, não apresentaram aumento de IFN- γ . A secreção desta citocina nos esplenócitos de grupos controle foi similar aos de animais imunossuprimidos e não imunossuprimidos, isto indica que mesmo tratados com dexametasona, as células de cultura continuam com a capacidade de produzir citocinas pró-inflamatórias, como IFN- γ .

Desta forma o tratamento com dexametasona reduz o numero de leucócitos levando a um estado de imunossupressão, mas não interfere na secreção de citocinas como pode ser observado na figura 28.

Figura 28 - Dosagem de IFN- γ , pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis*.

* $p < 0,05$ ** $p < 0,01$ quando comparado com os esplenocitos de animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

Na figura 29 pode-se observar que os níveis de IL-12 secretados pelos esplenócitos de animais imunizados com o P10 associado ou não ao tratamento quimioterápico, foram significativamente maiores do que os níveis secretados pelos esplenócitos de animais não imunizados.

Os animais que foram tratados somente com as drogas e com o adjuvante de Freund, não apresentaram aumento desta citocina pró-inflamatória (Figura 29).

Figura 29 - Dosagem de IL-12, pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis*.

* $p < 0,05$ ** $p < 0,01$ quando comparado com os esplenócitos de animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

Um resultado que chamou a atenção foi o aumento na secreção da interleucina IL-10 (IL-10) no sobrenadante de cultura celular, de esplenócitos de animais imunizados com o P10. Já que esta é uma citocina anti-inflamatória antagônica ao IFN- γ . Contudo, a IL-10 tem um importante papel regulador no desenvolvimento da resposta imune "T helper-cell".

O aumento na secreção desta citocina pode ser devido ao acréscimo na população de células Treg, que foi observado nos grupos de animais imunizados com o peptídeo (Figura 37 e 38), lembrando que esta população de células produz principalmente TGF- β e IL-10 para manter o equilíbrio na resposta imune do hospedeiro, evitando assim, os efeitos deletéreos no tecido devido à uma intensa resposta imune.

Figura 30 - Dosagem de IL-10, pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis*.

*** $p < 0,0001$ quando comparado com os esplenocitos de animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

Com relação à citocina IL-4, a qual está associada ao padrão Th2 da resposta imunológica, não foi observada diferença estatística na secreção desta citocina entre os grupos imunizados com P10 e os grupos não imunizados.

Além disso, pode-se perceber um padrão diferente ao observado no homogenato de pulmão, onde os animais imunossuprimidos, mesmo não infectados, mostraram maiores índices desta citocina. No sobrenadante de cultura celular podemos observar que os animais não imunossuprimidos apresentaram maior secreção desta citocina, quando comparado aos demais grupos de animais que receberam dexametasona.

Figura 31 - Dosagem de IL-4, pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados ou não com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis*.

Análise por One-way ANOVA seguida de pós-teste Tukey.

Na dosagem de outras importantes citocinas e pouco estudadas na PCM, encontramos um aumento significativo na secreção da interleucina 18 (IL-18), no sobrenadante de cultura celular dos camundongos anérgicos, infectados com Pb18 e imunizados com o peptídeo P10 (Figura 32). O aumento na secreção desta citocina pode ser muito importante no controle da doença, já que a IL-18 é uma citocina pro-inflamatória da superfamília das IL-1, com um importante papel na resposta imune inata e adquirida.

Em nosso modelo experimental de anergia, observamos que os animais imunizados com o P10, apresentaram uma menor carga fúngica nos pulmões e aumento na secreção de citocinas como IL-12 e IFN- γ , também apresentaram uma diminuição na disseminação da doença para outros órgãos como fígado e baço. No entanto foi observado um aumento significativo na secreção de IL-18, o que

provavelmente ajuda no desenvolvimento de uma resposta pro-inflamatória em resposta à infecção pelo fungo.

Também na figura 32 podemos observar que os animais que receberam dexametasona tiveram uma queda na secreção desta citocina, comparado com os animais não imunossuprimidos.

Figura 32 - Dosagem de IL-18, pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis*.

* $p < 0,05$ quando comparado com os esplenócitos de animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

Outra importante citocina dosada é a IL-1 β a qual tem como principal atividade, atuar como mediadora da inflamação.

Como podemos observar na figura 33, os animais imunizados somente com P10 e o grupo que foi imunizado associado ao tratamento com sulfametoxazol/trimetoprim, tiveram um aumento significativo na secreção de IL-1 β , o que está provavelmente relacionado com o aumento significativo na secreção de TNF- α , observado nos camundongos imunizados com o P10. Um mecanismo natural

de indução de células produtoras de IL-17 é a presença de IL-6 e IL-1 β no local da infecção, assim o aumento observado na secreção de IL-17 (Figura 34), pode estar relacionado de forma direta ou indireta com o aumento na secreção de IL-1 β , nos animais imunizados com o P10, o que desencadeia uma via de resposta pró-inflamatória.

Figura 33 - Dosagem de IL-1 β , pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis*.

* $p < 0,05$ ** $p < 0,01$ quando comparado com os esplenócitos de animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

A subpopulação de células Th17 é definida pela produção da família de citocinas IL-17, sendo a IL-17A a mais produzida, seguida por IL-17F, B, D, C, e E; as células T ativadas são a principal fonte de secreção destas citocinas.

Para a PCM, não está bem claro o papel da IL-17 no processo de infecção. No nosso modelo experimental de anergia, observamos um aumento na secreção desta citocina, para os animais imunizados somente com P10 (Figura 34), sendo que os grupos que receberam o peptídeo, associado ao tratamento quimioterápico não apresentaram secreção significativa desta citocina, quando comparado ao grupo controle imunossuprimido e infectado.

O aumento desta citocina pode estar relacionado também com o aumento de células Ly-6G/Ly-6C e L3T4+ nos pulmões dos camundongos anérgicos, imunizados com o P10, como podemos observar na marcação específica por imunohistoquímica nas figuras 24 e 25.

Figura 34 - Dosagem de IL-17A, pelo método de ELISA em sobrenadante de cultura de linfócitos esplênicos provenientes de animais imunizados com o P10, 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis*.

** $p < 0,01$ quando comparado com os esplenócitos de animais infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.11 Caracterização de células T com fenótipo de memória

A análise da população de células com fenótipo de memória foi realizada utilizando os esplenócitos após 144h de cultura, estimulados ou não com o P10, utilizando-se anticorpos anti-CD4/CD44, específicos para marcação de células de memória em camundongos (Figura 35 A, B, C e D). O gráfico da figura 35 A mostra que cerca de 3.81% das células presentes, co-expressam as moléculas CD4/CD44.

Por outro lado, foram detectadas quantidades significativas de células com fenótipo de memória expressando as moléculas CD4/CD44, no grupo de animais somente imunizados com o P10 (Figuras 35 B). Os resultados obtidos nos grupos de

camundongos tratados com as drogas antifúngicas itraconazol e sulfametoxazol-trimetoprim e imunizados com o P10 não mostraram aumento no número de células com fenótipo de memória. Isto ocorre provavelmente, devido a uma diminuição significativa da quantidade de leveduras, já que uma menor exposição ao antígeno induz a um número menor de células T efectoras e de memória. Lembrando que a resposta por estes dois tipos de células depende da duração e nível de estimulação antigênica.

Figura 35 - Percentual de linfócitos esplênicos com fenótipo de memória.

Esplenócitos de camundongos controle (sham) A; animais imunizados com o peptídeo (P10) B; animais imunizados com o peptídeo e tratados com Itraconazole C; animais imunizados com o peptídeo e tratados com Sulfametoxazole-trimetoprim D; as amostras foram analisadas após ensaio de proliferação celular. Resultados representam um experimento com pool de três animais por grupo.

Figura 36 - Percentual de linfócitos esplênicos com fenótipo de memória em camundongos BALB/c anérgicos.

O percentual de células de memória foi determinado 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis*, as amostras foram analisadas após ensaio de proliferação celular. Resultados representam um experimento com pool de três animais por grupo. * $P < 0,05$ quando comparado com animais sham não infectados, não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.12 Caracterização de células T com fenótipo de reguladoras (Treg)

No modelo experimental de paracoccidiodomicose no estado de imunossupressão, os resultados também mostram um maior percentual de células T $CD4^+Foxp3^+$ nos animais controle-positivo (apenas infectados com *P. brasiliensis*, sem ser imunizados com o peptídeo) quando comparados aos animais controle (sham).

Figura 37 - Percentual de linfócitos esplênicos com fenótipo de reguladora em camundongos BALB/c anérgicos.

O percentual de linfócitos esplênicos com fenótipo de reguladora foi determinado 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis*, as amostras foram analisadas após ensaio de proliferação celular. Resultados representam um experimento com pool de três animais por grupo.

Figura 38 - Percentual de linfócitos esplênicos com fenótipo de reguladora em camundongos BALB/c anérgicos.

As células Treg foram observadas 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis* e foram utilizados como controle animais sham. As amostras foram analisadas após ensaio de proliferação celular. Resultados representam um experimento com pool de três animais por grupo. * $P < 0,05$ ** $P < 0,001$ quando comparado com animais sham não infectados não imunizados e tratados com dexametasona. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.13 Contagem global de leucócitos

A contagem global de leucócitos do sangue, dos animais imunossuprimidos e infectados com Pb18, mostrou um aumento significativo dos glóbulos brancos nos animais imunizados com o P10, após 60 dias de infecção (Figura 39)

Figura 39 – Contagem global de leucócitos do sangue periférico de camundongos BALB/c anérgicos.

A contagem global de leucócitos foi realizada 60 dias após a infecção intratraqueal com 3×10^5 leveduras de *P. brasiliensis* e foram utilizados como controle animais somente infectados e não imunizados. ** $P < 0,001$ quando comparado com animais infectados e tratados com dexametasona não imunizados. Análise por One-way ANOVA seguida de pós-teste Tukey.

5.14 Curva de sobrevida de camundongos BALB/c anérgicos

Observamos uma melhor avaliação da capacidade protetora da imunização com o peptídeo P10 através da curva de sobrevida dos camundongos BALB/c anérgicos e infectados intratraquealmente com *P. brasiliensis*, onde a associação do peptídeo com as drogas Itraconazol e sulfametoxazol/trimetoprim levaram os animais anérgicos a atingir 100% de sobrevida (Figura 40). O grupo de animais somente infectados (controle) atingiu 100% de mortalidade no octagésimo primeiro dia após infecção, os dois grupos de animais que foram tratados com 10 mg/kg de itraconazol e 15-3 mg/kg de sulfametoxazol/trimetoprim, tiveram uma sobrevida entorno do 40% a 50%. Entretanto, a imunização com o P10 aumentou em 60% a sobrevivência dos animais. A imunização com o P10 associado ao tratamento com as duas drogas levou a 100% de sobrevida, demonstrando desta forma o efeito aditivo da imunização com o peptídeo associado ao tratamento quimioterápico.

Estes resultados obtidos na curva de sobrevida são muito importantes na estratégia para aperfeiçoar a formulação vacinal do peptídeo P10, o qual possui propriedades imunoprotetoras bem determinadas no modelo experimental de PCM.

Figura 40 - Curva de sobrevivência de camundongos BALB/c anérgicos infectados, por via intratraqueal, com 3×10^5 leveduras de *P. brasiliensis* isolado 18 e imunossuprimidos, com dexametasona.

(●) representa o grupo controle (PBS) infectado; (■) tratados com Itraconazol (10 mg/kg); (▲) tratados com Sulfametoxazol-trimetoprim (15-3 mg/kg); (▼) Imunizados com o peptídeo P10; (◆) Imunizados com o P10 e tratados com Sulfametoxazol-trimetoprim (15-3 mg/kg); (◻) Imunizados com o P10 e tratados com Itraconazol (10 mg/kg). Experimentos foram realizados em duplicatas, apresentando resultados similares. As curvas são diferentes entre si, Long-ranck (Matel-cox) test $p < 0,05$.

5.14.1 Curva de sobrevida de camundongos *Knockout* iNOS^{-/-}

Os camundongos C57/BL6 *Knockout* (iNOS^{-/-}) imunossuprimidos apresentaram 100% de mortalidade com 72 dias de infecção. Os animais imunizados com o P10 tiveram uma sobrevida de 80% com 200 dias de infecção (Figura 41). Após este período, os animais que sobreviveram foram sacrificados e as unidades formadoras de colônia observadas sendo que, os pulmões destes animais, com 200 dias de infecção, não apresentaram crescimento de leveduras.

Figura 41 - Curva de sobrevivência de camundongos C57/BL6 *Knockout* iNOS^{-/-} infectados por via intratraqueal com 3×10^5 leveduras de Pb18 e imunossuprimidos, com dexametasona.

Os animais foram imunossuprimidos com dexametasona e infectados com 3×10^5 leveduras de Pb18. (□) animais foram imunizados P10 e (•) grupo controle, animais infectados que receberam PBS. As curvas são diferentes entre si, Long-ranck (Matel-cox) test $p < 0,05$.

5.15 Histopatologia do pulmão de camundongos anérgicos infectados e imunizados, ou não, com o peptídeo 10

Na Figura 42, pode ser observada a comparação histopatológica em hematoxilina/eosina (HE) entre os diferentes grupos estudados. Nos grupos sham (imunossuprimido e não infectado) (Figura 42A), pode ser observado o pulmão em seu estado normal. O grupo de animais infectados, não tratados e imunossuprimidos (Figura 42B), apresentaram parênquima pulmonar muito comprometido, com grande infiltrado celular e muitos granulomas frouxos, com células fúngicas viáveis em seu interior, bem como muitas leveduras dispersas pelo tecido pulmonar. O grupo infectado e imunizado com P10 (Figura 42C) teve a presença de granulomas compactos e com poucas células fúngicas. O grupo que recebeu o tratamento com itraconazol, associado à imunização com P10 (Figura 42D), teve uma melhora importante no tecido pulmonar, haja visto a baixa recuperação de unidades formadoras de colônia. Isto também foi observado no grupo tratado com sulfametoxazol-trimetoprim, associado à imunização com o P10, conforme

apresentado nos gráficos acima descritos. Em todos os grupos foram observadas características similares, tanto após 45 como após 60 dias da infecção.

Figura 42 - Histopatologia dos pulmões dos camundongos BALB/c anérgicos infectados com Pb18 e imunizados, ou não, com P10.

(A) Sham (Controle negativo); (B) Controle positivo (somente infectado e tratado com dexametasona); (C) imunizado com P10; (D) Imunizado com P10 e associado à droga. Estes histológicos são de animais sacrificados 60 dias após a infecção. Coloração HE, 20X. As setas indicam granulomas e/ou leveduras.

5.16 Identificação da fibrose pulmonar nos animais imunizados com o peptídeo P10

Para identificar a formação de fibrose no tecido pulmonar dos camundongos BALB/c infectados e imunossuprimidos, os cortes histológicos foram submetidos à coloração de Tricom Masson's e à coloração de Gomori Silver Reticulina com o propósito de identificar as fibras de colágeno do tipo I e III respectivamente. O grupo controle, somente infectado, apresentou estrutura pulmonar comprometida, com muitos granulomas frouxos e muitas leveduras no seu interior. A formação destes granulomas no grupo infectado levou à formação de fibras de colágeno do tipo I nos pulmões, como pode ser observado na figura 43A.

Foi observada uma marcada diminuição na formação de fibras de colágeno nos animais que foram imunizados com o peptídeo P10 os quais não apresentaram

formação de fibras de colágeno do tipo I como pode ser observado na figura 43B igualmente ao ser associado com o tratamento medicamentoso (Figura 43 E, F) os animais que receberam só as drogas tiveram uma diminuição da fibrose pulmonar mas mesmo assim foram observadas alguns pontos com formação de fibras de colágeno do tipo I (Figura 43 C, D).

Figura 43 – Detecção da fibrose pulmonar através da histopatologia dos pulmões de camundongos BALB/c imunossuprimidos e infectados com Pb18, imunizados, ou não, com P10.

(A) Grupo infectado; (B) grupo infectado e imunizado com o peptídeo P10; (C) grupo infectado e tratado com STM; (D) grupo infectado e tratado com ITRA. (E e F) grupos que receberam STM e ITRA associado ao P10 respetivamente. Coloração Masson's Tricome, 20X. As setas indicam a formação de fibras de colágeno do tipo I.

Como pode ser observado na figura 44 ao comparar o grupo de animais somente infectados (A) e o grupo de animais que foram infectados e imunizados com o P10 (B), se observa que a imunização com o P10 levou a uma diminuição

significativa na formação de fibras de colágeno do tipo I, que causam a fibrose pulmonar uma importante secuela da PCM que pode levar a morte.

Figura 44 - Comparação da formação de fibras de colágeno do tipo I.

(A) Grupo só infectado; (B) grupo infectado e imunizado com o peptídeo P10. Coloração Masson's Tricome, 40X. As setas indicam a formação de fibras de colágeno do tipo I.

Para definir a formação de fibrose pulmonar foi necesario também que os tecidos fossem submetidos à coloração *Gomori Silver Reticulina* a qual nos ajuda a identificar a formação de fibras de colágeno do tipo III.

Como pode ser observado na figura 45 os animais imunizados com o peptídeo P10 apresentaram poucas fibras de colágeno do tipo III quando comparados aos tecidos pulmonares dos animais somente infectados.

Figura 45 - Comparação da formação de fibras de colágeno do tipo III.

(A) Grupo somente infectado; (B) grupo infectado e imunizado com o peptídeo P10. Coloração Masson's Tricome, 40X. As setas indicam a formação de fibras de colágeno do tipo III.

5.17 Biodistribuição do conjugado radiomarcado

A pureza radioquímica obtida foi de 92% por cromatografia de camada delgada. O produto radiomarcado e a impureza $^{99m}\text{TcO}_2$ ficavam no início da fita ($R_f=0$) quando metiletilcetona foi utilizado como solvente. Com acetonitrila 50 %, o produto se desloca ($R_f=1$) juntamente com a impureza de ^{99m}Tc livre na forma de pertecnetato de sódio.

Livre de impurezas, a radiomarcagem que foi realizada nos camundongos é mostrada na figura 46.

Figura 46 – Representação esquemática do desenho de um radiofármaco.

Fonte: Liu et al., 2003

Os resultados do estudo de biodistribuição do radiotraçador em animais BALB/c sadios estão demonstrados em porcentagem de dose injetada por grama

(%DI/g). Como podemos observar na figura 47, ao aplicar o P10 radiomarcado, começou a apresentar uma notável captação no rim e nos pulmões no primeiro tempo de avaliação (5 min), diminuindo em torno de 50% após os 30 min nos rins.

Foi observado também, que o traçador tem uma depuração mais lenta e captação maior nos pulmões, no tempo de 1h de avaliação. Em outros órgãos principalmente no baço e fígado, o radiofármaco teve boa captação no tempo de 4 h.

Figura 47 – Biodistribuição do ^{99m}Tc -HYNIC-P10 em camundongos BALB/c sadios

O produto HYNIC-P10- ^{99m}Tc (0,1 mL) com atividade aproximada de 74 MBq (2 mCi) foi administrado aos animais pela via intravenosa (veia caudal). Os animais (n=6) foram sacrificados por deslocamento cervical aos 5, 30, 60, 120, 240 e 360 minutos após a injeção do radioproduto. Setas pretas indicam o tempo de 4 h, seta vermelha o tempo de 1 h.

5.18 Aquisições de imagens cintilográficas

A última etapa do estudo proposto foi a aquisição de imagem cintilográfica (Figura 48). A partir dela podemos avaliar o comportamento do radiofármaco (neste caso o peptídeo P10) em um tempo conhecido. Quanto mais avermelhada a imagem, maior concentração do peptídeo radiomarcado, este resultado é importante porque nos mostra a biodistribuição do peptídeo P10 nas primeiras horas de inoculação no organismo, após 30, 60 e 120 min.

Como pode-se observar na figura 48, após 30 min da aplicação do peptídeo este estava em maior concentração na bexiga e no fígado. Após 120 min o P10 ainda pode ser detectado no fígado e nos linfonodos cervicais o que é determinante, na resposta imune direcionada contra o *P. brasiliensis*.

Figura 48 – Imagens cintilográficas em camundongos BALB/c 99mTc-HYNIC ligado no peptídeo P10.

Animais anestesiados 30, 60 e 120 minutos após a aplicação do P10 ligado ao 99mTc-HYNIC. A aquisição de imagem cintilográfica planar foi efetuada em gama-câmara equipada com um colimador de baixa energia e alta resolução, ângulo de 90° e uma matriz de 256 x 256 X 16, durante 180 segundos. Fígado (setas vermelhas), bexiga (setas pretas), linfonodo cervical (setas roxas).

5.19 Determinação da atividade citotóxica *in vivo* do peptídeo P10

No processo de imunização, os animais recebem 20 µg do peptídeo P10. Para observar se o P10 apresenta algum efeito citotóxico *in vivo*, os animais foram imunizados com uma dose 5 vezes maior (100 µg). A administração de uma maior concentração de P10 não mostrou alterações nos órgãos dos animais como, fígado, rins, baço e pulmões. Foram escolhidos estes órgãos, principalmente por dois motivos, primeiro pelo papel metabólico e de filtração do fígado e rins, órgãos que metabolizam e secretam substâncias inoculadas. Através da biomarcação, altas concentrações do peptídeo P10 foram observadas no fígado, mesmo assim o P10

não causou alterações neste órgão (Figura 49 B). Nos rins, também não foram observadas alterações (Figura 49 C).

Foram escolhidos órgãos onde o P10 tem uma alta atividade no processo de infecção como os pulmões e os baços. Nenhum destes órgãos apresentou alterações ao receber uma dose alta do P10 (Figura 49 A e D).

Ao observar que o peptídeo P10 não provocou nenhum efeito colateral em imunizações com doses de 100 ug, podemos descartar a possibilidade que cause algum efeito colateral em animais imunizados com 20 ug, dose normalmente utilizada nas imunizações com este peptídeo.

Figura 49 - Citotoxicidade *in vivo* do peptídeo P10.

O peptídeo foi aplicado na concentração de 100µg do peptídeo, pela via i.p. 3 vezes a cada 24 h, na ausência de adjuvante. No quinto dia os animais foram sacrificados e dissecados os órgãos (A) Pulmão, (B) Fígado, (C) Rins e (D) Baço, posteriormente foi realizada a coloração de H&E.

5.20 Determinação da atividade citotóxica *in vitro* do peptídeo P10

Para os ensaios de citotoxicidade *in vitro* do peptídeo P10, células das linhagens, endotelial (*Human Umbilical Vein Endothelial Cells*) HUVEC (5×10^3) e

fibroblasto murino MEFs (*mouse embryonic fibroblasts*), (1×10^4) foram estimuladas com 100, 80, 60, 40 e 20 $\mu\text{g/mL}$ de P10 por 24 h. Visivelmente as células estimuladas com P10 não apresentavam diferenças ao ser comparadas com as células que não foram estimuladas, para confirmar este resultado foi realizada contagem através do método de exclusão de Trypan Blue e efetivamente não foi observada uma diminuição significativa no número de células ao acrescentar o estímulo com o P10 (Figura 50).

Figura 50 - Citotoxicidade *in vitro* do peptídeo P10.

Citotoxicidade de diferentes concentrações de P10, avaliada *in vitro* nas culturas celulares das linhagens endotelial humana (*Human Umbilical Vein Endothelial Cells*) HUVEC (5×10^3) e fibroblasto murino (*mouse embryonic fibroblasts*) MEFs (1×10^4), foi realizado o teste de ANOVA o qual não mostrou diferença significativa entre os grupos.

6 DISCUSSÃO

Diferentes estudos tem demonstrado o papel imunoestimulador do peptídeo P10 na PCM experimental. O P10 (QTLIAHTLAIRYAN) da gp43, tem se tornado um forte candidato vacinal, considerando que este peptídeo leva a uma proliferação de células TCD4⁺, polarizando a resposta imune do tipo Th1, caracterizada pelo aumento na secreção de IFN- γ , que tem a função de estimular a formação de granulomas que podem conter as leveduras patogênicas (BRUMMER et al., 1988). O IFN- γ é capaz de ativar os macrófagos pulmonares, os quais tem sua atividade fungicida aumentada e, portanto, está envolvido na resistência à infecção por *P. brasiliensis*. Sua ausência contribui para determinar a suscetibilidade à infecção (BRUMMER et al., 1988; KASHINO et al., 2000; SOUTO et al., 2000).

A defesa contra as infecções fúngicas exige uma boa interação entre a resposta imune inata e adaptativa, desde que nenhuma delas seja exacerbada como foi observado por Pina et al. (2008), onde uma eficiente resposta inata está associada com susceptibilidade à PCM, no modelo experimental.

Nossos resultados demonstraram através de imunohistoquímica um aumento de células CD11b⁺, Ly-6G/Ly-6C⁺ e L3T4⁺, nos pulmões dos animais anérgicos imunizados com o P10. Entre as células que expressam estes receptores encontramos macrófagos, neutrófilos e Linfócitos TCD4⁺ respectivamente, os macrófagos, juntamente com os linfócitos T auxiliares, são os tipos celulares que mais produzem citocinas. Estes ativam uma rede de células e, entre as numerosas respostas fisiológicas, está a indução à resposta inflamatória e à proliferação celular (GOLDSBY et al., 2002).

Entre estas citocinas encontramos o IFN- γ , fundamental na resposta imune, contra vários patógenos, já que estimula e/ou ativa os macrófagos, os quais apresentam atividade fungicida contra conídios e leveduras do *P. brasiliensis* (CANO et al., 1998). O TNF- α , outra importante citocina pró-inflamatória, ajuda na formação de granulomas para conter o fungo. O aumento desta citocina no processo de infecção por *P. brasiliensis* foi observado em estudos experimentais e clínicos (CALVI et al., 2003).

Ao imunizar os animais com o P10 observamos aumento de IL-12 e IFN- γ tanto no homogenizado de pulmão como no sobrenadante de cultura celular, estas duas

citocinas pro-inflamatórias, atuam na efetiva resposta do hospedeiro contra fungo, o que demonstra a atividade do peptídeo em induzir resposta imune do tipo CD4⁺ Th1 produtor de IFN- γ (TABORDA et al., 1998).

A IL-8 é um potente quimiotático e ativador de neutrófilos (MONTON et al., 1998). Esta citocina é produzida por uma enorme variedade de células, entre elas podemos destacar os monócitos, linfócitos, células do endotélio ou epitélio e fibroblastos, em resposta a diferentes estímulos como lipopolissacarídeos e a outras citocinas (TNF- α , IL1- β). Embora nossos resultados não mostrem diferença significativa na secreção de IL-8 por ELISA, conseguimos observar por meio de imunohistoquímica, um recrutamento e aumento de neutrófilos no foco da infecção.

A quimiocina MCP-1 (*monocyte chemoattractant protein 1*) atua ligando-se à proteína G, presente nos leucócitos, para sua posterior ativação e migração destas células. Chensue et al. (1996), examinaram culturas de macrófagos, isolados de granulomas e nódulos linfáticos, revelando um aumento na produção da MCP-1 principalmente, quando o tipo de resposta imune é Th2. Este mesmo grupo de pesquisadores demonstrou que esta citocina ainda inibe a produção de IL-12, por macrófagos *in vivo* e *in vitro*, sendo que IL-12 é uma das citocinas pró-inflamatórias de grande importância, juntamente com IFN- γ na resposta celular – Th1.

O aumento na secreção destas citocinas estimula toda uma cascata na resposta imune, ativando outros leucócitos que secretam outras citocinas e assim sucessivamente. Nos animais imunizados com o P10 encontramos aumento significativo de citocinas pro-inflamatórias como a IL-1 β que é produzida por monócitos e macrófagos e sua ação mais importante na inflamação, se deve aos efeitos no endotélio, ativação de leucócitos e fibroblastos, bem como a indução das reações da fase aguda. Esta citocina atua na regulação da leucopoiese durante um processo infeccioso. A IL-1 β pode estimular a liberação de fatores de crescimento (CSF) para granulócitos, macrófagos e monócitos. Além disso, estimula a liberação do fator de necrose tumoral (TNF) que estimula os linfócitos T (OKAMURA et al., 1998).

A IL-18 é uma interleucina pouco estudada na PCM e que mostrou um aumento significativo nos animais imunizados com o P10, é secretada por macrófagos ativados, linfócitos T e B e células dendríticas (OKAMURA et al., 1995; OKAMURA et al., 1998). A principal função da IL-18 é induzir a proliferação dos

linfócitos Th1 e potencializar a produção de IFN- γ , aumentando assim a secreção de IL-12 (ROBINSON et al., 1997; YOSHIMOTO et al., 1998). A super expressão desta citocina pode promover uma rápida cura da infecção, mas pode levar a uma resposta imune exagerada, com danos teciduais na Paracoccidioidomicose (CORVINO et al., 2006).

Os camundongos imunossuprimidos e imunizados somente com o P10, apresentaram queda na secreção de IL-6 e os gupos de animais imunizados com o peptídeo associado ao tratamento quimioterápico não mostraram alterações na secreção desta citocina. Quando estudamos as funções da IL-6, encontramos que ela pode contribuir para o crescimento e/ou proliferação de linfócitos B, mas também pode ajudar no estímulo da unidade formadora de colônias de granulócitos-macrófagos (CSF-GM), o qual pode chegar a reverter o estado de imunossupressão por dexametasona (ROILIDES et al, 1996).

Nossos resultados mostraram uma diminuição significativa na secreção de algumas citocinas anti-inflamatórias nos animais imunizados com o P10, no homogeneizado de pulmão, o que demonstra a prevalência do perfil Th1.

No sobrenadante de cultura celular também foi observado um aumento significativo de algumas citocinas pro-inflamatórias como IFN- γ e IL-12. No entanto, houve aumento na secreção da IL-10, citocina imunoreguladora e antagonista do IFN- γ , este resultado não esperado após 144 horas de cultura dos esplenócitos. Contudo, a IL-10 tem um importante papel regulador no desenvolvimento da resposta imune “T helper-cell” e resposta imune inata (HUFFNAGLE; DEEPE 2003; MENCACCI et al., 2000; ROMANI, 2004).

O aumento na secreção de IL-10 pode ser devido ao pequeno mas, significativo aumento na população de células Treg, que foi observado nos grupos de animais imunizados com o peptídeo, lembrando que esta população de células produz principalmente TGF- β e IL-10 para manter o equilíbrio na resposta imune do hospedeiro, evitando assim, o dano no tecido (BELKAID, 2008).

Animais imunizados somente com P10 apresentaram um aumento na secreção de IL-17A. Tem sido descrito, que as células T CD8+ de memória, neutrófilos, monócitos e linfócitos T CD4+, são células produtoras destas citocinas principalmente IL-17A (FERRETTI et al., 2003; ZHOU et al., 2005). A IL-17 é uma citocina pro-inflamatória, importante no “*clearance*” de patógenos extracelulares e a

deficiência desta citocina pode aumentar a susceptibilidade à infecção, como foi descrito em *Candida albicans* (HUANG W et al., 2004).

Os resultados obtidos nos grupos de camundongos tratados com as drogas antifúngicas itraconazol e sulfametoxazol-trimetoprim e imunizados com o P10 não mostraram aumento no número de células com fenótipo de memória. Isto pode acontecer devido a uma diminuição significativa do patógeno, já que uma menor exposição ao antígeno induz um número menor de células T efectoras e de memória. Lembrando que a resposta por estes dois tipos de células depende da duração e nível de estimulação antigênica.

Nos animais que foram imunossuprimidos e sacrificados com 60 dias de infecção e que foram imunizados com o P10, foi possível observar um aumento significativo de células Ly-6G/Ly-6C⁺ (Figura 26), este receptor é principalmente expresso pelos neutrófilos. As primeiras características histopatológicas de pacientes com PCM mostram uma forte evidência do envolvimento dos neutrófilos na resposta primária do hospedeiro contra o fungo (COSTA et al., 2007). A presença destas células tem sido detectada no tecido infectado, sangue e medula de pacientes com paracoccidioidomicose (MARTINEZ, 1993).

As células polimorfonucleares (PMNs), entre as quais encontramos os neutrófilos, foram observadas nos pulmões de camundongos BALB/c infectados com *Paracoccidioides brasiliensis*, até um período de 24 semanas, sendo este número decrescente ao aumentar o tempo de infecção (McEWEN et al., 1987).

O aumento na secreção de citocinas pro-inflamatórias e na produção de óxido nítrico nos pulmões dos camundongos BALB/c anérgicos, infectados com o isolado virulento Pb18, e imunizados com o P10, está direta ou indiretamente relacionado ao aumento na população das células CD11b⁺, Ly-6G/Ly-6C⁺ e L3T4⁺, nos animais imunizados.

Os animais imunizados com o P10 tiveram aumento significativo na produção de óxido nítrico, este gás solúvel é fundamental na resposta imune contra diversos patógenos e é sintetizado pela óxido nítrico sintase (iNOS). Quando esta enzima é depletada nos camundongos *Knockout* para iNOS^{-/-}, conseguimos observar melhor o papel do óxido nítrico no processo de imunização. Observamos que mesmo os animais onde a iNOS foi depletada, a imunização com P10 mostrou uma sobrevida maior do que nos animais não imunizados. Isto significa que o óxido nítrico é

importante na resposta ao *P. brasiliensis*, mas não é fundamental, já que por outras vias ativadas o sistema imune consegue evitar a infecção causada pelo fungo. Uma destas possibilidades pode ser a morte do fungo diretamente pelos linfócitos T CD4+, nos quais foi comprovada a capacidade citotóxica destes, atuando diretamente no patógeno (LEVITZ et al., 1995).

É importante ter um aumento na resposta inflamatória, para conter a infecção e disseminação do fungo no hospedeiro, mas é importante também evitar os efeitos deletérios por uma resposta imune exacerbada. A fibrose pulmonar é uma severa e progressiva seqüela da PCM. O desenvolvimento da fibrose pulmonar é atribuída a um prolongado estímulo inflamatório (NARANJO et al., 2010) o qual é comum na PCM por ser uma doença crônica. Os animais imunizados somente com o P10 ou associado às drogas, apresentaram uma diminuição da fibrose pulmonar, uma provável explicação é a conseqüente diminuição da carga fúngica nos pulmões dos animais imunizados, o que levou a uma queda no estímulo e conseqüente diminuição da fibrose pulmonar.

Neste trabalho observamos o efeito aditivo da imunização com o P10 no modelo de anergia na PCM experimental. Verificamos que os animais anérgicos infectados com o isolado Pb18 e imunizados somente com P10 apresentavam redução significativa da carga fúngica nos pulmões e menor disseminação da doença, quando comparados com os animais-controle. Os animais que foram tratados com as drogas sulfametoxazol/trimetoprim e itraconazol, associadas ao P10, mostraram 100% de sobrevida por um período de 200 dias e as melhoras foram evidentes. As características fenotípicas destes animais eram bem diferentes quando comparados aos animais não imunizados. Avaliando as unidades formadoras de colônias, constatamos que o P10 foi capaz de diminuir o avanço da doença para outros órgãos. Além disso, o aumento nos níveis de citocinas, pro-inflamatórias, nos pulmões, indicando uma restauração da imunidade celular desses animais. TNF- α , juntamente com IL-12 e IL-18, potencializam a atividade fungicida de macrófagos. Na PCM, o TNF- α está diretamente relacionado com a formação de granulomas compactos (MAMONI; BLOTTA, 2006).

A análise histopatológica, com coloração por hematoxilina/eosina (HE), dos animais anérgicos, mostrou um parênquima pulmonar comprometido, com bastante infiltrado celular e a coloração de Gomori-Grocott demonstrou um número elevado

de leveduras dispersas pelo tecido. Nos animais anérgicos imunizados com o P10 foi observado um parênquima pulmonar mais conservado, com formação de granulomas pequenos, compactos e com poucas leveduras.

Atualmente, o tratamento da PCM está relacionado à gravidade da doença, ao tipo de droga utilizada e o tempo de uso. Toxicidade das drogas e relatos de recidivas, devido à resistência do fungo, tem sido observados, o que torna importante a pesquisa de novos adjuvantes terapêuticos com o propósito de diminuir o tempo de tratamento e reduzir as recidivas evitando a disseminação do fungo para outros sítios anatômicos.

O uso de vacinas preventivas ou terapêuticas em doenças infecciosas tem se expandido e exige a seleção de componentes imunogênicos que possam levar a proteção do hospedeiro. Resultados obtidos em nosso laboratório reforçam a conclusão de que o P10 protege contra a infecção experimental e abre perspectivas para o estudo em humanos, tornando-se um importante candidato vacinal para ser testado na paracoccidiodomicose humana. A eficácia da imunização do P10, associado a outros adjuvantes, ou mesmo sozinho, abre novas perspectivas.

Diferentes formulações vêm sendo testadas com o propósito de encontrar a melhor proteção na PCM experimental. O peptídeo P10 é um importante candidato vacinal, o que nos leva a pesquisar cada vez mais a interação deste peptídeo com outras células e novos adjuvantes que potencializem o efeito protetor deste peptídeo, como foi observado com a Flagelina (APÊNDICE A, Artigo 4), o uso de nanopartículas (APÊNDICE A, Artigo 5), o lipídeo catiônico (APÊNDICE A, Artigo 9) entre outros.

E quando utilizadas como adjuvante, elas são mais eficientes na apresentação de um peptídeo, por este motivo foram realizados experimentos com estas células pulsadas com o peptídeo P10 (MAGALHAES et al., 2011), e foi observado que, os animais vacinados, apresentaram uma diminuição significativa da carga fúngica nos pulmões, o que evidencia o potencial das células dendríticas em atuar como adjuvante na formulação de uma vacina utilizando o peptídeo P10 para o tratamento e cura da PCM.

Nossos resultados sugerem que o peptídeo P10 é um forte candidato vacinal que pode atuar como adjuvante ao tratamento medicamentoso, representando uma

alternativa promissória na geração de uma vacina anti-PCM e evitando possíveis recidivas no hospedeiro imunossuprimido.

7 CONCLUSÕES

- Verificamos através da contagem de leucócitos e do teste de hipersensibilidade do tipo tardia (HTT) que os camundongos BALB/c apresentam estado de anergia a partir de 30 dias de tratamento com o fosfato de dexametasona.
- Observamos que a imunização com o peptídeo P10, associado ao tratamento medicamentoso, apresentou um efeito aditivo, levando à redução da carga fúngica e impediu a disseminação do fungo para outros órgãos como o baço e o fígado dos animais imunossuprimidos infectados com o isolado virulento Pb18.
- A pesquisa de nitrito, no homogeneizado pulmonar, de camundongos BALB/c anérgicos, indicou a presença de altos títulos, nos animais imunizados com o P10 quando comparados ao grupo controle.
- Os camundongos anérgicos imunizados com P10 apresentaram um aumento na secreção de citocinas pro-inflamatórias, como IFN- γ , TNF- α e IL-12.
- O P10 foi capaz de manter uma linfoproliferação de TCD4⁺ elevada em camundongos infectados com Pb18, em estado anérgico induzido pela administração de fosfato de dexametasona.
- Análise por imunohistoquímica demonstrou um aumento das populações celulares CD11b⁺, Ly-6G/Ly-6C⁺ e L3T4⁺ nos pulmões dos animais anérgicos imunizados com o P10. Entre células que expressam estes marcadores encontramos macrófagos, neutrófilos e linfócitos TCD4⁺ respectivamente.
- Verificamos, através dos cortes histológicos, que os pulmões dos animais imunizados com o peptídeo P10 associado às drogas apresentaram um parênquima pulmonar conservado, com uma diminuição significativa de células fúngicas.
- O efeito aditivo da imunização com o P10 levou a uma sobrevivência de 100% dos animais anérgicos durante um período de 200 dias. Todos os animais (100%) não imunizados morreram com 80 dias de infecção.
- A biodistribuição do P10 mostrou captação nos pulmões e nos linfonodos cervicais, nas primeiras horas após imunização.

- Ensaio de citotoxicidade *in vitro* e *in vivo* mostraram que o peptídeo P10 não possui toxicidade em células endoteliais humanas e em fibroblastos murinos, o que torna este peptídeo uma vacina promissora na PCM.

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APÊNDICES

APÊNDICE A - Artigos Publicados em Periódicos Indexados

- Artigo 1** - MUÑOZ, J. E.; LUFT, V. D.; AMORIM, A.; MAGALHÃES, A.; THOMAZ, L.; NOSANCHUK, J. D.; TRAVASSOS, L. R.; TABORDA C. P. Immunization with P10 peptide increases specific immunity and protects immunosuppressed BALB/c mice infected with virulent yeasts of *Paracoccidioides brasiliensis*. **Microbes and Infection**, p. xx, 2013.
- Artigo 2** - MARQUES, A.; DA SILVA, M.; JULIANO, M.; MUÑOZ, J. E.; TRAVASSOS, L. R.; TABORDA, C. P. Additive effect of P10 immunization and chemotherapy in anergic mice challenged intratracheally with virulent yeasts of *Paracoccidioides brasiliensis*. **Microbes and Infection**, p. 1, 2008.
- Artigo 3** - BUISSA-FILHO, R.; PUCCIA, R.; MARQUES, A. F.; PINTO, F. A.; MUÑOZ, J. E.; NOSANCHUK, J. D.; TRAVASSOS, L. R.; TABORDA, C. P. The Monoclonal Antibody against the Major Diagnostic Antigen of *Paracoccidioides brasiliensis* Mediates Immune Protection in Infected BALB/c Mice Challenged Intratracheally with the Fungus. **Infection and Immunity**, v. 76, p. 3321-3328, 2008.
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- Artigo 5** - AMARAL, A. C.; MARQUES, A. F.; MUÑOZ, J. E.; BOCCA, A. L.; SIMIONI, A. R.; TEDESCO, A. C.; MORAIS, P. C.; TRAVASSOS, L. R.; TABORDA, C. P.; FELIPE, M.S.S. Poly (lactic acid-glycolic acid) nanoparticles markedly improve immunological protection provided by peptide P10 against murine paracoccidioidomycosis. **British Journal of Pharmacology**, p. 1-7, 2010.
- Artigo 6** - DEGOBBI, C.; LOPES, F. D. T. Q. S.; CARVALHO-OLIVEIRA, R.; MUÑOZ, J. E.; SALDIVA, P. H. N. Correlation of fungi and endotoxin with PM2.5 and meteorological parameters in atmosphere of Sao Paulo, Brazil. **Atmospheric Environment**, v. 45, p. 2277-2283, 2011.
- Artigo 7** - ROSSI, D. C. P.; MUÑOZ, J. E.; BELMONTE, R.; CARVALHO, D. D.; FAINTUCH, B.; BORELLI, P.; MIRANDA, A.; TABORDA, C. P.; DAFFRE, S. Therapeutic use of a cationic antimicrobial peptide from the spider *Acanthoscurria gomesiana* in the control of experimental candidiasis. **BMC Microbiology**, v. 12, p. 1-12, 2012.

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1. SANTOS, D. A.; SILVA, F. D.; MUÑOZ, J. E.; SOARES, M. A.; CISALPINO, P. S.; TABORDA, C. P. *Paracoccidioides lutzii*: exoantigenic profile and immunotherapy based on anti-gp43 monoclonal antibodies and P10 peptide.
2. FONSECA, F. L.; GUIMARÃES, A. J.; DUTRA, F. F.; SILVA, F. D.; MUÑOZ, J. E.; TABORDA, C. P.; BOZZA, M. T.; NIMRICHTER, L.; CASADEVALL, A.; RODRIGUES, M. L. Binding of the wheat germ lectin to *Cryptococcus neoformans* chitooligomers impairs extracellular polysaccharide release, TLR2-mediated interaction with phagocytes, and brain colonization in mice.
3. MUÑOZ, J. E.; ROSSI, D. C.; ARRUDA, C. A.; NADER, O. M.; CORTE, M. D.; TABORDA, C. P. Inhibitory activity of limonene against isolated virulent for *Candida albicans* in vitro and in vivo.

APÊNDICE C – Patente.

1. AMARAL, A.; MARQUES, A. F.; **MUÑOZ, J. E.**; BOCCA, A. L.; SIMIONI, A. R.; TITZE-DE-ALMEIDA, R.; TEDESCO, A. C.; MORAIS, P. C.; TABORDA, C. P.; TRAVASSOS, L. R.; FELIPE, M. S. S. Formulação nanoestruturada do peptídeo imunoprotetor P10 com ácido dimercaptosuccínico em polímero de ácido polilático-poliglicólico para o tratamento de micoses. 2008.

Artigo 1

MUÑOZ, J. E.; LUFT, V. D.; AMORIM, A.; MAGALHÃES, A.; THOMAZ, L.; NOSANCHUK, J. D.; TRAVASSOS, L. R.; TABORDA C. P. Immunization with P10 peptide increases specific immunity and protects immunosuppressed BALB/c mice infected with virulent yeasts of *Paracoccidioides brasiliensis*. **Microbes and Infection**, p. xx, 2013.

**Immunization with P10 peptide increases specific immunity and protects
immunosuppressed BALB/c mice infected with virulent yeasts of *Paracoccidioides*
*brasiliensis***

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Running title: P10 protects anergic mice infected with *P. brasiliensis*

SUMMARY

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*. The acute/subacute forms of the disease are most severe and are frequently lethal without antifungal treatment. A peptide from the major diagnostic antigen gp43, named P10, induces a T-CD4⁺ helper-1 immune response in mice of different haplotypes and protects against intratracheal challenge with virulent *P. brasiliensis*. In the present study, we evaluated the efficacy of the P10 peptide alone or combined with antifungal drug treatments in mice immunosuppressed with dexamethasone and infected with a highly virulent human isolate of *P. brasiliensis*, Pb18. P10 immunization led to an effective cellular immune response augmenting T cell proliferative responses, and the stimulated splenocytes produced higher levels of IFN- γ , IL-1 β and IL-12 as well as increased NO concentrations. P10 immunization also led to a minimization in fibrosis in response to Pb18 infection. The addition of antifungal drugs to P10 immunization most significantly impacted survival after lethal challenge with Pb18 as P10 immunization with the administration of either itraconazole or sulfamethoxazole/trimethoprim resulted in 100% survival at 200 days post-infection, whereas untreated mice died within 80 days and P10 alone produced a survival rate of 60%. The present study shows that P10 immunization promotes a strong specific immune response even in immunocompromised hosts. P10 is a promising vaccine candidate for use in immunocompromised hosts.

Key words: *P. brasiliensis*; Anergy; Chemotherapy; Immunization.

INTRODUCTION

The thermally dimorphic fungus *Paracoccidioides brasiliensis* is the etiological agent of paracoccidioidomycosis (PCM). PCM is the most frequent systemic mycosis in Latin America, with the highest incidence of diagnosis in Brazil, Argentina, Colombia, and Venezuela (22). The main route of acquisition occurs by the inhalation of fungal particles, which usually leads to an asymptomatic infection (4). There are two main clinical forms of PCM, acute/subacute and chronic. The acute and subacute form is characterized by a rapid course (weeks to months), impaired cellular immunity, an absence of delayed-type hypersensitivity reactions and a high mortality rate. The chronic form affects mainly adult males with ages between 30–50 years-old and primarily manifests with predominant pulmonary and/or mucocutaneous involvement (10).

An effective cellular immune response is essential for the control of experimental and human PCM (6). Several studies have shown that high levels of specific antibodies and polyclonal activation of B cells are associated with the severe form of the disease whereas inflammatory cytokines, such as IFN- γ , IL-12 and TNF- α , have an important protective role in the host resistance (29, 8). Enhancement of these cytokines elicits a strong cell-mediated immune response that can combat PCM. Notably, IFN- γ has been used to activate macrophages, which augments their fungicidal activity against *P. brasiliensis* (5).

The glycoprotein of 43 kDa (gp43) of *P. brasiliensis* is the main diagnostic antigen for PCM as it is recognized by nearly 100% of sera from patients (20). The gp43 binds laminin, a protein component of the extracellular matrix of mammalian tissues, facilitating fungal invasion and the subsequent destruction of tissues (32). The gp43 carries an immunodominant epitope that induces a predominant IFN- γ -mediated Th-1 response (31). It is primarily responsible for delayed type sensitive (DTH) reactions in infected animals (24). A 15-amino-acid peptide of gp43 (QTLIAHTLAIRYAN), designated as P10, presents a CD4 T-cell

specific epitope that is recognized by the molecules of the major histocompatibility complex class II. P10 is the main epitope in gp43 responsible for activating the cellular immune response in BALB/c mice (31).

Treatment of PCM requires intensive and prolonged antifungal chemotherapy. Treatment with sulfonamides (either sulfamethoxazole or sulfadiazine) combined with trimethoprim, amphotericin B, or azoles is typically administered from 2 to 6 months, although extended periods of treatment (years) are often necessary depending on the drug employed and the disease severity (26). Unfortunately, relapses are particularly common in patients receiving short durations of treatment (13).

Host immune status can increase the risk for and severity of PCM. For example, severe cases of PCM involving immunosuppressed patients with cancer or after renal transplantation have been described (27), presumably due to reactivation of a latent lesion in the setting of cytotoxic drugs. PCM occurs in patients with HIV infection, although PCM has a significantly lower incidence in patients with HIV infection compared to disease due to *Cryptococcus* or *Histoplasma* (23). Notably, PCM patients with HIV infection frequently present with rapidly progressive, often multi-focal disease and they also are more likely to relapse after initial treatment (17) and HIV patients coinfectd with *P. brasiliensis* have less immunoreactivity to antigens from this fungus (2).

Corticosteroids such as Dexamethasone (DEX) can be used to treat many different diseases including cancer. DEX is effective reducing airway inflammation through multiples mechanisms, including the modulation in the synthesis of many inflammatory cytokines (9). Influence multiple transduction pathways led at immune suppression, condition which exposes people to contract a fungal disease. Although *P. brasiliensis* is not an opportunistic fungus has been observed some cases of infection in patients undergoing treatment with immunosuppressive drugs. Consequently, the aim of this work is to improve the

chemotherapy treatment through the additive effect induced by the immunization of P10 peptide associated with antifungal drugs in an anergic model of paracoccidioidomycosis (16). In of the future, to turn the P10 a therapeutic vaccine in human PCM associated with drug treatment could reduce time to treatment and avoid patient relapses and drug side effects.

MATERIAL AND METHODS

Animals. 5 Male BALB/c mice per group (6 to 8 weeks old), were housed in polypropylene cages under Specific Pathogen Free conditions and all materials were sterilized prior to use. Animals used in this study were bred at Institute of Biomedical Sciences of University of São Paulo animal facility. All experiments involving animals were conducted and approved by Ethics Committee of Institute of Biomedical Sciences of University of São Paulo and conducted in accordance with international recommendations.

Immunosuppression of mice. Dexamethasone phosphate (Sigma, St Louis, MO) was used to induce immune suppression, this corticoid was added to the drinking water of animals twenty days before infection and treatment remained until the day of sacrifice sixty days post infection. Assuming an average water intake of 5 ml per day for 30 days, the daily dexamethasone phosphate dose was calculated as 0.15 mg kg⁻¹ (16). Control animals (non-Dex) did not receive dexamethasone phosphate.

Fungal strain. Virulent *P. brasiliensis* Pb18 yeast cells were used to infect the animals. The fungal strain was maintained by weekly passage on solid Sabouraud medium at 37°C. After 7 to 10 days of growth, yeast cells were cultivated in modified McVeigh-Morton medium at 37°C for 5 to 7 days (21). The fungal cells were then collected, washed in phosphate-buffered saline (PBS pH 7.2) and counted in a hemocytometer. The viability of fungal suspensions was determined by staining with trypan blue (Sigma, St. Louis, MO) and was always higher than 90%. The virulence of the Pb18 strain was checked in each experiment by infecting BALB/c mice i.t. and recovering the yeast cells from the infected organs.

Intratracheal infection. BALB/c mice were inoculated i.t. with 3x10⁵ yeast cells/animal of virulent *P. brasiliensis* Pb18 in sterile saline (0.85% NaCl). A maximum volume of 50 µl was inoculated per mouse. Briefly, the mice were anesthetized i.p. with 200 µl of a solution containing 80 mg/kg ketamine and 10 mg/kg of xylazine (both from União Química

Farmacêutica, Brazil). After approximately 5 min, their necks were hyperextended, and the tracheas were exposed at the level of the thyroid and injected with 3×10^5 yeast cells.

Peptide synthesis and purification. P10 peptide used in this study was purchased from Peptide 2.0 (Chantilly, VA). HPLC and MS analyses showed that the synthetic P10 was 98% pure.

Immunization of mice. Anergic BALB/c mice (6- to 8-week old males) were immunized for with 20 μ g of P10 once weekly for four weeks 30 days after i.t. infection. The first immunization was subcutaneous with P10 in complete Freund's adjuvant (CFA) and subsequently peptide immunization was i.p. with incomplete Freund's adjuvant (IFA). Control mice were injected with CFA and IFA alone without the presence of the peptide.

Treatment with Antifungal Drugs. Antifungal drug treatment started 30 days after i.t. infection. Thereafter, mice received doses of 10 mg/kg itraconazole (ITC) (Janssen Pharmaceutica, NV), or 15-3 mg/kg sulfamethoxazole/trimethoprim (SMT/TMP); (Bac-sulfitrin, Ducto - Brazil) every 24h for 30 days (to day 60 post infection). All drug administrations were i.p.

Groups studied. Ten groups of mice (with 6 animals each) were used. Four controls were included: **sham non-DEX**, mice that not receive any intervention; **infected non-DEX**, mice infected with Pb 18, but not treated with steroids; **Sham**, uninfected mice immunosuppressed with dexamethasone phosphate; **Infected**, immunosuppressed mice infected with Pb18 strain; **CFA/IFA**, mice immunosuppressed, infected and immunized with CFA/IFA; **P10**, immunosuppressed, infected and immunized with P10 peptide; **(ITC)**, immunosuppressed, infected and treated with itraconazole; **(SMT/TMP)**, immunosuppressed, infected and treated with sulfamethoxazole/trimethoprim; **Itra+P10**, immunosuppressed, infected, immunized with P10 and treated with itraconazole; **Sulfa+P10**, immunosuppressed, infected, immunized with P10 and treated with SMT/TMP.

Cellular proliferation assay. Spleen cells were collected from mice belonging to the different groups to assess cellular proliferation to P10 stimulation. Spleen cells were collected, dispersed manually and washed in RPMI 1640 (Cultilab, Brazil) supplemented with 20mM NaHCO₃, 10mM HEPES, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 5mM sodium pyruvate, 100mM non-essential amino acids (Sigma Chemical Co., St. Louis, MO), and 10% fetal bovine serum (FBS). Cells were washed twice in FBS-free RPMI, counted, added to 96-well plates at a cell density of 4×10^5 cells/well at a final volume of 200 µl/well. Subsequently, the splenocytes were stimulated with P10 peptide for 144 h at 37 °C in a humidified 5% CO₂ incubator. As positive control, splenocytes from mice of the sham group were stimulated with ConA (4µg/ml, Sigma Chemical Co., St. Louis, MO). Naïve splenocytes from sham group mice were used as a negative control. To determine proliferation, MTT (1mg/ml, Thiazolyl Blue Tetrazolium Bromide – Sigma, St Louis) was added to each well during the final 4 h of culture. The reaction was stopped with 100 µl/well of isopropanol – HCl 0,04N, and plates were read in a ELISA reader (Titertek Multiskan EIA reader) at 590 nm wavelength. Data were expressed as the means and standard deviations (SD) of triplicate cultures.

Cytokine analysis. Cytokine profiles were determined in the supernatants of the splenocyte cultures obtained in the cellular proliferation assay. Interleukin-4 (IL-4), interleukin-12 (IL-12), and interferon-gamma (IFN-γ) were measured using ELISA kits (BD Biosciences, San Diego, CA) and interleukin-1β (IL-1β) was measured using an ELISA kit by eBiosciences, Inc. (San Diego, CA). The limit of detections for the assays were 7.8 pg/ml for IL-4, 31.25 pg/ml for IFN-γ, 62.5 pg/ml for IL-12p40, and 8 pg/ml for IL-1β, as previously determined by the manufacturer.

Production of nitric oxide. Supernatants of the splenocyte cultures obtained in the cellular proliferation assay were used to determinate the production of nitric oxide (NO), in a NO

chemiluminescence analyzer (NOATM280, Sievers Inc., USA). A calibration curve was determined using sodium nitrate standards. Using the NOATM280 analyzer, nitrate was reduced to NO with vanadium (III) at 90 °C, and the NO formed was detected by gaseous phase chemiluminescence after reaction with ozone.

Immunohistochemical analysis. Lung tissue samples from infected mice were submerged in liquid nitrogen then stored at -80 °C until analysis. The frozen sections were cut on a cryostat (Leica CM1850) and sections of 5 micrometers were applied to poly-L-lysine microscope slides (Star Frost) and fixed with acetone to perform immunohistochemical analyses. After washing with buffer, endogenous peroxidase was blocked with a 3% solution of hydrogen peroxide (30%) for 5 minutes. Nonspecific protein binding was blocked with NORMAL SERUM (Vector Laboratories Vectastain ABC Kit) and BSA 2% (Bovine Serum Albumin, pH 7.4; Sigma Chemical Co., St. Louis, MO) was used to block endogenous biotin. Slides were separately incubated for an hour with a dilution of 1:50 (BSA 1%/Tween 20) with rat polyclonal antibody anti-mouse CD11b, Ly-6G/Ly-6C and L3T4 (BD Pharmingen™ San Diego, CA). Biotinylated goat anti-rat IgG (1:500) (Vector Laboratories, Burlingame, CA, USA) was used to bind rat polyclonal antibody anti-mouse CD11b, Ly-6G/Ly-6C and L3T4, this was applied for 1 h at room temperature, followed by the addition of streptavidin-peroxidase (1:50) (Vector Laboratories, Burlingame, CA, USA) for an hour at room temperature. Chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) was used to localize peroxidase in tissue sections. Finally, the slides were counterstained with Mayer's haematoxylin and examined using a light microscope (Nikon Eclipse E200, Japan).

Fibrosis detection. Lung tissues from infected mice were fixed in 10% buffered formalin, and then embedded in paraffin for sectioning. Tissue sections were stained with Gomori's silver reticulin-stain to assess the changes occurring in the organization of reticulin fibers (collagen

III), and Masson's trichrome-stained preparations were used to identify collagen I type fibers. The slides were evaluated under light microscopy.

Survival curve. Six groups of mice (with 6 animals each) pre-treated with DEX were infected as described. (1) the control group was only infected and get PBS daily (2) group infected and immunized with P10 (The first immunization was subcutaneous with CFA and subsequently peptide immunization was i.p. with IFA) (3) infected and treated with ITC, (4) infected and treated with SMT/TMP, (5) a group infected, immunized with P10 and also treated with ITC and (6) a group infected, immunized with P10 and also treated with SMT/TMP. Deaths were registered daily for a 200 day period and the results were statistically analyzed.

Statistical analysis. Statistics were performed using GraphPad Prism5 software (San Diego, CA). The results were expressed as the means the standard deviations (SD) of the indicated numbers of animals or experiments. The nonparametric Tukey's honestly significant difference test was employed. *P* values of ≤ 0.05 indicated statistical significance. In the case of the survival curve, we performed the Log-rank (Mantel-Cox) Test with *P* values of ≤ 0.0001 indicating statistical significance.

RESULTS

Splenocytes from immunosuppressed mice immunized with P10 peptide develop a lymphoproliferative response when stimulated with the P10 *in vitro*.

Splenocytes from untreated uninfected and infected mice produced greater proliferative responses than uninfected or infected mice treated with DEX (Fig. 1). Treatment of immunosuppressed and infected animals with CFA/IFA or antifungal drugs alone did not alter the responsiveness of the spleen cells. In contrast, the splenocytes of immunosuppressed mice that were immunized with P10 peptide produced high levels of cellular proliferation in comparison to unimmunized infected immunosuppressed mice. Interestingly, the addition of antifungal therapy to P10 immunization did not further increase splenocyte responsiveness. These results indicate that P10 alone is a strong proliferation-inducing immunostimulator of splenocytes in of immunosuppressed mice, and the response is neither impeded or further stimulated by the inclusion of antifungal drugs.

P10 peptide induces proinflammatory cytokine production.

Previous studies with corticosteroids suggested that DEX directly inhibit cytokine production in T cells (2). We showed whether supernatants of spleen cells cultures had high levels of inflammatory cytokines in mice subjected to DEX and immunized with P10. DEX treated mice infected with Pb18 and immunized with P10 produced increased amounts of IL-12, IFN- γ , and IL-1 β compared to controls (Fig. 2). Notably, immunosuppressed and infected mice treated with IFA/CFA also had an increase of IFN- γ that was similar to that achieved with P10. Cytokine profiles in the mice treated with P10 and antifungal drugs were similar to that achieved with P10 alone. Mice immunosuppressed with DEX, infected with Pb18 and immunized with P10 showed similar levels of secretion of IL-4 compared to controls (Fig. 2).

Nitric Oxide production.

Since previous studies indicated that corticosteroids such as DEX inhibit nitric oxide production in macrophages (14), we measured the concentrations of NO in supernatants from P10 stimulated spleen cell cultures. Significant concentrations of NO were detected in all groups immunized with P10 compared to controls (Fig. 3). Notably, treatment with either ITC or SMT/TMP produced results similar to that achieved with P10 alone. Combination treatments with P10 and antifungal drugs did not further increase NO levels. Immunosuppressed, infected mice treated with CFA/IFA also displayed an increase in NO production, albeit significantly less than P10 or antifungal treated animals. The immunocompromised uninfected or infected but untreated mice had lower levels of NO than the competent controls, confirming the suppressive effects of DEX on macrophage NO production.

Phenotypic characterization of CD11b⁺, Ly-6G/Ly-6C⁺ and L3T4⁺ cells in lungs from immunosuppressed BALB/c mice.

We determined the phenotype of CD11b⁺, Ly-6G/Ly-6C⁺ and L3T4⁺ cells in the lesions obtained from immunosuppressed BALB/c mice 60 days after infection with Pb18 (Fig. 4). We observed a significant increase ($p < 0.05$) in the number of cells CD11b⁺ in the pulmonary tissue of animals immunized with P10 with dense clustering of these cells around rare yeast cells. In contrast, the tissue sections from infected and non-immunized animals had few CD11b⁺ cells that were not tightly associated with dispersed yeast cells. These data strongly indicated that macrophages accumulated in *P. brasiliensis* induced lesions of immunosuppressed mice that were immunized with P10 peptide. Within small compact granulomas of animals immunized with P10, we also found a significant increase ($p < 0.05$) in the number Ly-6G/Ly-6C⁺ cells, which were in close proximity to the rare yeast cells. The L3T4⁺ receptor is expressed in a subpopulation of mature T lymphocytes, as MHC class II-

restricted T cells, including most T helper cells that exert an important role in host defense against fungi. In our experiment, we noted a significant increase ($p < 0.05$) in the number of L3T4+ cells in the pulmonary tissue of mice immunized with P10 compared with infected animals non-immunized (Fig. 4). In the P10 immunized mice, the L3T4+ cells were again clustered around rare yeast cells.

Immunization with P10 minimizes production of fibrosis in the lung tissue.

We analyzed the quantity and appearance of reticulin and collagen fibers in lungs from immunosuppressed BALB/c mice 60 days after infection with Pb18 (Fig. 5). In mice treated only with DEX (controls), the architecture of the lung tissues was significantly disrupted with large aggregates of yeast cells as well as single fungal cells present diffusely. Moreover, we observed enough fibers of collagen and reticulin in the pulmonary tissue of these animals (Fig. 5 A and C). DEX treated animals that received immunization with P10 displayed a conserved pulmonary tissue architecture with a few compacted granulomas and rare yeast cells. In contrast with the dense fibrosis in the controls, the lungs of the P10 immunized animals had significantly less collagen and reticulin (Fig. 5 B and D).

P10 immunization increases survival rates of immunosuppressed mice.

The survival rates of DEX treated, anergic BALB/c mice infected with Pb18 were significantly impacted by P10 and antifungal therapy (Fig. 6). In untreated controls, 100% mortality occurred within the first eighty days post-infection. Animals treated with 10 mg/kg of itraconazole or 15-3 mg/kg of sulfamethoxazole/trimethoprim, had significantly increased survival rates of 40% to 50%, respectively. Mice immunized with P10 peptide presented a 60% of survival rate, which was not significantly different from antifungals alone. However, there were no deaths in mice that were immunized with P10 and treated with either antifungal drug over a period of two hundred days post-infection. These results confirmed the protective capacity of immunization with the P10 peptide associated with chemotherapy.

DISCUSSION

Our previous results demonstrate that immunization with P10 peptide (QTLIAIHTLAIRYAN), led to an additive effect when the immunization was associated with different drug treatments, inducing a decrease in fungal burden and preventing its spread to other anatomical sites in the experimental model of paracoccidioidomycosis (16). In this study we showed the ability of P10 peptide to stimulate and induce specific immune response against *P. brasiliensis* in anergic animals infected with the virulent isolate Pb18. P10 has become a strong candidate vaccine, leading to a predominant Th1-type immune response. This type of response is the main against *P. brasiliensis* infection characterized by increased secretion of IFN- γ , which stimulates the granuloma formation that may contain pathogenic yeasts (5). IFN- γ is capable to activate lung macrophages, which increases its fungicidal activity and, therefore, are involved in the resistance to infection by *P. brasiliensis*. Their absence contributes to determine the susceptibility to infection (5, 30). Defense against fungal infections requires a good interaction between the innate and adaptive immune responses. This report adds new data on the induction of immune responses in mice vaccinated with P10 and shows that, under experimental conditions, this peptide vaccine represents a promising therapeutic approach for the control of paracoccidioidomycosis.

Cellular immunity is essential in host defense against fungal infection in humans and in experimental models (25). Immunization with P10 restored the ability of lymphoproliferation of immunosuppressed animals. Spleen cells from anergic animals infected with Pb18 and immunized with P10, when stimulated with P10 peptide *in vitro* showed a significant higher lymphoproliferation than the splenocytes of non-immunized animals. Therefore, we think that this increase in lymphoproliferation in vaccinated animals helps to prevent the fungal spread to other organs. However, in cell culture supernatant of splenocytes used in the lymphoproliferation assay, we observed an increased secretion of

proinflammatory cytokines such as IL-12, TNF- α , IFN- γ and IL-1 β . Previous studies have demonstrated the important role of these cytokines, especially IFN- γ , which plays an important role in the protection and resistance to PCM (7). This result tells us that immunosuppressed animals, when immunized with P10 peptide have a predominant cellular response when compared to non-immunized animals, thus leading to a reduction in fungal burden and fall in the spread of the fungus to other organs.

Macrophages are important producers of nitric oxide. Previous studies have shown that nitric oxide is involved in the control of *P. brasiliensis* as mycelium or yeast forms (12). Anergic animals infected with *P. brasiliensis* and then immunized with P10 showed an increased production of nitric oxide in the splenocyte culture supernatants. This increase can be directly or indirectly related to the increase in CD11b⁺ cells.

Through immunohistochemistry, our results demonstrate a significant increase in the number of CD11b⁺, Ly-6G⁺/Ly-6C⁺ and L3T4⁺ cells in the lung tissue of immunosuppressed animals that were previously immunized with P10. The CD11b receptor may be expressed by macrophages, dendritic cells, natural killer, microglia, and B-1 cells, (1). Dendritic cells and macrophages are part of the first line of defense against *P. brasiliensis* (28). Neutrophils and monocytes are Ly-6G⁺/Ly-6C⁺ cells that may be involved in the first host response against infection by *P. brasiliensis* (19). T lymphocytes (MHC class II-restricted T cell, including most T helper cells) and the NKT cells are L3T4⁺ cell type, important in the cellular response, the major mechanism of host defense in PCM.

It is important to have an increased inflammatory response to contain the infection and the spread of the fungus in the host, but it is also important to avoid the deleterious effects of an exacerbated immune response. Pulmonary fibrosis is a severe and progressive sequel of PCM. Development of pulmonary fibrosis is probably attributed to a prolonged inflammatory stimulus which is common in PCM (18). Animals immunized with P10 showed a decrease in

pulmonary fibrosis. A likely explanation is the consequent reduction in fungal burden in the lungs of immunized animals, which led to a decrease in stimulation and consequent reduction of pulmonary fibrosis.

In addition, we detected increased levels of cytokines such as IFN- γ , TNF- α , IL-1 β and IL-18 in the lungs (data not shown), indicating a restoration of cellular immunity of anergic animals immunized with P10. In paracoccidioidomycosis TNF- α is directly related to the formation of granulomas (15) and with IL-12 and IL-18, potentiates the fungicidal activity of macrophages.

The 100% of the animals immunosuppressed and infected with the virulent isolate Pb18, died within 80 days after infection. Animals immunized with P10 showed a survival of 50% and there was a 40% survival in those animals treated only with drugs. Mice that were treated with the drugs sulfamethoxazole/trimethoprim and itraconazole, associated with the immunization with P10 showed 100% survival for a period of 200 days.

Currently, treatment of PCM is related to the severity of the disease, type of drug utilized and the time of use. The toxicity of drugs, combined with reports of relapses due to the resistance of the fungus has been observed (26). This fact makes important to search for new therapeutic adjuvants in order to decrease the duration of treatment and relapses, preventing the spread of the fungus to other anatomical sites.

In conclusion we demonstrated that the peptide P10 restores specific immunity against *P. brasiliensis* even in animals subjected to immunosuppressive treatment with synthetic corticosteroids (DEX) in an experimental model of PCM. Use of preventive or therapeutic vaccines in infectious diseases has expanded and requires the selection of immunogenic components that may lead to the protection of the host. Efficiency of immunization with the P10 peptide, as well as the additive effect of the treatment associate

with conventional medications, open new perspectives and makes the P10 an important vaccine candidate to be tested in human paracoccidioidomycosis.

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CONFLICTS OF INTEREST

None to declare.

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FIGURES

Figure 1: Immunosuppressed mice immunized with P10 peptide develop a lymphoproliferative response of splenocytes when stimulated with P10 *in vitro*. Splenocytes were isolated from experimental groups after 60 days i.t. infection with 3×10^5 yeast of *P. brasiliensis*. The splenocytes were stimulated with P10 peptide in PRMI for 144 hours. Splenocytes from the uninfected mice that didn't receive dexamethasone were also incubated in RPMI alone (negative control) or with ConA (positive control, 4 μ g/ml). * $P < 0.05$ significance relative to the control group.

Figure 2: Detection of cytokines in supernatants of spleen cells cultures of immunosuppressed BALB/c mice after 60 days i.t. infection with 3×10^5 yeast of *P. brasiliensis*. Asterisks indicate statistically significant differences between results detected in mice immunized with P10 and mice that received only PBS. * $P \leq 0.05$, ** $P \leq 0.01$.

Figure 3: Nitric oxide measurements in spleen cells culture of immunosuppressed BALB/c mice after 60 days i.t. infection with 3×10^5 yeast of *P. brasiliensis*. Splenocytes were cultured in RPMI medium in the presence of P10 peptide for 144 hours. NO levels were detected using a chemiluminescence analyzer (NOATM280, Sievers Inc., USA). * $P \leq 0.05$, *** $P \leq 0.001$ relative to the infected group treated with Dex. Error bars denote SD.

Figure 4: CD11b⁺, Ly-6G/Ly-6C⁺ and L3T4⁺ cells in the lung tissues from immunosuppressed and infected BALB/c mice. Tissue sections were obtained 60 days after infection with 3×10^5 yeast cells of *P. brasiliensis*. (A) CD11b⁺ cells in lung tissue of control group and immunized mice with P10. (B) Ly-6G/Ly-6C⁺ cells in lung tissue of control group and immunized mice with P10. (C) L3T4⁺ cells in lung tissue of control group and immunized mice with P10. Diaminobenzidine (DAB) was

used as the peroxidase substrate to generate a brown-staining signal and the sections were counterstained with Mayer hematoxylin. Magnification, X 200.

Figure 5: Evaluation of pulmonary fibrosis in the lungs of immunosuppressed BALB/c mice infected with 3×10^5 yeast cells the *P. brasiliensis* 60 days post-infection. (A, C) Untreated group. (B, D) Immunized with P10 peptide group. (A, B) Masson's Trichrome staining to detect the fibers of collagen type I and (C, D) Gomori's silver reticulin staining to detect collagen type III fibers. Magnification 100X.

Figure 6: Survival curve of immunosuppressed BALB/c mice i.t. infected with 3×10^5 yeast cells of *P. brasiliensis*. (●) Control group (PBS). (■) Treated with ITC (10 mg/kg); (▲) Treated with SMT/TMP (15-3 mg/kg); (▼) Immunized with P10; (◆) Immunized with P10 and treated with SMT/TMP (15-3 mg/kg); (□) Immunized with P10 and treated with ITC (10 mg/kg); the results are representative of two independent experiments. $p \leq 0.0001$ between different groups.

Artigo 2

MARQUES, A.; DASILVA, M.; JULIANO, M.; MUÑOZ, J. E.; TRAVASSOS, L. R.; TABORDA, C. P. Additive effect of P10 immunization and chemotherapy in anergic mice challenged intratracheally with virulent yeasts of *Paracoccidioides brasiliensis*. **Microbes and Infection**, p. 1, 2008.

Original article

Additive effect of P10 immunization and chemotherapy in anergic mice challenged intratracheally with virulent yeasts of *Paracoccidioides brasiliensis*

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Abstract

Paracoccidioidomycosis is a systemic granulomatous disease manifested in the acute/subacute or chronic forms. The anergic cases of the acute/subacute form are most severe, leading to death threatening conditions. Drug treatment is required to control the disease but the response in anergic patients is generally poor. A 15-mer peptide from the major diagnostic antigen gp43, named P10, induces a T-CD4⁺ helper-1 immune response in mice of different haplotypes and protects against intratracheal challenge with virulent *P. brasiliensis*. Presently, P10 immunization and chemotherapy were associated in an attempt to improve antifungal treatment in Balb/c mice made anergic by adding dexamethasone to the drinking water. The combined drug/peptide treatment significantly reduced the lung CFUs in infected anergic mice, largely preserved lung alveolar structure and prevented fungal dissemination to liver and spleen. Results recommend that a P10-based vaccine should be associated to chemotherapy for improved treatment of paracoccidioidomycosis aiming especially at anergic cases.

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Keywords: *P. brasiliensis*; Anergy; Dexamethasone; Chemotherapy; Immunization; P10; gp43

1. Introduction

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by *Paracoccidioides brasiliensis*, a thermally dimorphic fungus widespread in Latin America mainly affecting rural workers. An estimate on the incidence of PCM in Latin America showed that approximately 10 million people may be infected and 2% of them may develop the disease [1]. The incidence may increase in recent areas of deforestation and development with the practice of soil churning, as well as in immunocompromised individuals [2]. In the 1980–1995 period

there occurred 3181 lethal cases only in Brazil, but this is probably underestimated, since the disease is not classified as of mandatory notification by the governmental health authority [3].

The disease can exhibit two forms, acute/subacute and chronic. The former equally affects both genders and primarily involves the reticuloendothelial/lymphatic system. The chronic form affects mainly adult males with predominant pulmonary and/or mucocutaneous involvement [4]. Activation of an immune cellular response is the primary effective mechanism of control of experimental and human PCM and a correlation has been found between the severity of the disease and impaired delayed-type hypersensitivity (DTH) response [5].

Antifungal chemotherapy is required to control the disease. Initial treatment with sulfonamides, amphotericin B, or azoles may last from 2 to 6 months. Extended periods of treatment are often necessary, up to 2 or more years [6]. Significant frequency of relapsing disease is observed in patients

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submitted to short periods of treatment (reviewed in ref. [7]). Anergic conditions are death threatening due to poor response to chemotherapy when the immune system collapses.

The gp43 is the major diagnostic antigen of *P. brasiliensis*. Antibodies to the glycoprotein can be used in the follow-up of patients using a variety of serological tests [8,9]. Also, it was demonstrated that gp43 can elicit delayed hypersensitivity reactions in guinea pigs [10] and humans [11], implying the presence of T-CD4⁺ reacting epitopes.

The gp43 gene was cloned and sequenced encoding a polypeptide of 416 amino acids (Mr 45,947) with 56–58% similarity with exo-1,3- β -D-glucanases from *Saccharomyces cerevisiae* and *Candida albicans* [12]. The mature protein has a single high mannose N-glycosylated site [13]. Investigation of the gp43 gene polymorphism showed that the P10 encoding sequence is highly conserved with rare mutations in this region [14].

The H-2^d-restricted T-cell epitope was mapped to a 15-mer peptide called P10. An inner core of P10 formed by HTLAIR is the essential domain of the epitope inducing proliferation of lymph node cells from mice sensitized to the gp43 or infected with *P. brasiliensis*. Lymphoproliferation induced by either P10 or gp43 involved CD4⁺T-helper lymphocytes producing IL-2 and IFN- γ . Immunization of mice with both gp43 and P10 in the presence of complete Freund's adjuvant (CFA) caused a 200-fold decrease in CFU from the lung of mice challenged intratracheally with virulent yeasts of *P. brasiliensis* [15].

Recently we have shown that P10 immunization associated to chemotherapy improved antifungal treatment and prevented relapses. In all cases the combined immunochemotherapy showed an additive protective effect clearly superior to drug and P10-vaccine administered alone. P10-vaccination could successfully reverse the relapsing infection observed in sulphamethoxazole/trimethoprim treated mice. Generally, drug treatment favored a Th-2 response producing IL-4 and IL-10, whereas immunization with P10 stimulated a pro-inflammatory Th-1 response with increased IL-12 and IFN- γ . In successfully treated mice both Th-1 and Th-2 cytokines were present. The combined drug/vaccine treatment significantly reduced the lung CFUs, largely preserved lung alveolar structure and prevented fungal dissemination to liver and spleen [16].

In the present work we investigated the effectiveness of P10 in an experimental model that aimed at mimetizing the anergic state, common in patients with the acute and sub acute forms. In general, we studied the behavior of immunosuppressed animals, infected and treated with itraconazole or sulfamethoxazole and trimethoprim, associated or not with the immunization with P10. This novel treatment was shown to be quite effective in mice intratracheally infected with this fungus and with the perspective of potentially reducing the time of treatment in human patients, prevent relapsing disease and eventually reverse life threatening anergic cases.

2. Materials and methods

2.1. Animals

Balb/c mice (6- to 8-week-old males) were bred at University of São Paulo animal facility under specific-pathogen-free

conditions. Procedures involving animals and their care were conducted in conformity with the local Ethics Committee and international recommendations.

2.2. Fungal strain

Virulent *P. brasiliensis* Pb18 yeast cells were maintained by weekly passage on solid Sabouraud medium at 37 °C and were used after 7–10 days of growth. Before experimental infection, the cultures were grown in modified McVeigh–Morton medium (MMcM) at 37 °C for 5- to 7-days [17]. The fungal cells were washed in phosphate-buffered saline (PBS, pH 7.2) and counted in a hemocytometer. The viability of fungal suspensions determined by staining with trypan blue (Sigma, St Louis, MO) was always higher than 90%. The virulence of isolate Pb18 was checked in each experiment by infecting intratracheally Balb/c mice and recovering the yeast cells from the organs of mice.

2.3. Immunosuppression of mice

To examine the effects of immunosuppression in mice intratracheally infected with *P. brasiliensis*, dexamethasone phosphate (Sigma, St Louis, MO) was added to the drinking water of infected animals. Assuming an average water intake of 5 ml per day for 30 days, the daily dexamethasone phosphate dose was calculated as 0.15 mg kg⁻¹.

2.4. Delayed-type hypersensitivity (DTH) reaction

To verify the effect of dexamethasone administration the DTH reactions against Pb-18 antigen were measured as well the evolution of the infectious process was evaluated. The left hind footpad was injected subcutaneously with 25 μ l of Fava Netto's antigen every 3 days [18] and variations in size (diameters) were measured after 24 and 48 h.

2.5. Peptide synthesis and purification

Peptide synthesis and purification was carried out at the Department of Biophysics, UNIFESP as described previously [15]. HPLC analysis showed that the synthetic P10 in the amidated form was 90% pure.

2.6. Immunization of mice

Anergic Balb/c mice (6- to 8-week old males) were weekly immunized for 4 weeks with 20 μ g of P10 each. The first immunization was subcutaneous with complete Freund's adjuvant (CFA) and subsequently peptide immunization was i.p. with incomplete Freund's adjuvant (IFA). Control mice were injected with CFA alone.

2.7. Intratracheal infection of Balb/c mice

Balb/c mice were inoculated intratracheally with 3×10^5 yeast cells of virulent *P. brasiliensis* Pb18, grown on

Sabouraud agar and suspended in sterile saline (0.85% NaCl), per animal. A maximum volume of 50 µl was inoculated per mouse. Briefly, mice were anesthetized i.p. with 200 µL of a solution containing 80 mg/kg of ketamine and 10 mg/kg of xylazine (both from União Química Farmacêutica, Brazil); after approximately 10 min, their necks were hyper-extended, and the trachea was exposed at the level of the thyroid and injected with 3×10^5 yeast cells in PBS using a 26-gauge needle. The incisions were sutured with 5-0 silk.

2.8. Fungal burden in organs of infected mice

Mice were sacrificed 45 days after i.t. infection and the fungal burden was measured by colony forming units (CFU). Sections of the lung, liver and spleen were removed, weighed, homogenized and then washed three times with PBS. The corresponding pellets were re-suspended and homogenized each in 1 ml of PBS. A 100 µl-sample of this suspension was plated on solid brain–heart infusion (BHI) medium supplemented with 4% fetal calf serum (Gibco, NY, USA), 5% spent *P. brasiliensis* isolate-192 culture supernatant, streptomycin/penicillin 10 IU/ml (Cultilab, Brazil) and cycloheximide 500 mg/ml (Sigma, St Louis, MO). Petri dishes were incubated at 37 °C for at least 10 days, and colonies were counted (1 colony = 1 CFU).

2.9. Chemotherapy of anergic infected mice

The anergic state was obtained 20 days after the administration of dexamethasone. Animals were then infected and drug treatment started 15 days after i.t. infection. The treatment was held for 30 days during which groups of mice received doses every 24 h of itraconazole (10 mg/kg), or sulfamethoxazole/trimethoprim (15 mg and 3 mg/kg). All drug administrations were i.p.

2.10. Groups studied

Eight groups of mice (with 10 animals each) were used. Three controls were included: sham, a group that was neither infected nor treated with dexamethasone, antifungals or with P10; a second control group was infected with Pb 18, received dexamethasone in the drinking water but was not further treated with either an antifungal drug or P10; a third control group included mice infected but not treated with dexamethasone, antifungal drugs or P10. Five anergic groups included: (a) a group infected and immunized with P10; (b) two groups of mice infected and treated with either itraconazole or sulfamethoxazole/trimethoprim; and (c) two groups treated with antifungal drugs and also immunized with P10. All five groups (anergic) received dexamethasone in the drinking water. The whole experiment was repeated twice with reproducible results.

2.11. Cytokine analysis

Mice were sacrificed 45 days after infection and sections of the lung (right and left alternatively), liver and spleen were

homogenized in 2 ml of PBS in the presence of protease inhibitors (Complete Mini; Boehringer Mannheim, Indianapolis, IN). The homogenates were centrifuged, and the supernatants frozen at –80 °C until tested. The supernatants were assayed for IL-2, IL-4, IL-10, IL-12, and IFN-γ using ELISA kits (BD PharMingen, San Diego, CA). The detection limits of such assays were as follows: 3.1 pg/ml for IL-2; 7.8 pg/ml for IL-4; 31.25 pg/ml for IL-10 and IFN-γ; and 62.5 pg/ml for IL-12p40, as previously determined by the manufacturer.

2.12. Antibody response to gp43

The antibody response to gp43 was determined 45 days after infection. Microtiter plates sensitized with 500 ng of purified gp43 were incubated at 37 °C for 1 h with 100 µl of mouse serum, serially diluted starting at 1:200. The reaction was quantified with goat anti-mouse Ig for 1 h at 37 °C, followed by donkey anti-goat Ig-biotin conjugate and reaction with streptavidin-peroxidase for 30 min at 37 °C. After addition of the orthophenylenediamine reagent (500 µg/ml), the reaction was read at 492 nm. The antibodies, conjugates, and substrate were from Sigma Chemical Co (St Louis, MO).

2.13. Histopathology

The lungs were excised, fixed in 10% buffered formalin, and embedded in paraffin for sectioning. The sections were stained with hematoxylin-eosin or silver nitrate and examined microscopically at 25× magnification (Optiphot-2; Nikon, Tokyo, Japan).

2.14. Statistical analysis

Statistical analysis was done using GraphPad Prism5 software. The results were expressed as means ± SD. The non-parametric Tukey test was used. Unpaired Student's *t*-test with Welch's correction (two-tailed) was used for the comparison of two groups when the data met the assumptions of the *t* tests. *p* < 0.05 indicated statistical significance. Survival experiments were compared by log rank analysis (SigmaStat; SPSS, Chicago, IL).

3. Results

3.1. Determination of the anergic state in Balb/c mice

A group of infected animals were treated with dexamethasone in the drinking water and every 3 days the animals were submitted to a DTH test to determine the time necessary for complete anergy. No DTH reaction was observed after 20 days of treatment with dexamethasone (Fig. 1A). The mortality in groups of infected mice submitted or not to dexamethasone treatment was scored up to 100 days after infection. It was restricted to the group of animals that received dexamethasone phosphate in the drinking water in the time range of the experiment (Fig. 1B). Unlike the control group, all animals

Fig. 1. Delayed-type hypersensitivity and Survival curve of Balb/c mice. (A) Follow-up of DTH reactions in infected Balb/c mice (○) and infected, immunosuppressed Balb/c mice (●). Dotted line represents averages of uninfected Balb/c mice. Error bars denote standard deviations. (B) Survival of Balb/c mice infected with 3×10^5 yeast cells of *P. brasiliensis* Pb18 treated with dexamethasone (0.15 mg/kg) in the drinking water for 30 days (●); untreated control (○). The experiments were repeated twice with similar results.

were killed by day 70. After determining the time necessary for anergy induction, all subsequent experimental infections were carried out after 20 days of exposure to dexamethasone in the drinking water. Infected anergic mice submitted to P10 vaccination and/or antifungal drug treatment were all alive in the 100-day period of the experiment much like the control mice untreated with dexamethasone (data not shown).

3.2. Organ CFU from intratracheally infected anergic Balb/c mice immunized with P10 and/or treated with antifungal drugs

Our previous work has shown that P10 could be used as adjuvant in the treatment of established experimental infection [16]. To explore the effects of P10 immunization in anergic Balb/c mice, animals' immunization started 15 days after infection. Analysis of organ CFUs was done after 45 days of infection. A significant reduction of fungi recovered from lung, spleen and

liver of animals (CFU) was obtained in mice immunized with P10. The treatment with itraconazole and sulfamethoxazole/trimethoprim also reduced the fungal burden; a more significant reduction, however, was obtained when itraconazole or sulfamethoxazole/trimethoprim and P10 immunization were combined in the treatment of anergic Balb/c mice (Fig. 2).

3.3. Lung histopathology from anergic Balb/c mice vaccinated with P10 only, after intratracheal infection

Lungs of infected animals submitted to dexamethasone treatment showed multiple pulmonary foci of epithelioid granulomatous inflammation, with a loose architecture containing a great number of neutrophils and fungal cells (Fig. 3A). Lungs of animals submitted to P10 immunization showed few well demarcated epithelioid granulomas, compact and with few fungal cells (Fig. 3B). A similar pattern of lung histopathology was observed in the tissues of mice treated with itraconazole (Fig. 3C) or sulfamethoxazole/trimethoprim (Fig. 3E). The association of drugs and immunization with P10 resulted in areas with very few or undetectable yeast cells (Fig. 3D,F). Clearly, in this case, P10-immunization was an essential adjuvant therapy that reduced the infection. Few untreated animals showed, during the experiment, brain dissemination of fungal elements that was absent in the P10 immunized mice (data not shown).

3.4. Cytokine detection in the lungs of anergic infected Balb/c mice immunized with P10 and/or treated with antifungal drugs

Previous studies have established that P10 was able to activate a Th-1 immune response with the known pattern of cytokine production *in vitro* and *in vivo* [15,16]. Presently, lung cytokine levels were measured in anergic mice infected with *P. brasiliensis* and treated with itraconazole or sulfamethoxazole/trimethoprim combined or not with P10 immunization. A sham group was evaluated for basal levels of cytokines as well as a control group of infected-only mice untreated with dexamethasone, antifungal drugs or peptide as shown in Table 1. The comparative cytokine levels among infected Balb/c mice treated or not with dexamethasone were then evaluated. Results showed that infected mice treated with dexamethasone had a much higher concentration of IL-4 and IL-10 than of IL-12 and IFN- γ . Treatment of mice with antifungal drugs led to partial reduction of IL-4 and IL-10 levels which were then further reduced with the association of P10 immunization and the antifungal drug. P10 immunization as well as its association with antifungal treatment markedly enhanced IL-12 and IFN- γ levels. Thus, although both Th-1 and Th-2 types of immune response seemed present in all cases, incorporation of P10 greatly stimulated a Th-1 response with increased production of IL-12 and IFN- γ , clearly associated with protection.

3.5. Antibody titers against gp43 after 45 days of infection

Before sacrifice for other determinations, blood was collected from infected animals and the serum antibodies to

Fig. 2. Colony forming units from organs of infected mice. Colony forming units (CFU) from lungs (L), spleen (S) and liver (V) of Balb/c mice infected intratracheally with 3×10^5 yeast cells of *P. brasiliensis* Pb18 (non-anegetic) or infected and treated with dexamethasone (anegetic). Groups of anergic animals were also submitted to P10 immunization (P10), itraconazole (Itra) or sulfamethoxazole/trimethoprim (SMT/TMP) treatment, or the combined P10 immunization and chemotherapy (itraconazole + P10 and SMT/TMP + P10). Bars represent the CFU means and standard deviations from organs of five to 10 animals in each group. * significant ($p < 0.05$) difference in relation to the group of mice infected and treated only with dexamethasone in drinking water. ** significant ($p < 0.05$) differences between the combined treatment of P10 and antifungal drug, and the individual treatments with each agent.

the gp43 major antigen were titrated. Animals treated with dexamethasone and infected, showed after 45 days by ELISA an antibody titer of 1/6400 compared to 1/200 in mice infected and submitted to immunization with P10. Anergic mice immunized with P10 and treated with either itraconazole or sulfamethoxazole/trimethoprim also produced fewer specific antibodies than those treated only with antifungal drugs.

4. Discussion

The main clinical forms of PCM are: acute/subacute and chronic. The acute form is more severe and represents 3–5% of all cases of PCM affecting mainly children and teenagers but eventually also adults up to 35 years old (reviewed in ref. [6]).

Clinical evidence as well as experimental studies have shown that cell-mediated immunity is the chief mechanism of host defense. Acute/subacute forms are more frequently associated with anergic immune response whereas the chronic form is hyperergic. Acute/subacute forms tend to show necrotizing lesions with abundant fungal cells, impairment of cell-mediated immunity and high titers of circulating antibodies. In contrast, the chronic form is seldom disseminated, and is associated with tuberculoid granulomata and few fungal cells. The cell-mediated immunity is better preserved with low antibody levels [19,20].

The control of the disease is made by antifungal drugs such as sulphonamides, amphotericin B and azoles (reviewed in ref. [7]). Itraconazole is a good option to start the treatment but sulphamethoxazole/trimethoprim therapy is still being used in public hospitals in Brazil [6].

The use of peptides as therapeutic vaccines in the treatment of fungal infections as they have being used in cancer [21] could be an adjuvant to chemotherapy potentially able to reduce the toxicity of conventional drugs as well as the time of

treatment, prevent relapsing disease and even help to treat anergic cases [22,23].

The immunoprotective properties of P10 were well determined in a murine model of PCM. Injection of P10 emulsified in complete Freund's adjuvant (CFA) protected mice against intratracheal challenge with virulent *P. brasiliensis* isolate. Disease progression was followed by counting the CFUs in tissues and by histopathology. The protective effect of P10 is undoubtedly related to the induction of an IFN- γ -dependent Th-1 immune response [15]. A P10-derived multiple-antigen-peptide construction (MAP) administered without CFA conferred equal protection in i.t. infected mice [24]. We have shown then that an additive protective effect was achieved by the combination of drug treatment and P10 immunization [16]. Aiming at defining the role of P10 as an adjuvant immune treatment in drug treated, infected animals, we presently extended this study to determine the capacity of P10 to reverse the anergic state of Balb/c mice induced by dexamethasone. The recognized anergic state was observed after 20 days of treatment with dexamethasone in the drinking water a condition that caused death of all infected mice after 70 days of infection. The use of dexamethasone to induce the anergic state in mice has been described previously [25].

Anergic animals i.t. infected with virulent *P. brasiliensis* showed high CFU in the lungs and also in the liver, spleen and brain after 45 days of infection. Cytokine determination showed increased IL-4 and IL-10 and low levels of IFN- γ and IL-12, a pattern that was reversed by P10 immunization and less efficiently by antifungal drugs. Combination of P10 and antifungal drugs promoted the best Th-1 type immune conversion. Anti-gp43 IgG titers were higher in the dexamethasone and antifungal treated groups than in the P10-immunized ones. Immunization of anergic Balb/c mice with P10 led to significant reduction of CFU in the lung, spleen and liver. The protective properties of IFN- γ in the murine model

Fig. 3. Histopathology of lungs from intratracheally infected anergic Balb/c mice submitted to immunization with P10 and/or chemotherapy. Additive protective effect of P10 immunization and antifungal drug treatment is shown after 45 days of infection. (A) Lung section from infected mouse treated only with dexamethasone; (B) same as (A) but immunized with P10, showing a single granuloma; (C) lung section from anergic mouse treated with itraconazole; (D) same from anergic mouse immunized with P10 and treated with itraconazole; (E) lung section from anergic mouse treated with sulfamethoxazole/trimetoprim (SMT/TMT) and (F) same from anergic mouse immunized with P10 and treated with (SMT/TMT). Hematoxylin eosin staining; magnification, $\times 25$.

have been established previously. Survival analysis of mice deficient in IFN- γ or IFN- γ receptor (but not IFN- α -R or IFN- β -R), and IRF-1 showed 100% mortality 3–4 weeks after intratracheal challenge with a virulent isolate. Consistent with the need of IFN- γ for effective immunization with P10, non-immunized infected IFN- γ -R knock-out mice displayed the same mortality rate as of P10-immunized KO mice [9].

Histopathology of infected organs confirmed the protective effect of P10, either by significantly reducing the number of granulomas or, when associated with antifungal drugs, leading to fungal elimination but not sterility because of the short time of the experimental design [26].

The use of P10 as an adjuvant to antifungal treatment is a promising tool in the treatment of experimental

Table 1

Lung cytokine levels after 45 days of infection in anergic Balb/c mice infected with 3×10^5 yeast cells of *P. brasiliensis* Pb18

Cytokine	Cytokine levels (ng/ml of tissue)							
	Sham ^a	Infected only ^b	Infected dexamethasone ^c	P10 ^d	Itraconazole ^e	Itraconazole and P10 ^f	SMT/TMP ^g	SMT/TMP and P10 ^h
IL-2	1.71 ± 0.7	2.51 ± 0.56	1.98 ± 0.94	8.09 ± 0.32	3.16 ± 1.0	8.07 ± 1.98*	3.4 ± 1.35	6.51 ± 0.56*
IL-4	0.2 ± 0.06	3.37 ± 0.69	10.9 ± 1.86	1.14 ± 0.46	4.86 ± 0.38	0.68 ± 0.19*	5.56 ± 0.61	0.62 ± 0.12*
IL-10	4.39 ± 1.87	12.52 ± 1.76	20.16 ± 5.24	8.16 ± 2.99	8.66 ± 2.52	3.64 ± 2.04*	13.28 ± 2.64	6.53 ± 2.16*
IL-12	0.58 ± 0.23	2.12 ± 0.50	1.52 ± 0.25	10.48 ± 1.53	2.81 ± 0.43	11.6 ± 1.38*	2.2 ± 0.48	10.05 ± 0.63*
IFN-γ	0.38 ± 0.26	0.31 ± 0.11	1.1 ± 0.7	4.5 ± 0.88	1.18 ± 0.30	7.39 ± 1.34*	0.41 ± 0.22	6.54 ± 0.77*

*Significant differences ($p < 0.05$) relative to anergic mice submitted to chemotherapy only. Values are means for 10 animals per group with standard deviations.

The whole experiment was repeated twice with reproducible results.

^a Uninfected, non-immunized and untreated animals ($n = 10$).^b Infected only, untreated with dexamethasone, antifungal drugs or P10.^c Infected only, but treated with dexamethasone.^d Treated with dexamethasone (anergic), infected and immunized with P10.^e Anergic, treated with itraconazole.^f Anergic, treated with itraconazole and P10 immunization.^g Anergic, treated with sulfamethoxazole/trimetoprim (SMT/TPM).^h Anergic, with SMT/TPM and P10 immunization.

paracoccidioidomycosis with a role also in the prevention of relapses. The combined treatment could be important even in cases of clinical resistance to azoles and sulfamethoxazole/trimethoprim [7,16]. Presently, we report on the additive protective effect of itraconazole or sulfamethoxazole/trimethoprim and P10 immunization in anergic animals. Anergic Balb/c mice treated only with antifungal drugs still showed high levels of IL-4 and IL-10 possibly due to increased fungal destruction and high B-cell stimulation. The combined treatment with a P10-vaccine clearly stimulated a protective Th-1 response rich in IL-12 and IFN-γ. A predominant Th-1 immune response simultaneous with a persisting but less intense Th-2 response seems to represent the adequate balance leading to protection of infected animals. Most probably T-dependent antibodies are also implicated in the immunoprotection but with the exception of anti-gp43 antibodies which may include protective and non-protective types [27] this was not further explored presently.

Several features have been examined for validation of P10 as a candidate vaccine antigen including: (a) the presentation of P10 by different mouse haplotypes [15]; (b) its conservation in nature determined by examining gp43 molecules from different isolates [28]; (c) its immunogenicity in formulations that do not require complete Freund's adjuvant [24,29]; (d) its presentation by human HLA-DR molecules and that of other promiscuous peptides derived from the gp43 [30]; and (e) additive effect when administered with antifungal drugs [16] and finally, its role in the immunoprotection of anergic mice infected with *P. brasiliensis* and submitted to chemotherapy which was the focus of the present work. The present data strongly suggest that a P10-based vaccine may be used to enhance antifungal protection by chemotherapy even in cases of anergy.

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Artigo 3

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The Monoclonal Antibody against the Major Diagnostic Antigen of *Paracoccidioides brasiliensis* Mediates Immune Protection in Infected BALB/c Mice Challenged Intratracheally with the Fungus[▽]

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The protective role of specific antibodies against *Paracoccidioides brasiliensis* is controversial. In the present study, we analyzed the effects of monoclonal antibodies on the major diagnostic antigen (gp43) using in vitro and in vivo *P. brasiliensis* infection models. The passive administration of some monoclonal antibodies (MAbs) before and after intratracheal or intravenous infections led to a reduced fungal burden and decreased pulmonary inflammation. The protection mediated by MAb 3E, the most efficient MAb in the reduction of fungal burden, was associated with the enhanced phagocytosis of *P. brasiliensis* yeast cells by J774.16, MH-S, or primary macrophages. The ingestion of opsonized yeast cells led to an increase in NO production by macrophages. Passive immunization with MAb 3E induced enhanced levels of gamma interferon in the lungs of infected mice. The reactivity of MAb 3E against a panel of gp43-derived peptides suggested that the sequence NHVRIPIGWAV contains the binding epitope. The present work shows that some but not all MAbs against gp43 can reduce the fungal burden and identifies a new peptide candidate for vaccine development.

Paracoccidioides brasiliensis is a thermally dimorphic fungus that is the etiological agent of paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in Latin America that is endemic in areas of Brazil, Argentina, Colombia, and Venezuela. The impact of the disease is demonstrated by Coutinho et al. (14), who reported that 3,181 lethal cases of PCM occurred in the 1980 to 1995 period in Brazil. The acute and subacute forms of PCM affect both genders and primarily involve the reticuloendothelial/lymphatic system. The chronic form affects mainly adult males with predominant pulmonary and/or mucocutaneous involvement (20). The activation of the immune cellular response is the primary effective mechanism to control experimental and human PCM (4, 25). A correlation has been found between the severity of the disease and an impaired delayed-type hypersensitivity response (30).

Antifungal chemotherapy is required for PCM treatment, though even after prolonged administration, there is no assurance of the complete destruction of the fungus. The period of treatment depends on the drug used and disease severity. Standard drugs include sulfonamides, amphotericin B, and azoles (44).

gp43, first described by Puccia et al. (35), is the major diagnostic antigen of *P. brasiliensis* used in a variety of serological tests (16, 50). Antibody titers to gp43 have been used to mon-

itor the response to treatment in patients (6). gp43 has also proven to be immunodominant in a crude antigenic preparation, eliciting delayed hypersensitivity reactions in guinea pigs (40) and humans (42), which indicated the presence of T-CD4⁺-reacting epitopes.

The gp43 gene has been cloned and sequenced (13). It encodes a polypeptide of 416 amino acids (M_r , 45,947) with a leader peptide of 35 residues; the mature protein has a single high-mannose N-glycosylated chain (1). The H-2^d-restricted T-cell epitope has been mapped to a 15-mer peptide called P10 (48). Different 12-mer sequences, all containing the hexapeptide HTLAIR, induce the proliferation of lymph node cells from mice sensitized to gp43 or infected with *P. brasiliensis*. Lymphoproliferation induced by either P10 or gp43 involves CD4⁺ T-helper lymphocytes producing interleukin-2 (IL-2) and gamma interferon (IFN- γ). The immunization of mice with either gp43 or P10 in complete Freund's adjuvant significantly protected them from intratracheal (i.t.) infection with virulent yeasts of *P. brasiliensis*. After 3 months of infection, the lung CFU were >200-fold fewer than in the untreated control mice.

The role of antibody-mediated immunity in host resistance to *P. brasiliensis* is less certain (10). In several systems, however, there is considerable evidence that the administration of monoclonal antibodies (MAbs) can modify the course of disease in mice infected with fungi such as *Cryptococcus neoformans* (28), *Candida albicans* (15, 24), *Histoplasma capsulatum* (31), *Pneumocystis* spp. (51), *Fonsecaea pedrosoi* (2), *Aspergillus* spp. (12), and, more recently, *P. brasiliensis* (17).

The mechanisms of antibody action in combatting an infectious disease include antigen neutralization, cooperative effects

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with cellular immunity by the enhancement of phagocytosis or mediating-cell cytotoxicity, complement activation, growth inhibition, adherence, and biofilm or direct antimicrobial effects (reviewed in reference 11). With *C. neoformans*, it is clear that antibody-mediated immunity can be decisive for host defense. The mechanisms involved are complex and dependent on several parameters, such as antibody isotype (26), T-cell function (53), *C. neoformans* strain (29), antibody quantity (49), expression of inducible NO synthase (39), Fc region, and complement activation (43).

The control of PCM is classically associated with a vigorous Th1 response and granuloma formation. There is evidence, however, that antibody-mediated immunity can contribute to infection clearance. In the present study, a panel of MABs against gp43 was utilized for evaluating the effect of passive immunization in mice intravenously (i.v.) or i.t. infected with a virulent strain of *P. brasiliensis*.

MATERIALS AND METHODS

Animals. BALB/c mice (6- to 8-week-old males) were bred at the University of São Paulo, São Paulo, Brazil, animal facility under specific pathogen-free conditions. The procedures involving animals and their care were conducted according to the local ethics committee and international rules.

Fungal strain. Virulent *P. brasiliensis* Pb18 yeast cells were maintained by weekly passage on solid Sabouraud medium at 37°C and were used after 7 to 10 days of growth. Before the experimental infection, the fungus was grown in modified McVeigh-Morton medium at 37°C for 5 to 7 days (38). The fungal cells were washed in phosphate-buffered saline (PBS; pH 7.2) and counted in a hemocytometer. The viability of the fungal suspensions, determined by staining with Janus B (Merck, Darmstadt, Germany), was always higher than 90%.

MAbs. The MAbs against gp43—19G, 10D, 32H, and 17D (immunoglobulin G2a [IgG2a]) and 21F and 3E (IgG2b)—were previously characterized (36). IgG ascites was generated in BALB/c mice given intraperitoneal (i.p.) injections of MAb hybridomas. IgG MAbs were purified from ascitic fluid by protein A affinity chromatography (Pierce, Rockland, IL) as per the manufacturer's instructions. Endotoxin (lipopolysaccharide) concentration was <1 ng/ml, measured by the *Limulus* amebocyte test (BioWhittaker, Walkersville, MD). The antibody concentration was determined by enzyme-linked immunosorbent assay (ELISA) relative to isotype-matched standards. Irrelevant IgG MAb was used as a control and was provided by Elaine G. Rodrigues, Universidade Federal de São Paulo.

Endotoxin-free preparations. Disposable pyrogen-free plasticware and endotoxin-free water or PBS were used in all experiments. Ascitic fluid and isolated MAb were further purified in Detoxi-Gel columns (Pierce, Rockland, IL) to remove any endotoxin contamination.

Phagocytosis assay. Peritoneal and alveolar macrophages from BALB/c mice were harvested by washing the abdominal cavities or the lungs and culturing adherent cells in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum and 1% nonessential amino acids (Cultilab, Brazil). Other phagocytosis experiments were carried out with the J774.16 macrophage-like cell line, derived from a reticulum cell sarcoma (37), or with the MH-S line, originated from an SV40-transformed adherent cell-enriched population of alveolar macrophages (23). The protocol for in vitro phagocytosis was that described in earlier studies (46, 47) with minor modifications. Primary cells were plated on 96-well tissue culture plates (Switzerland) at a final density of 1.6×10^5 cells/well, and macrophage-like cells were plated at a density of 0.8×10^5 to 1×10^5 macrophages in order to obtain the same density after overnight incubation. The cells were stimulated with 50 U/ml recombinant murine IFN- γ (PeproTech, Rock Hill, NJ) and incubated at 37°C overnight. Phagocytosis was measured in the presence or absence of purified MAbs (0 to 100 μ g/ml). *P. brasiliensis* cells were added at a ratio of 5:1 macrophages to fungal cells and incubated at 37°C for 6, 12, and 24 h. The cells were then washed several times with sterile PBS, fixed with cold absolute methanol, and stained with a 1/20 solution of Giemsa (Sigma, St. Louis, MO). Phagocytosed yeasts were counted by light microscopy at $\times 400$ magnification. The phagocytic index (PI) is defined by the equation $PI = P \times F$, where P is the percentage of macrophages with internalized yeast and F is the average number of yeast cells per macrophage. Phagocytosis was confirmed by transmission electron microscopy that showed the internalization of yeast cells

in macrophages (data not shown). Experiments were carried out in triplicate, and five to eight different fields were counted.

Intratracheal infection of BALB/c mice. BALB/c mice were inoculated i.t. with 3×10^5 yeast cells of virulent *P. brasiliensis* Pb18, grown on Sabouraud agar and suspended in sterile saline (0.85% NaCl), per animal. Briefly, the mice were anesthetized i.p. with 200 μ l of a solution containing 80 mg/kg ketamine and 10 mg/kg of xylazine (both from União Química Farmacêutica, Brazil). After approximately 10 min, their necks were hyperextended, and the tracheas were exposed at the level of the thyroid and injected with 3×10^5 yeast cells in PBS using a 26-gauge needle. The incisions were sutured with 5-0 silk.

Fungal burden in organs of i.t. infected mice. For the fungal burden analysis, two protocols were utilized: (i) 1 mg of MAbs was administered i.p. 24 h before the infections, and the mice were sacrificed 15 and 30 days after i.t. infection, and (ii) 1 mg of MAbs was administered i.p. 30 days after the infections, and the mice were sacrificed 45 and 60 days after i.t. infection. The fungal burden was measured by CFU. Sections of the lungs, livers, and spleens were removed, weighed, homogenized, and then washed three times with PBS. The corresponding pellets were resuspended and homogenized, each in 1 ml of PBS. A 100- μ l sample of this suspension was plated on solid brain heart infusion medium supplemented with 4% fetal calf serum (Gibco, NY), 5% spent *P. brasiliensis* (strain 192) culture supernatant, 10 IU/ml streptomycin/penicillin (Cultilab, Brazil), and 500 μ g/ml cycloheximide (Sigma, St. Louis, MO). The petri dishes were incubated at 37°C for at least 10 days, and colonies were counted (1 colony = 1 CFU).

Histopathology. The lungs of i.t. infected mice were excised, fixed in 10% buffered formalin, and embedded in paraffin for sectioning. The sections were stained with hematoxylin-eosin or silver nitrate and examined microscopically at $\times 25$ magnification (Optiphot-2; Nikon, Tokyo, Japan).

Fungal burden in organs of i.v.-infected mice. MAbs 3E and 32H and irrelevant MAb were administered (1 mg) i.p. 24 h before i.v. infection with 3×10^5 yeast cells of virulent *P. brasiliensis* Pb18. A maintenance dose of 100 μ g of each MAb was given every week for a month. The mice were sacrificed 30 days after infection and the fungal burden measured as described for the i.t. infection.

Cytokine analysis. Sections of the lungs (alternating right and left lungs) were homogenized in 2 ml of PBS in the presence of protease inhibitors (Complete Mini; Boehringer Mannheim, Indianapolis, IN). The homogenates were centrifuged, and the supernatants frozen at -80°C until tested. The supernatants were assayed for IL-2, IL-4, IL-10, IL-12, and IFN- γ using ELISA kits (BD Pharmingen, San Diego, CA). The detection limits of such assays were as follows: 3.1 pg/ml for IL-2, 7.8 pg/ml for IL-4, 31.25 pg/ml for IL-10 and IFN- γ , and 62.5 pg/ml for IL-12p40, as previously determined by the manufacturer.

Production of nitric oxide. The levels of nitric oxide metabolite (nitrite) were determined by Griess reaction (33) in the culture supernatant of macrophages challenged with opsonized yeasts. All determinations were performed in triplicate.

Peptide synthesis and purification for screening. Peptide synthesis and purification were carried out at the Department of Biophysics, Universidade Federal de São Paulo. The amino acid sequence of *P. brasiliensis* gp43 glycoprotein (GenBank accession number AY005437) was used to synthesize the peptides by solid-phase technology using the 9-fluorenylmethoxy carbonyl strategy on an automated benchtop simultaneous multiple solid-phase peptide synthesizer PSSM8 (Shimadzu, Tokyo, Japan) with 9-fluorenylmethoxy carbonyl-protected amino acid residues and TentaGel Rink resin (Novabiochem, San Diego, CA). Therefore, all peptides were obtained with the C-terminal carboxyl group in an amide form. All peptides were deprotected and cleaved from the resins by treatment with K reagent composed of 80% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% ethanedithiol, 5.0% anisole, 5.0% water, and 5.0% phenol. The resulting peptides were analyzed by reverse-phase high-performance liquid chromatography (Shimadzu, Tokyo, Japan) on a C_{18} column eluted at 1 ml/min using a 5% to 95% gradient of 90% acetonitrile in 0.1% trifluoroacetic acid over 30 min. The peptide quality was assessed by a matrix-assisted laser desorption/ionization-time of flight instrument (Micromass, Manchester, United Kingdom) using α -cyano-4-hydroxy cinnamic acid as the matrix.

Peptide containing the reactive epitope of the protective MAb 3H. The peptide NHVRIPIGYWAV was synthesized at Peptides International (Louisville, KY) with 95% purity, in both the carboxy and amidated forms.

MAb reactivity with peptides. Antibody reactivity with peptides from gp43 was determined by ELISA. Microtiter plates coated with 100 ng of each peptide/well were incubated with 100 μ l of MAb serially diluted, starting at 10 μ g/ml, at 37°C for 1 h. The plates were washed three times and incubated with 100 μ l of goat anti-mouse IgG peroxidase (Sigma, St. Louis, MO) for 1 h at 37°C. The plates were washed, and the reaction was developed with a solution of *o*-phenylenediamine (0.5 mg/ml; Sigma, St. Louis, MO) and 0.005% H_2O_2 (Sigma, St. Louis, MO). The reaction was terminated with 4 N H_2SO_4 after 8 to 10 min of incu-

FIG. 1. Phagocytosis of yeast cells by J774.16 cells after 12 h in the presence of MABs against gp43 (3E, 10D, 17D, 19G, 21F, and 32H) compared to that of controls of yeast incubated without MAB (PBS) or with an irrelevant MAB (MAB A4). Each bar represents the average of three measurements, and error bars indicate SD. Experiments were done in triplicate, and different fields were counted. *, significant difference ($P < 0.05$, determined by analysis of variance and Tukey's honestly significant difference test) compared to the control without MAB.

bation in the dark. The optical densities were measured at 492 nm in an ELISA reader (Titertek Multiskan EIA reader).

Direct effect of MABs on *P. brasiliensis*. The growth rate of Pb18 in the presence of protective and nonprotective MABs was compared to that in the presence of irrelevant antibodies or medium alone. Yeast cells were grown in Sabouraud medium at 37°C, and 100 µg/ml of each MAB was added at 96-h intervals. Culture samples were taken at 48-h intervals, and cell numbers were counted with a hemocytometer.

Statistical analysis. Statistical analysis was done using GraphPad Prism5 software. The results were expressed as the means $\pm$ the standard deviations (SD) of the indicated numbers of animals or experiments. The nonparametric Tukey's honestly significant difference test was used. The unpaired Student's *t* test with Welch's correction (two-tailed) was used for the comparison of the two groups when the data met the assumptions of the *t* tests. *P* values of <0.05 indicated statistical significance.

RESULTS

In vitro phagocytosis mediated by MABs. We investigated the effect of MABs to gp43 on *P. brasiliensis* phagocytosis in vitro using both primary macrophages (lung and peritoneal) and cell lines J774.16 and MH-S. Yeast cells opsonized with MABs 19G, 21F, 10D, 17D, and 3E were at least twofold more internalized by J774.16 cells than by nonopsonized or 32H-opsonized yeast cells (Fig. 1). The same result was obtained with primary macrophages. The MH-S cell line and the lung primary macrophages showed similar results. Phagocytosis was observed regardless of whether or not the macrophages were activated with IFN- γ , but the indices were significantly higher with cytokine-stimulated cells (data not shown).

Yeast cells opsonized with MABs to gp43 promote macrophage fungicidal activity in vitro. MABs that increase *P. brasiliensis* phagocytosis by macrophages also induce an increase in nitric oxide metabolite (nitrite) in the supernatants of the phagocytic cells (Fig. 2).

Passive immunization reduces fungal burden. To evaluate whether the administration of MABs could reduce lung CFU from mice infected with *P. brasiliensis*, two protocols were followed. In the first protocol, BALB/c mice were immunized with different MABs against gp43 24 h prior to i.t. infection with 3×10^5 yeast cells of *P. brasiliensis*. After 15 days of infection, the number of CFU in the lungs of mice that received MABs 19G, 21F, 10D, 3E, and 17D but not 32H showed a significant reduction compared with that of the controls (Fig. 3). Additionally, MABs 19G, 10D, and 3E maintained significant CFU reductions after 30 days of infection (Fig. 3). In the second protocol, two MABs were selected, 32H that did not reduce the lung CFU and 3E that reduced the CFU. BALB/c mice were infected as indicated above, and after 30 days, 1 mg of MABs 32H or 3E was injected i.p. The CFU were measured after 45 and 60 days of infection, and MAB 3E but not 32H was able to reduce the CFU from the lungs of the mice at day 60 (Fig. 4).

FIG. 2. Nitrite measurements in supernatants from phagocytosis assays. *P. brasiliensis* yeast was cultured with J774.16 cells for 12 h in the presence of MABs against gp43 (3E, 10D, 17D, 19G, 21F, and 32H), PBS (without MAB), or the irrelevant MAB (A4). Nitrite levels were detected using a Griess assay. *, significant difference ($P < 0.05$, determined by analysis of variance and Tukey's honestly significant difference test) relative to the PBS control. Error bars denote SD.

FIG. 3. Lung CFU from mice infected i.t. with 3×10^5 yeast cells and treated with MAbs against gp43 (3E, 10D, 17D, 19G, 21F, and 32H) 24 h prior to infection. Mice were sacrificed after 15 (black bars) or 30 (gray bars) days of infection. Control mice were infected and received PBS (infected only) or an irrelevant MAb (A4). Each bar represents the average count of fungi in the lung, and error bars indicate SD. *, significant difference ($P < 0.05$, determined by analysis of variance and Tukey's honestly significant difference test) relative to the PBS control.

To evaluate the effect of MAbs in mice, an intravenous infection model was used. Groups of animals were passively immunized i.p. with 1 mg of MAb 3E, MAb 32H, or the irrelevant control MAb 24 h prior to infection with 5×10^6 yeast cells and were given 0.1 mg of the MAbs every week for 1 month before the animals were sacrificed. For the CFU determinations, the lung, spleen, and liver tissues were homogenized separately and plated on solid medium. The mice immunized with MAb 3E had significantly lower lung and spleen CFU compared to the CFU of the mice receiving MAb 32H, the irrelevant MAb, or PBS (Fig. 5). Liver CFU were below the detection threshold.

Viability of *P. brasiliensis* yeast cells in the presence of MAbs 3E and 32H. The viability of yeast cells incubated with MAbs 3E or 32H or the irrelevant MAb was evaluated during 30 days; no direct inhibitory effects by the MAbs were observed (data not shown).

Lung histopathology of treated, i.t. infected BALB/c mice (30 days). The lungs of the untreated control animals showed intense infiltration, mainly by macrophages, lymphocytes, and epithelioid cells. Around the foci of the epithelioid granulomas, giant cells were also observed. Multiplying fungal cells were seen. Using protocol 1 (for which the mice received MAbs 24 h before i.t. infection), we observed that the animals

FIG. 4. Lung CFU from mice infected i.t. with 3×10^5 yeast cells and treated with MAbs against gp43 (3E or 32H) 30 days after infection. Mice were sacrificed after 45 (black bars) or 60 (gray bars) days of infection. Control mice were infected and received PBS (infected only) or an irrelevant MAb (A4). Each bar represents the average count of fungi in the lung, and error bars indicate SD. *, significant difference ($P < 0.05$) relative to the PBS control.

FIG. 5. CFU from lungs (black bars) or spleens (gray bars) of mice infected i.v. with 3×10^5 yeast cells and treated with 1 mg of MABs against gp43 (3E or 32H) 24 h prior to infection and with 100 μ g every week for a month as a maintenance dose. Mice were sacrificed after 30 days of infection. Control mice were infected and received PBS (infected only) or an irrelevant MAB (A4). Each bar represents the average count of fungi in the organ, and error bars indicate SD. *, significant difference ($P < 0.05$, determined by analysis of variance and Tukey's honestly significant difference test) relative to the PBS control.

immunized with MAB 32H were pathologically similar to the infected control mice. In contrast, the animals that received MAB 3E had fewer epithelioid granulomas with a reduced number of yeast cells and large areas of preserved lung tissue (Fig. 6).

MAB treatment alters cytokine expression. Cytokine levels were detected in the lung tissue of i.t. infected mice treated with MAB 3E and 32H in both protocols. Groups of 10 mice were used in each protocol; all experiments were repeated twice with similar results. As shown in Table 1, with the first protocol, animals immunized with MAB 3E had higher levels of IFN- γ and reduced levels of IL-4 after 15 and 30 days of infection compared with those of the animals immunized with MAB 32H. The other cytokines (IL-10, IL-12, and TNF- α) showed less variability. With the second protocol, the levels of IL-12 after 45 days of infection and IFN- γ after 45 and 60 days of infection were higher than those of MAB 32H (Table 2).

Identification of the epitope of MAB 3E. The reactivity of MAB 3E was evaluated against a panel of gp43-derived peptides previously described (48). The analysis of the results suggested that the recognized epitope is within the sequence NHVRIPIGWAV (data not shown). The MAB 3E but not 32H

recognized this peptide using ELISA (Fig. 7). This peptide sequence was found to be conserved in the internal sequences of β -1,3-glucanases of *Aspergillus fumigatus*, *Aspergillus oryzae*, and *Blumeria graminis*.

DISCUSSION

The protective role of antibodies against fungal infections is in many cases controversial. Recently, several studies have established that some antibodies are protective against fungi (31, 41, 52). MABs or fragments of MABs have been approved for the clinical evaluation of cryptococcosis (21) and candidiasis (32). Murine MAB 18B7 directed against the capsular polysaccharide of *C. neoformans* was used to evaluate the safety and maximum tolerated dose of a therapeutic MAB. The study used human immunodeficiency virus-infected patients who had successfully been treated for cryptococcal meningitis. The MAB infusion had a half-life in the serum of approximately 53 h and reduced the fungal circulating antigen (21). In another study, a human recombinant MAB to heat shock protein 90 was used in the treatment of patients with invasive candidiasis (32). A consensus has now emerged that the inabil-

FIG. 6. Histopathology of representative lung sections from mice i.t. infected according to protocol 1 and euthanized at 30 days after infection. (A) Lung section from an untreated infected mouse; (B) lung section from a mouse infected and treated with MAB 32H; (C) lung section from an infected mouse treated with MAB 3E. Hematoxylin-eosin staining, $\times 10$ magnification.

TABLE 1. Protocol 1 cytokine levels in the lungs of mice in response to the passive transfer of MAbs 24 h before i.t. infection with *P. brasiliensis*

Cytokine	Days of infection	Cytokine level (pg/g of tissue) ^a				
		Sham	Infected only	Irrelevant MAb A4	Treated with MAb 3E	Treated with MAb 32H
IL-4	15	248.2 ± 63.1	248.7 ± 43.6	327.1 ± 56	102.8 ± 52 ^b	107.7 ± 49.1 ^b
	30	322.5 ± 70.7	380.4 ± 81.9	422.4 ± 87.8	120.6 ± 67 ^b	559.5 ± 76.9 ^b
IL-10	15	4,204 ± 269.4	6,786.9 ± 1,250.1	8,062.5 ± 926.4	11,027.8 ± 1,321.8 ^b	12,852 ± 1,560 ^b
	30	3,920 ± 330	9,033.3 ± 978.4	9,357.1 ± 1,077.8	15,804 ± 2,135 ^b	24,155 ± 1,398 ^b
IL-12	15	4,682.1 ± 303	8,648.6 ± 218.2	8,543.7 ± 743	12,829.9 ± 1,422.5 ^b	8,724.3 ± 1,403.4
	30	4,880 ± 335.8	7,598 ± 838	7,050 ± 765	6,535.3 ± 834.2	10,128.4 ± 988 ^b
IFN-γ	15	101 ± 13	300 ± 35.1	240 ± 38.3	1,100.8 ± 115.6 ^b	150.4 ± 87.8
	30	108 ± 10.1	260.9 ± 8.3	301.3 ± 50.8	780 ± 99.3 ^b	155.7 ± 93.4
TNF-α	15	2,142.8 ± 505	12,616.7 ± 918.7	11,725 ± 1,426.6	6,944.4 ± 1,387.5 ^b	4,217.9 ± 657.5 ^b
	30	2,680 ± 622.2	25,794.8 ± 2,492.2	22,857.1 ± 2,412	14,456.5 ± 2,224.4 ^b	11,662.2 ± 2,380.1 ^b

^a Values are means ± standard deviations of measurements from 10 animals per group. The experiment was repeated twice with reproducible results.

^b Statistically significant ($P < 0.05$, determined by analysis of variance and Tukey's honestly significant difference test) relative to the value for the untreated, infected mice.

ity of immune sera to mediate protection against fungi reflects inadequate amounts of protective antibody and/or the simultaneous presence of protective and nonprotective antibodies rather than a fundamental inability of antibody to protect against fungal pathogens (11).

The first evidence of an antibody-mediating protection against *P. brasiliensis* was described by de Mattos Grosso et al. (17). The authors showed that the passive transfer of two murine MAbs against a glycoprotein of 70 kDa, which is recognized in 96% of the sera from PCM patients, led to a significant reduction in the number of CFU in the lungs and fewer granulomas within the organs of experimentally infected mice (17). More recently, another MAb that recognizes a secreted 75-kDa protein with phosphatase activity inhibited *P. brasiliensis* growth in vitro and reduced lung CFU in vivo (52).

Although the MAbs to gp43 have not directly affected the growth of *P. brasiliensis*, the protective action of the MAbs was directly associated with their capacity to enhance phagocytosis. Since macrophages are effector cells capable of exerting yeast fungicidal effects, we investigated NO production in macrophages that had phagocytosed MAb-opsonized yeast cells. The NO results showed an increase for all "protective" MAbs, but

the response was variable, with MAb 3E demonstrating the most impressive effect. Previous data from our group and others clearly demonstrated that activated macrophages kill the fungus in vitro by the production of nitric oxide and hydrogen peroxide (7, 8).

Using a panel of six MAbs against gp43, including IgG2a (10D, 17D, 19G, and 32H) and IgG2b (3E and 21F), we identified the protective and nonprotective MAbs by the passive administration of the antibodies into mice infected i.t. or i.v. with *P. brasiliensis*. The presence of protective and nonprotective antibodies against the same antigen is a conceptually relevant finding that has previously been described for cryptococcosis (27). The histopathology of lung sections from i.t. infected animals treated with the protective MAb showed a reduction in the number of yeast cells and epithelioid granulomas in comparison with the untreated control mice.

The role of IFN-γ in mediating activated macrophages in a systemic fungal infection has been reported. Murine peritoneal macrophages activated by IFN-γ show enhanced fungicidal activity with both yeast and conidial forms of *P. brasiliensis* (7–9). The production of IL-10 has been associated with more severe disease in susceptible mice (19). Alveolar macrophages

TABLE 2. Protocol 2 cytokine levels in the lungs of mice in response to the passive transfer of MAbs 30 days after i.t. infection with *P. brasiliensis*

Cytokine	Days of infection	Cytokine level (pg/g of tissue) ^a				
		Sham	Infected only	Irrelevant MAb A4	Treated with MAb 3E	Treated with MAb 32H
IL-4	45	208.2 ± 73.1	589.2 ± 81.9	342.3 ± 36.2	2,151.6 ± 352 ^b	1,370.6 ± 134 ^b
	60	254.3 ± 85.7	862.8 ± 185.5	764.8 ± 107.8	1,633.2 ± 188 ^b	1,832.9 ± 287.6 ^b
IL-10	45	1,685.7 ± 252.5	2,541.9 ± 172.2	1,666.7 ± 250	3,245.4 ± 318.1 ^b	1,790.6 ± 240.6 ^b
	60	2,360 ± 494.7	2,760.2 ± 612.3	3,238.1 ± 798.4	3,583.3 ± 279 ^b	2,500 ± 839.9
IL-12	45	3,257.6 ± 370.4	4,912.5 ± 817.4	8,362.5 ± 1,969.6	21,337.9 ± 3,475.9 ^b	12,470.1 ± 1,815.5 ^b
	60	3,243.7 ± 1,885.6	6,987.4 ± 891.5	9,509.5 ± 3,249.4	14,699.3 ± 2,981.1 ^b	11,630.6 ± 2,350.8 ^b
IFN-γ	45	99.41 ± 33.1	280.9 ± 41.2	106.6 ± 29.2	1,328.6 ± 207.2 ^b	162.5 ± 42.1 ^b
	60	90.2 ± 25.4	382.4 ± 39.2	133.3 ± 33	1,229.8 ± 192.3 ^b	416.4 ± 59.3
TNF-α	45	1,980.5 ± 359	22,870.5 ± 1,057.6	22,100 ± 3,225.5	16,985.4 ± 1,698.2 ^b	18,798.5 ± 2,785.2 ^b
	60	2,010.5 ± 438.8	30,021 ± 3,777.8	26,789.2 ± 1,469.3	14,892.4 ± 2,001 ^b	21,364.7 ± 3,911.2 ^b

^a Values are means ± standard deviations of measurements from 10 animals per group. The experiment was repeated twice with reproducible results.

^b Statistically significant ($P < 0.05$, determined by analysis of variance and Tukey's honestly significant difference test) relative to the value for the untreated, infected mice.

FIG. 7. Reactivity of peptides NHVRIPIGYWAV(R-CONH₂), -(R-COOH), and P10 (QTLAIAHTLAIRYAN) with MAb 3E by ELISA. Microtiter plates were sensitized with 100 ng of each peptide, and the reaction was developed with MAb 3E. The diamond symbol indicates the background measurement with buffer alone, and the square symbol corresponds to the reactivity of the peptides against the irrelevant antibody.

from i.t. infected IL-4-deficient mice more efficiently control fungal growth than wild-type macrophages (34), and also IL-4-deficient mice have increased levels of pulmonary IFN- γ (34). The passive administration of irrelevant MAb, 3E, or 32H in i.t. infected BALB/c mice showed a mixed Th1/Th2 pattern of activation. Nevertheless, the cytokine changes associated with protective MAb administration were more Th1-like, given the increased levels of IFN- γ and IL-12 in the lungs of i.t. infected mice. A discreet but statistically significant increase of IL-10 and a reduction of IL-4 and TNF- α were noted compared with the levels of IL-10, IL-4, and TNF- α in the control infected mice. The passive transfer of MAb 32H induced lower levels of lung IFN- γ and IL-12 than MAb 3E treatment. In fungal disease, the effects of antibody treatment on cytokine production are poorly understood and may depend on several features, including the fungus, target organ, timing of fungal dissemination, and antibody quantity, isotype, and specificity (18, 39, 49). Antibody-mediated protection has been associated with lower levels of IFN- γ in A/J mice infected with *C. neoformans* (18), but both Th1- and Th2-related cytokines are necessary for antibody protection in cryptococcosis (3). Strong Th1 responses can result in reduced survival and tissue damage (22, 39). In our studies, we found that MAb treatment did not have the polarity of the Th1/Th2 response and that IFN- γ appears to be beneficial.

Attempts at determining the epitope of protective MAb 3E led to the sequence NHVRIPIGYWAV shared with the *A. fumigatus*, *A. oryzae*, and *B. graminis* internal sequences of β -1,3-glucanases. The opsonizing effect of MAb 3E could then involve the recognition of this peptide sequence in the gp43 accumulated on the cell wall of *P. brasiliensis*. In fact, gp43 is stored inside a vacuole, migrates to the plasma membrane, and spreads into the *P. brasiliensis* cell wall. The secretion of this antigen occurs at discrete sites along the cell surface (45).

Historically, protection against PCM has been attributed to a vigorous cellular immune response, whereas specific high levels of antibodies have been associated with disease severity, as observed in patients with acute and subacute forms. Here we

show that there are protective and nonprotective anti-gp43 antibodies and that the former play an important role in disease control. Antibodies against gp43 are found in almost 100% of patients with PCM (5). Probably, there is a low concentration of protective antibodies in the serum samples of these patients, and they are not sufficient to control the disease. To overcome this, passive immunization with MAbs to gp43 was used and shown to be protective in mouse models of PCM. The present work also raises the potential therapeutic use of the peptide carrying the B epitope of MAb 3E in active immunization adjuvant to chemotherapy and/or in association with a peptide sequence containing the T-cell epitope of gp43, known as P10, that elicits a strong cell-mediated immunity and is protective against experimental infection.

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Artigo 4

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Paracoccidioides brasiliensis Vaccine Formulations Based on the gp43-Derived P10 Sequence and the *Salmonella enterica* FliC Flagellin[▽]

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Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*. Anti-PCM vaccine formulations based on the secreted fungal cell wall protein (gp43) or the derived P10 sequence containing a CD4⁺ T-cell-specific epitope have shown promising results. In the present study, we evaluated new anti-PCM vaccine formulations based on the intranasal administration of *P. brasiliensis* gp43 or the P10 peptide in combination with the *Salmonella enterica* FliC flagellin, an innate immunity agonist binding specifically to the Toll-like receptor 5, in a murine model. BALB/c mice immunized with gp43 developed high-specific-serum immunoglobulin G1 responses and enhanced interleukin-4 (IL-4) and IL-10 levels. On the other hand, mice immunized with recombinant purified flagellins genetically fused with P10 at the central hypervariable domain, either flanked or not by two lysine residues, or the synthetic P10 peptide admixed with purified FliC elicited a prevailing Th1-type immune response based on lung cell-secreted type 1 cytokines. Mice immunized with gp43 and FliC and intratracheally challenged with *P. brasiliensis* yeast cells had increased fungal proliferation and lung tissue damage. In contrast, mice immunized with the chimeric flagellins and particularly those immunized with P10 admixed with FliC reduced *P. brasiliensis* growth and lung damage. Altogether, these results indicate that *S. enterica* FliC flagellin modulates the immune response to *P. brasiliensis* P10 antigen and represents a promising alternative for the generation of anti-PCM vaccines.

Paracoccidioidomycosis (PCM) is a granulomatous disease caused by the thermodimorphic fungus *Paracoccidioides brasiliensis*, which is prevalent in Brazil and other Latin American countries (5, 6, 33). Some individuals develop one of the two main clinical forms of PCM. The acute form is characterized by impaired cellular immunity, negative delayed-type hypersensitivity reactions, increased systemic proliferation of the fungus, and high mortality rate. The chronic form, ranging from mild to severe chronic disease, shows exacerbated host cellular immune responses and formation of granulomas containing fungal cells and may evolve to develop extensive sequelae, including fibrotic lesions and impairment of lung function (33, 39).

Vaccines against PCM are still not available for human use, but promising formulations have been experimentally tested during the last few years. Irradiated *P. brasiliensis* or cellular antigens fractionated by anion-exchange chromatography conferred partial protection against fungal proliferation in the murine model (11). The extracellular gp43 glycoprotein, the major diagnostic antigen of *P. brasiliensis*, is the most intensively studied component aimed at a vaccine for PCM control. Previous reports have shown that mice immunized with the purified protein, DNA, or anti-idiotypic monoclonal antibody

were partially protected against challenges by *P. brasiliensis* (28, 36, 37, 40). A 15-amino-acid peptide (QTLIAIHTLAIRYAN), designated P10, contains the gp43 immunodominant CD4⁺ T-cell-specific epitope presented by major histocompatibility complex class II molecules from three different mouse haplotypes (37) and most human HLA-DR alleles (17, 18). Indeed, parenteral immunization with P10 in complete Freund adjuvant (CFA), or in the form of a truncated multiple-antigen peptide (MAP) complex, induced protective Th1 cellular immune responses in mice against intratracheal (i.t.) challenge with a virulent *P. brasiliensis* isolate (37, 38, 41).

The rational use of vaccines has been significantly improved after elucidation of innate immune mechanisms in mammalian cells. The recognition of distinct pathogen-associated molecular patterns by members of the Toll-like receptor (TLR) family initiates a signaling cascade mediated by adaptor proteins, including MyD88 and interleukin-1 (IL-1) receptor-associated kinase, that culminates in the production of proinflammatory cytokines, such as tumor necrosis factor alpha and IL-12, and increased expression of cell surface molecules involved in epitope presentation by antigen-presenting cells (APC) (1, 19). Proper APC activation by TLR agonists represents a key step for an effective adaptive immune response induced by pathogens or vaccines and explains, at least in part, the marked adjuvant effects of several bacterial molecules, including lipopolysaccharides, lipoproteins, peptidoglycan fragments, and flagellins (2).

Flagellin, the structural subunit of bacterial flagellum, is a

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>Salmonella</i> Dublin		
SL5928	<i>aroA his fliC::Tn10</i>	24
SL5930	SL5928 with pLS408; mobile	24
SLP10	SL5928 with pLSP10	This study
SLP10L	SL5928 with pLSP10L	This study
<i>P. brasiliensis</i>		
Pb18	Highly virulent human isolate of <i>P. brasiliensis</i>	35
Plasmids		
pLS408	pUC19 derivative carrying the <i>fliCd</i> gene from <i>S. München</i> with 48-bp deletion in the hypervariable region of the <i>FliC</i> gene <i>bla</i>	24
pLSP10	pLS408 derivative with a 45-bp insert encoding the P10 epitope	This study
pLSP10L	pLS408 derivative with a 57-bp insert encoding the P10 epitope with two flanking lysines	This study

highly conserved protein that induces TLR5-dependent inflammatory responses and exerts strong adjuvant effects on both antibody and cellular immune responses (12, 13, 30). Flagellin has been successfully used as a vaccine adjuvant to generate antigen-specific antibodies and T cells either when administered to mice as the native purified protein (4, 14, 20, 27) or as a hybrid protein genetically fused to the target antigen (10, 15, 16). Additionally, in contrast to other vaccine adjuvants, such as CFA, flagellin may exert strong adjuvant effects following administration through mucosal routes (14, 27).

In the present study, we have evaluated the adjuvant effects and protective efficacy of intranasal (i.n.) anti-PCM vaccine formulations based on *Salmonella enterica* serovar Dublin FliC flagellin and purified recombinant *P. brasiliensis* gp43 or the synthetic P10 peptide. In addition, recombinant chimeric flagellins genetically fused to P10 were also tested as potential anti-PCM vaccine antigens. The results demonstrate that *S. enterica* FliC flagellin modulates the murine immune system favoring either the generation of antibodies (gp43 plus FliC) or activation of cellular immune responses. In accordance with the administered vaccine formulation, mice challenged with *P. brasiliensis* were differentially protected against exacerbated fungal proliferation. The present results indicate that *Salmonella* FliC has an important role in the generation of mucus-delivered anti-*P. brasiliensis* peptide-based vaccine formulations. Furthermore, flagellin-based adjuvants may contribute to the understanding of immune mechanisms involved in PCM development.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. The strain *Salmonella enterica* pv. Dublin SL5928 is a nonflagellated *aroA* attenuated vaccine strain (34). *Salmonella* pv. Dublin SL5930 is a SL5928 derivative transformed with the plasmid pLS408 encoding the gene *fliCd*, originally derived from *Salmonella*

enterica serovar München, with a 48-bp deletion generated after removal of a EcoRV-EcoRV fragment from the central hypervariable domain leading to a single EcoRV site used for in-frame insertion of nucleotide sequences encoding heterologous epitopes. All *S. Dublin* and *Escherichia coli* strains were routinely cultivated at 37°C with shaking (200 rpm) in Luria-Bertani (LB) broth or on LB agar plates supplemented with ampicillin (100 µg/ml).

Fungal strain. The virulent *P. brasiliensis* Pb18 strain was maintained by weekly passages on solid Sabouraud medium at 36°C. Before experimental infection, cultures were grown in modified McVeigh-Morton medium at 36°C for 5 to 7 days (31). The fungal cells were washed in phosphate-buffered saline (PBS; pH 7.2) and counted in a hemocytometer. The upper part of cell suspensions that contained isolated or single budding cells was used in the infection experiments and for cell counting after decanting the clusters of yeast cells. The viability of fungal suspensions was determined by staining with trypan blue (Sigma, St. Louis, MO) and was always higher than 90%. The virulence of the Pb18 strain was checked in each experiment by infecting BALB/c mice i.t. and recovering the yeast cells from the infected organs.

Generation of hybrid flagellins genetically fused to P10. Chimeric *Salmonella* flagellins were generated after cloning the complementary oligonucleotides into the single EcoRV site of the pLS408-cloned FliC-encoding gene (24). Complementary 45-base oligonucleotides P10fw (5'-GAA ACC CTG ATT GCG ATT CAT ACC CTG GCG ATT CGC TAT GCG AAC-3') and P10rv (5'-CTT TGG GAC TAA CGC TAA GTA TGG GAC CGC TAA GCG ATA CGC TTG-3'), encoding P10 (QTLIAHTLAIRYAN), were melted at 65°C, annealed by slowly cooling at room temperature, and blunt end ligated with T4 DNA ligase to the EcoRV-cleaved pLS408. The same procedure was repeated with P10KKfw and P10KKrv oligonucleotides that, in addition to the sequence encoding the P10 peptide, carried on both 5' and 3' ends the sequence "AAA AAA," encoding two additional lysine residues flanking the heterologous epitope (KKQTLIAHTLAIRYANKK) genetically fused to FliC flagellin, in order to improve the proteolytic processing by cathepsin B and enhance epitope processing and presentation by APC (42). The resulting plasmids (pLSP10, encoding the recombinant FliC genetically fused to P10, and pLSP10L, in which the P10 peptide is flanked by two lysine residues) were introduced by electroporation (using 0.2-cm electroporation cuvettes at 600 Ω, 25 µF, and 1.75 kV; Gene-Pulser [Bio-Rad, Hercules, CA]) into *E. coli* strain DH5α, and transformants were selected on LB plates containing ampicillin. Recombinant plasmids with the right inserts were screened by EcoRV digestion and sequenced with the BigDye Terminator DNA sequencing kit (PerkinElmer Applied Biosystems, Waltham, MA) using a 15-mer primer (5'-CCA GGT GCC TAC ACC CCG-3') corresponding to a sequence located 50 bp downstream of the EcoRV insertion site in pLS408. The recombinant plasmids encoding *fliCd* genetically fused to P10 or the sequence flanked by two additional lysines were named pLSP10 and pLSP10L, respectively. Finally, the plasmids pLSP10 and pLSP10L were introduced into the flagellin-negative *S. Dublin* SL5928 strain by electroporation and the recombinant vaccine strains named SLP10 and SLP10L, respectively.

Purification of *Salmonella* flagellins. *Salmonella* flagellins, comprising FliC, FliCd-P10, and FliCd-P10L, were harvested from the respective *S. Dublin* SL5930, SLP10, and SLP10L strains cultivated in LB broth, according to a previously described procedure (4). Briefly, flagellins were obtained after centrifugation of cells, suspended in PBS (pH 7.4), and sheared in a bench mixer at maximal speed (a 1-min treatment repeated three times), followed by another centrifugation step to remove the bacterial cells. Broken flagellar fragments were precipitated with acetone, suspended in PBS, and finally, submitted to heat treatment (65°C for 30 min) to dissociate the flagellin monomers. The protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL), and the purity of the protein preparations was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Removal of contaminating lipopolysaccharide was accomplished with Detoxi-Gel columns (Pierce, Rockford, IL) according to the manufacturer's instructions. Endotoxin levels, determined with the chromogenic *Limulus* amoebocyte lysate assay (Cambrex Bio Science, Walkersville, MD), were always below 3.0 endotoxin units/µg of protein.

Purification of gp43 antigen. *P. brasiliensis* Pb18 was cultivated in yeast Sabouraud liquid medium for 7 days at 36°C with shaking. The culture was inactivated by adding 0.2 g of thimerosal (Merthiolate)/liter filtered through filter paper, concentrated in a vacuum at 40°C, and dialyzed against distilled water. Purification of gp43 was obtained by affinity chromatography on Affi-Gel (Bio-Rad, Hercules, CA) bound to anti-gp43 monoclonal antibody as previously described (29). Elution was carried out with 50 mM citrate buffer, pH 2.8. The eluate was concentrated in Amicon 10K cells, and the antigen preparation was monitored by SDS-PAGE revealed with silver staining. The protein content was determined by the Bradford method (3).

FIG. 1. Characterization of the specific serum IgG subclass response elicited in mice i.n. immunized with gp43 or gp43 admixed with FliC. (A) Anti-gp43-specific IgG subclass detected 1 week after the last immunization dose. (B) Anti-gp43-specific IgG subclass detected in vaccinated mice 2 months after i.t. challenge with the Pb18 strain. gp43, mice immunized with 3 doses of purified gp43; gp43 + FliC, mice immunized with purified gp43 admixed with FliC. For the other groups (FliCd, FliCd-P10, FliCd-P10L, and P10 + FliCd), a gp43-specific antibody response was not detected. Values are means of endpoint titers plus standard deviations for serum pools ($n = 8$) prepared from each mouse group. Data are representative of two independent experiments with similar results. The IgG1/IgG2a ratio of each immunization group is indicated at the top of the figure. Asterisks indicate a statistically significant difference observed between mice immunized with gp43 and mice immunized with gp43 admixed with FliC ($P \leq 0.01$).

SDS-PAGE. SDS-PAGE was performed following standard procedures using a Mini-Protein II vertical electrophoresis unit (Bio-Rad, Hercules, CA). Proteins sorted in 12% polyacrylamide gels were stained with Coomassie blue.

Immunization regimens. Isogenic pathogen-free 8- to 12-week-old male BALB/c ($H-2^d$) mice were supplied by the Isogenic Mouse Breeding Facility of the Department of Parasitology, Institute of Biomedical Sciences (ICB), University of São Paulo (USP). Animal handling was carried out in accordance with the Brazilian code for use of laboratory animals and was approved by the Ethics Committee of ICB, USP. Groups of 8 to 10 animals were immunized via the i.n. route with 15 μ g of each fusion protein (FliCd-P10 or FliCd-P10L) or with a mixture of 20 μ g P10 peptide or 25 μ g gp43 protein with 5 μ g FliC flagellin. Control groups were immunized with sterile PBS, 25 μ g of gp43, 5 μ g FliC flagellin, or 20 μ g of P10 peptide in Freund adjuvant 1:1 (vol/vol). The formulations were instilled into the nostrils (5 μ l/nostril) with a micropipette on days 0, 21, and 28. The control group immunized with P10 in Freund adjuvant received 1 dose (20 μ g of the peptide plus CFA in a total volume of 50 μ l) via the subcutaneous route (rear footpads) and 3 doses (20 μ g of the peptide in incomplete Freund adjuvant; a total volume of 200 μ l) administered intraperitoneally at 1-week intervals. Immune responses were evaluated 7 days after immunization and 60 days after fungal challenge.

Challenge with *P. brasiliensis* yeast cells. Eighty days after the last immunization, mice were inoculated i.t. with 3×10^5 yeast cells/animal of virulent *P. brasiliensis* Pb18 grown on Sabouraud agar and suspended in sterile saline (0.85% NaCl). A maximum of 50 μ l was inoculated per mouse. Briefly, mice were anesthetized intraperitoneally with 200 μ l of a solution containing 80 mg of ketamine/kg of body weight and 10 mg/kg of xylazine (both from União Química Farmacêutica, Brazil). After approximately 10 min, the necks were extended and the tracheas exposed at the level of the thyroid. For the i.t. inoculation, a 26-gauge needle was used and the incisions were sutured, right afterwards, with a 5 to 0 silk thread.

Fungal burden in organs of infected mice. Mice were sacrificed 60 days after i.t. infection, and the fungal burden was measured by CFU. Sections of the lungs, livers, and spleens were removed, weighed, and homogenized using a tissue grinder in 10 ml of sterile PBS. The corresponding pellets were resuspended and homogenized each in 1 ml of PBS. A 100- μ l sample of this suspension was plated on solid brain heart infusion medium supplemented with 4% fetal calf serum (Gibco, NY), 5% spent *P. brasiliensis* (strain 192) culture supernatant, streptomycin/penicillin (10 IU/ml) (Cultilab, Brazil), and cycloheximide (500 mg/ml) (Sigma, St. Louis, MO). Petri dishes were incubated at 36°C for at least 20 days, and colonies were counted (1 colony = 1 CFU) (35).

Lung histopathology. Following immunization with different vaccine formulations, BALB/c mice were i.t. infected and sacrificed after 2 months. The lungs were excised, fixed in 10% buffered formalin, and embedded in paraffin for sectioning. The sections were stained with hematoxylin-eosin and examined microscopically (Optiphot-2; Nikon, Tokyo, Japan).

Cytokine determination by enzyme-linked immunosorbent assay. Cytokine analysis was performed 7 days after the immunizations and 2 months after infection of the animals. Lung sections (right and left alternating) of mice were homogenized in 2 ml of PBS in the presence of protease inhibitors (Boehringer Mannheim, Indianapolis, IN). The homogenates were centrifuged, and the supernatants were frozen at -80°C until tested. The supernatants were assayed for IL-4, IL-10, IL-12, and gamma interferon (IFN- γ) using enzyme-linked immunosorbent assay kits (BD PharMingen, San Diego, CA). The detection limits of such assays were as follows: 7.8 pg/ml for IL-4, 31.25 pg/ml for IL-10 and IFN- γ , and 62.5 pg/ml for IL-12p40, as indicated by the manufacturer.

Statistical analysis. Data were analyzed by one-way analysis of variance and Student's *t* test followed by Tukey's honestly significant difference test and Dunnett's multiple comparison tests to compare the differences between the mean values of the immunization groups studied.

RESULTS

Mice immunized with purified gp43 admixed with *Salmonella* FliC flagellin develop specific antibody responses. Male BALB/c mice received three i.n. doses of purified gp43 alone or admixed with purified FliC, and the serum gp43-specific immunoglobulin G (IgG) responses were measured 7 days after the last immunization and 60 days after the i.t. challenge with *P. brasiliensis* strain Pb18. Enhanced gp43-specific IgG1 antibodies were detected in noninfected mice immunized with the FliC-containing vaccine formulation (IgG1 titer, $3,804 \pm 28.83$) but not in animals immunized with purified gp43 (IgG1 titer, 207 ± 75.12) (Fig. 1A). A further 6.2-fold increase in the anti-gp43 serum IgG1 was detected in vaccinated mice following challenge with Pb18 (average IgG1 titer, $23,924 \pm 21.00$) in mice immunized with the FliC-containing vaccine formulation

FIG. 2. Generation of recombinant hybrid flagellins genetically fused to the CD4⁺ T-cell-specific gp43-derived P10 epitope. (A) Schematic representation of the recombinant flagellins after P10 in-frame insertion at FliC central hypervariable domain. The two recombinant flagellins carried the P10 peptide (FliCδ-P10) or the P10 peptide with lysine residues on each side of the fusion site (FliCδ-P10L). (B) Coomassie blue-stained 12% polyacrylamide gel loaded with flagellins extracted from different *Salmonella* strains. Lane 1, molecular mass markers (Fermentas); lane 2, FliCδ flagellin extracted from *S. Dublin* strain SL5930 (with no insert); lane 3, FliCδ-P10 flagellin extracted from *S. Dublin* strain SLP10; lane 4, FliCδ-P10L flagellin extracted from *S. Dublin* strain SLP10L; lane 5, purified gp43. Each well was loaded with approximately 2 µg of purified flagellins or gp43.

(Fig. 1B). Determination of the specific IgG subclasses elicited in mice immunized with gp43/FliC vaccine formulation revealed a biased type 2 response with IgG1/IgG2 ratios of 7.5 and 14.3 before and after the fungus challenge, respectively (Fig. 1). These results indicate that *Salmonella* FliC flagellin acted as a strong antibody-inducing adjuvant when admixed with purified *P. brasiliensis* gp43 following mucosal administration in BALB/c mice.

Generation of vaccine formulations based on P10 genetically fused or admixed with *Salmonella* FliC. Additional anti-*P. brasiliensis* vaccine formulations were tested and employed the P10 peptide in combination with FliC. The first approach relied on the generation of hybrid flagellins in which the P10 minigene was wedged in frame at the central hypervariable domain of FliC, a permissive insertion site that did not affect both the inflammatory and adjuvant properties of *Salmonella* flagellin (Fig. 2A). The resulting recombinant *S. Dublin* SLP10 (encoding FliCδ-P10) and SLP10L (encoding FliCδ-P10L) strains were nonmotile in semisolid agar plates but expressed abundant extracellular flagella composed by hybrid flagellins with slightly altered molecular weights, as demonstrated by SDS-PAGE of purified flagella (Fig. 2B). After purification, the flagellin preparations were >95% pure and endotoxin levels were below 3.0 endotoxin units/µg of protein. Another

P10-based vaccine formulation involved admixing of purified native FliC (5 µg) with the synthetic P10 peptide (20 µg).

Cytokine expression patterns in mice immunized with different vaccine formulations containing *Salmonella* FliC flagellin. The cytokine (IL-4, IL-10, IL-12, and IFN-γ) expression pattern in the lung tissue of mice immunized with the different vaccine formulations was determined 7 days after the last immunization dose and 60 days following the i.t. challenge with *P. brasiliensis* Pb18 yeast forms. As shown in Table 2, the concentrations of IL-4 and IL-10 were low in lung extracts from all immunized groups except in animals immunized with purified gp43 admixed with FliC, in which IL-4 and mainly IL-10 appeared in higher concentrations than in nonimmunized mice or mice vaccinated with FliC only. This picture changed considerably when the same cytokines were measured in vaccinated mice 2 months after the challenge with the Pb18 strain. The IL-4 and IL-10 concentrations in mice immunized with gp43 were approximately 30% lower than the values detected in nonimmunized animals, but the addition of FliC enhanced production of cytokines to levels similar to those found in the nonimmunized control group. Mice immunized with the recombinant hybrid P10/flagellins had IL-4 and IL-10 levels similar to animals immunized only with gp43 or FliC. On the other hand, mice immunized with FliC admixed with the synthetic

TABLE 2. Cytokine levels detected in lungs of mice immunized with different vaccine formulations^a

Vaccine group	Level (ng/ml) (mean ± SD) of cytokine:							
	IL-4		IL-10		IL-12		IFN-γ	
	BC	AC	BC	AC	BC	AC	BC	AC
PBS	0.08 ± 0.03	6.22 ± 0.41	0.07 ± 0.07	14.42 ± 1.95	0.25 ± 0.44	25.90 ± 3.86	0.05 ± 0.03	4.37 ± 0.53
FliCδ	1.50 ± 0.24	2.93 ± 0.64	2.76 ± 0.29	7.52 ± 1.97	5.33 ± 1.34	22.00 ± 1.56	1.23 ± 0.07	9.96 ± 1.08
gp43	0.20 ± 0.01 ^b	4.77 ± 0.55 ^b	0.18 ± 0.10 ^b	10.25 ± 2.09	3.07 ± 0.46 ^b	30.07 ± 1.44 ^b	0.66 ± 0.09 ^b	12.62 ± 0.44 ^b
gp43 + FliCδ	3.01 ± 0.82 ^b	6.05 ± 1.16 ^b	4.10 ± 0.39 ^c	14.40 ± 4.90 ^b	7.46 ± 0.46 ^b	36.24 ± 2.05 ^c	2.25 ± 0.49 ^b	20.61 ± 4.13 ^c
FliCδ-P10	0.82 ± 0.56	3.21 ± 0.83	2.39 ± 0.15	9.48 ± 2.30	4.38 ± 0.97	30.67 ± 3.10 ^b	1.26 ± 0.25	4.89 ± 1.05
FliCδ-P10L	0.60 ± 0.30 ^b	3.16 ± 0.22	1.00 ± 0.70 ^b	8.95 ± 0.93	3.65 ± 0.83	22.76 ± 2.26	0.44 ± 0.06 ^b	7.16 ± 1.40 ^b
P10 + FliCδ	1.50 ± 0.49	2.80 ± 0.06	1.47 ± 0.88 ^b	1.46 ± 0.66 ^b	9.40 ± 0.86 ^c	36.63 ± 2.37 ^c	2.35 ± 0.19 ^b	18.10 ± 0.25 ^b

^a Immunization groups (5 to 8 animals/group) are as described in the legend to Fig. 2. BC, before challenge; AC, after challenge.

^b *P* < 0.05; statistically significant difference relative to that of mice immunized only with the FliCδ flagellin.

^c *P* < 0.01; statistically significant difference relative to that of mice immunized only with the FliCδ flagellin.

FIG. 3. Cytokine relative ratios measured in lungs of mice after challenge with the Pb18 strain. All cytokines were measured in whole extracts of lung cells. Immunization groups and specific cytokine values used in the cytokine ratio determination were as depicted in Table 2.

P10 peptide had IL-4 and IL-10 values equal to those in non-challenged vaccinated animals, which were statistically different from the values in animals immunized only with FliC.

Measurements of IL-12 and IFN- γ in vaccinated mice showed that all tested vaccines led to enhanced cytokine expression in lung cells, but statistically significant differences with regard to nonimmunized animals were observed only in animals immunized with gp43 and P10 admixed with FliC (Table 2). Interestingly, there was no significant enhancement in IL-12 and IFN- γ levels detected in mice immunized with the recombinant flagellins compared to mice immunized only with FliC, both before and after i.t. challenge with Pb18. However, mice immunized with the P10 and FliC mixture, as well as those immunized with gp43 plus FliC, produced enhanced IL-12 and IFN- γ levels similar to those detected in mice immunized with gp43 admixed with FliC. On the other hand, the IL-10 levels of P10 plus FliC-vaccinated mice remained lower than those detected in mice immunized with FliC, particularly after challenge with Pb18. Collectively, these data suggest that the vaccine formulation based on P10 admixed with FliC induced a predominant Th1 immune response compared to mice immunized with the other tested vaccine formulations. Determination of the IFN- γ /IL-4 (or IFN- γ /IL-10) and IL-12/IL-4 (or IL-12/IL-10) ratios clearly demonstrated that mice immunized with P10 admixed with FliC developed a more pronounced Th1-biased immune response compared to animals immunized with the other vaccine formulations (Fig. 3).

Growth of *P. brasiliensis* in lung tissues of mice vaccinated with the different vaccine formulations. The protective effects of the vaccine formulations were determined 2 months after the i.t. challenge with the virulent Pb18 strain. CFU counts were determined in homogenized lung tissue from vaccinated animals as well as from nonimmunized mice and those inoculated only with purified FliC (Fig. 4). In mice i.n. immunized with gp43 only, there was a significant reduction in the number of fungal colonies compared to the nonimmunized (PBS) or the FliC-immunized groups, in agreement with previously described results based on parenteral immunizations (37). Mice immunized with gp43 admixed with FliC, however, showed enhanced fungal proliferation in the lung tissues compared to the nonimmunized (PBS) or the FliC-immunized groups. Mice immunized with the hybrid flagellins (FliCd-P10 or FliCd-P10L) showed a reduction in the number of viable yeast cells similar to that observed in mice immunized with gp43 without adjuvant. Furthermore, mice immunized with the vaccine formulation prepared with the synthetic P10 peptide admixed with FliC had a drastically reduced number of viable fungal cells recovered from their lung tissues (<100 CFU/g lung tissue) 2 months after challenge with the Pb18 strain. In contrast to mice immunized with gp43 or gp43 plus FliC, no viable yeast cells were recovered from spleen and liver homogenates from mice vaccinated with P10 admixed with FliC or the hybrid flagellins (data not shown).

FIG. 4. Detection of viable fungal cells in lung tissues of vaccinated mice after i.t. challenge with *P. brasiliensis* Pb18. (A) Detection of viable fungi in mice i.n. immunized with purified gp43 or gp43 admixed with FliC. Immunization groups are as described in the legend to Fig. 2. (B) Detection of viable fungal cells in mice i.n. immunized with P10 epitope genetically fused with flagellin (FliCd-P10 or FliCd-P10L) or synthetic P10 peptide admixed with FliC flagellin. PBS, mice immunized only with PBS; FliCd, mice immunized only with FliC; FliCd-P10 and FliCd-P10L, mice immunized with purified recombinant FliCd-P10 and FliCd-P10L, respectively; P10+FliCd, mice immunized with P10 admixed with FliC. All mice groups were i.t. challenged with the Pb18 strain and sacrificed 2 months later for determination of CFU in homogenized lung tissue. The same experiments were repeated three times. Each bar represents the medium number plus standard deviation in organs collected from 8 to 10 animals in each group. Asterisks indicate statistically significant differences between results detected in mice immunized with gp43 and P10 and those in mice immunized only with FliC (*, $P \leq 0.05$; **, $P \leq 0.01$).

Histological analysis of lung tissue damage in immunized mice challenged with *P. brasiliensis*. Histopathological analyses of lung tissue samples collected from mice submitted to different vaccine formulations showed that mice immunized with gp43 admixed with FliC had extensive tissue destruction, with abundant cellular infiltration (Fig. 5B). Exudative epithelioid granulomas containing giant cells with multiplying fungal cells were also observed in this vaccination group to a higher extent than in nonimmunized animals (Fig. 5A). Mice immunized with the hybrid recombinant FliCd-P10 and FliCd-P10L flagellins showed lower numbers of exudative lesions than those detected in animals immunized with FliC only or nonimmunized animals (Fig. 5C). The number of visible, detectable, and viable fungi was also lower than in the control groups. In contrast, lung tissues of mice immunized with P10 admixed with flagellin were virtually devoid of granulomas and viable fungal cells. The lung tissues of mice immunized with P10 plus FliC showed preserved alveolar organization with no indication of phagocytic cell infiltration (Fig. 5D).

DISCUSSION

In the present study, mucus-delivered vaccine formulations based on the *Salmonella* FliC flagellin revealed contrasting, but rather interesting, results regarding induction of specific immune response and prophylactic protection against a virulent *P. brasiliensis* strain in the murine model. While i.n. administration of purified gp43 admixed with FliC induced a Th2-biased immune response, leading to exacerbated fungal multiplication and tissue damage, vaccine formulations based on hybrid flagellins genetically fused with the gp43-derived P10 sequence, and mainly purified FliC admixed with synthetic P10 peptide, resulted in a Th1 predominant immune response and

FIG. 5. Representative histopathology of lung lesions caused by *P. brasiliensis* strain Pb18 in mice immunized with different vaccine formulations. Tissue samples were collected 2 months after i.t. challenge with strain Pb18. (A) Lung section from PBS-immunized mouse with granuloma containing multiple viable fungal cells. (B) Lung section from a mouse immunized with gp43 admixed with FliC. Observe the extensive granulomatous lesions with intense cellular infiltration and large number of multiplying fungal cells. (C) Lung section from mouse immunized with the hybrid FliCd-P10L. (D) Lung section from mouse immunized with P10 admixed with FliC showing preserved alveolar structure and absence of granulomatous lesions and fungal cells. All sections were amplified 40-fold and stained with hematoxylin-eosin.

enhanced protection to the fungal challenge in vaccinated mice. The present evidence represents the first attempt to develop experimental anti-PCM mucosal vaccines and extends the knowledge about *Salmonella* flagellin adjuvant effects associated with different antigens.

The mucus-delivered vaccines have several advantages over conventional parenteral vaccines. For example, mucosal vaccines are easier to administer, lack iatrogenic infection risks, and more importantly, induce broader immunity, including activation of both systemic and local immune responses, a feature that may be particularly relevant for airborne infections. Therefore, the incorporation of *Salmonella* flagellin, a potent adjuvant known to act both parenterally and at mucosal sites, to P10-based vaccine formulations represented an alternative to the Freund adjuvant previously used in other anti-PCM vaccines. Indeed, it opens a renewed perspective for a future clinical use.

The epitope-based vaccine concept was designed as a strategy to preserve antigen immunogenicity but avoid potential undesirable effects, such as activation of suppressive immune responses or induction of self-reacting antibodies (21). The low immunogenicity of synthetic peptides, which represents a major drawback in the development of effective peptide-based vaccines, stimulated parallel experimental procedures, such as the use of potent adjuvants, synthesis of tandem repeats or MAPs, and genetic fusion with carrier proteins (25). In the present study, we have shown that the combination of the synthetic P10 peptide and the *Salmonella* FliC flagellin elicited strong activation of CD4⁺ T-cell-dependent immune responses leading to the efficient control of fungal infection in vaccinated mice. The best results were achieved with the pep-

tide admixed with the adjuvant, thus avoiding complex and expensive chemical synthetic procedures or generation and purification of hybrid peptides by genetic engineering methods. The rather promiscuous binding of P10 to several major histocompatibility complex class II molecules, both from mice and humans (17, 37), in combination with the strong mucosal adjuvant effects of *Salmonella* flagellins resulted in enhanced vaccine efficacy, leading to more efficient control of fungus multiplication.

The genetic fusion of ovalbumin or the influenza virus M2 protein to *Salmonella* flagellins was required to generate antigen-specific B- and T-cell-dependent responses (15, 16). In our hands, coadministration of flagellin and the P10 peptide resulted in a higher Th1-biased immune response than that in mice immunized with hybrid flagellin genetically fused with P10. Linking antigens to flagellin would supply in a single molecule the signals required for activation and maturation of APC, but the present results based on a mucus-delivered formulation as well as other parenterally delivered vaccines (4, 23) clearly show that genetic fusion of flagellin to the target antigen does not represent a requirement for proper stimulation of the immune system by flagellin-containing vaccine formulations.

Quantification of IFN- γ and IL-12 indicated that mice immunized with P10 admixed with FliC developed a more pronounced Th1 immune response than mice immunized with the recombinant hybrid flagellins (FliCd-P10 and FliCd-P10L). Additionally, determination of the IFN- γ /IL-4 and IFN- γ /IL-10 ratios (as well as the IL-12/IL-4 and IL-12/IL-10 ratios) showed that mice immunized with FliC and P10 elicited a predominant Th1 immune response to other immunization groups, including mice immunized with gp43 and FliC and those immunized with the recombinant hybrid P10-containing flagellins. Indeed, induction of a Th1-biased immune response positively correlated with asymptomatic and mild forms of PCM in humans as well as resistance to *P. brasiliensis* infection in mice (9, 22, 26, 32). Additionally, the lack of anti-gp43 antibodies in animals immunized with the P10/FliC formulation further showed that immunization with P10 peptide avoids other gp43 sequences involved in nonprotective anti-*P. brasiliensis* immune responses. The possibility to add another gp43-derived peptide, which reacts with a protective monoclonal antibody (7), may further enhance the efficacy of the vaccine formulation by means of a proper Th1 response in combination with a protective antibody response.

Innate immunity has a pivotal role on the control of *P. brasiliensis* replication, as well as other microbial pathogens, in different mammalian hosts (8). TLR ligands directly interact with macrophages and dendritic cells, leading to inflammatory responses required for the direct elimination of the pathogen and generation of protective adaptive responses. In this study, we demonstrated that incorporation of *Salmonella* FliC, a TLR-5 ligand, may trigger anti-PCM immune responses ranging from complete prophylactic protection to exacerbated parasite multiplication according to the nature of the antigen tested, thus offering new tools for the understanding of the immunological mechanisms leading to resistance or sensitivity to *P. brasiliensis*. In addition, the evidence that the immunogenicity of epitope-based vaccines, when associated with *Salmonella* FliC, may elicit a protective immune response in vac-

inated mice raises new perspectives for the development of improved vaccine formulations and warrants further studies aimed at the prophylactic and therapeutic control of PCM.

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Artigo 5

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RESEARCH PAPER

Poly(lactic acid-glycolic acid) nanoparticles markedly improve immunological protection provided by peptide P10 against murine paracoccidioidomycosis

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Background and purpose: The present study reports on the preparation and testing of a sustained delivery system for the immunomodulatory peptide P10 aimed at reducing the *in vivo* degradation of the peptide and the amount required to elicit a protective immune response against paracoccidioidomycosis.

Experimental approach: BALB/c mice were infected with the yeast *Paracoccidioides brasiliensis* to mimic the chronic form of paracoccidioidomycosis. The animals were treated daily with sulfamethoxazole/trimethoprim alone or combined with peptide P10, either emulsified in Freund's adjuvant or entrapped in poly(lactic acid-glycolic acid) (PLGA) nanoparticles at different concentrations (1 µg, 5 µg, 10 µg, 20 µg or 40 µg·50 µL⁻¹). Therapeutic efficacy was assessed as fungal burden in tissues and the immune response by quantitative determination of cytokines.

Key results: Animals given combined chemotherapy and P10 nanotherapy presented a marked reduction of fungal load in the lungs, compared with the non-treated animals. After 30 days of treatment, P10 entrapped within PLGA (1 µg·50 µL⁻¹) was more effective than 'free' P10 emulsified in Freund's adjuvant (20 µg·50 µL⁻¹), as an adjuvant to chemotherapy. After treatment for 90 days, the higher doses of P10 entrapped within PLGA (5 or 10 µg·50 µL⁻¹) were most effective. Treatment with P10 emulsified in Freund's adjuvant (20 µg·50 µL⁻¹) or P10 entrapped within PLGA (1 µg·50 µL⁻¹) were accompanied by high levels of interferon-gamma in lung.

Conclusions and implications: Combination of sulfamethoxazole/trimethoprim with the P10 peptide entrapped within PLGA demonstrated increased therapeutic efficacy against paracoccidioidomycosis. P10 incorporation into PLGA nanoparticles dramatically reduced the peptide amount necessary to elicit a protective effect.

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Keywords: immunomodulatory peptide; antifungal therapy; biodegradable polymers; drug delivery; nanobiotechnology

Abbreviations: CFUs, colony-forming units; DMSA, dimercaptosuccinic acid; Gp43, 43 kDa glycoprotein; IFN-γ, interferon-gamma; IL-4, interleukin-4; IL-10, interleukin-10; IL-12, interleukin-12; MAP, multiple antigenic peptide; P10, peptide P10; PBS, phosphate-buffered saline; PCM, paracoccidioidomycosis; PGA, poly-glycolic acid; PLA, poly-lactic acid; PLGA, poly(lactic acid-glycolic acid)

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Introduction

Extensive research has focused on the design of efficient adjuvants to vaccines for immunological protection in several diseases. More recently, because of the dramatic increase in the incidence of systemic mycoses, attention has been given

to the development of antifungal vaccines (Cutler *et al.*, 2007). Peptide antigens, among other immunogens, are especially promising for triggering effective immune protective responses against these infections (Taborda *et al.*, 1998; Travassos *et al.*, 2008a). Incorporation of peptides into controlled release systems is an approach used to avoid degradation and promote their release at predetermined rates (Johansen *et al.*, 2000).

Biodegradable polymers are attractive delivery systems for vaccines because of their property of slowly and gradually controlling the release of an antigen (Commandeur *et al.*, 2006). After *in vivo* administration of biocompatible polymers, they are broken down into molecules that take part in normal metabolic pathways and are then eliminated. Any polymer-entrapped molecule (a drug or a peptide) is released upon polymer biodegradation, which depends on polymer constitution and occurs under certain conditions, such as pH and temperature changes (Commandeur *et al.*, 2006; Vicent and Duncan, 2006).

Polymeric systems, such as nano- and micro-particles, are appropriate as adjuvants to prepare a single-shot vaccine. Antigens encapsulated within polymers can be released for a prolonged period at a controlled rate of polymer degradation (Vicent and Duncan, 2006). Polymers have been shown to be effective adjuvants for various antigenic proteins, including ovalbumin, cholera and tetanus toxoid, and malarial and pneumotrophic bacterial antigens (Dhiman and Khuller, 1998; Jaganathan *et al.*, 2005). Poly(lactic acid-glycolic acid) (PLGA) is a polymer whose degradation rate and antigen release can be predicted (Jiang *et al.*, 2005). By releasing an immunomodulatory peptide at a predetermined rate, it can significantly reduce the amount and number of doses of antigen required for protection.

Taborda *et al.* (1998) identified a 15-amino acid peptide that carries the T-cell epitope of the glycoprotein 43 kDa glycoprotein (Gp43), the major diagnostic antigen secreted by *Paracoccidioides brasiliensis* (Puccia *et al.*, 1986). This peptide, named P10, elicits an interferon-gamma (IFN- γ)-dependent immune protection against experimental paracoccidioidomycosis (PCM; Taborda *et al.*, 1998; Travassos *et al.*, 2004). Immunization with P10 improved the therapeutic efficacy against PCM when combined with sulfamethoxazole/trimethoprim, among other antifungals, suggesting an important contribution of P10 in improving outcome and reducing the time of treatment against PCM (Marques *et al.*, 2006).

Paracoccidioidomycosis is a health problem in Latin America, where around 10 million individuals may be infected by the dimorphic human pathogenic fungus *P. brasiliensis* and 2% of them may develop acute or chronic forms of PCM (Brummer *et al.*, 1993). The acute form involves the lymphoreticular system and may be lethal; in chronic PCM, the lung is mainly affected with a granulomatous inflammatory response, which represents an effective defence against fungal spread (Brummer *et al.*, 1993; de Camargo and de Franco, 2000). The usual therapy for PCM is based on polyenes, azoles and sulphonamides. Amphotericin B is indicated in severe disseminated cases and must be followed by a prolonged treatment with azoles and sulfamethoxazole/trimethoprim (Brummer *et al.*, 1993; Lortholary *et al.*, 1999).

The present study reports on the design, preparation and *in vivo* testing of a sustained delivery system containing the immunoprotective peptide P10 loaded on nanoparticles of the biodegradable polymer PLGA. This preparation was administered in combination with sulfamethoxazole/trimethoprim during the treatment of a murine model of systemic PCM.

Methods

Preparation of PLGA particles loaded with P10 peptide

The particles were prepared using PLGA polymeric blends with 50:50 poly-lactic acid (PLA) : poly-glycolic acid (PGA). The polymers were first dissolved in dichloromethane and then added to an aqueous solution containing 1% polyvinyl alcohol and the P10 peptide, aiming for the final preparation to contain 1, 5, 10, 20 or 40 $\mu\text{g}\cdot 50\ \mu\text{L}^{-1}$ of the peptide. The mixture was submitted to vigorous agitation in a blender (10 000–15 000 rpm) to obtain the water-in-oil emulsification. The organic solvent was removed from the solution by stirring at room temperature and evaporation under reduced pressure. The particles were centrifuged (4–10°C; 1100–4600 $\times$ g.) for 10- to 20-min intervals. The preparation was washed three times in distilled water, suspended in 1.0 mL phosphate-buffered saline (PBS), stored at 4°C and used for up to 1 week. All procedures were performed in a sterile room with all the manipulations in a sterile hood.

Animals

All animal handling and experimental procedures performed in this study were approved by the Animal Care and Use Committee of the Universidade de São Paulo. Male BALB/c mice (6–8 weeks old) from the Universidade de São Paulo (USP), Brazil, were used in this study. Animals were housed in polypropylene cages under specific pathogen-free conditions and were provided with food and water *ad libitum*.

Fungal inoculum for in vivo experiments

P. brasiliensis, isolate Pb18, was used to infect the animals. The strain was sub-cultured in defined liquid medium McVeigh-Morton culture medium at 35°C in a rotatory shaker (220 rpm) (Restrepo and Arango, 1980). After 5 or 7 days of culture, the yeast cells were collected by centrifugation, the supernatant was discarded, and the cells were washed three times in sterile PBS, pH 7.4. We determined the cell count in a haemocytometer and adjusted the inoculum suspension to 3×10^5 viable fungi per 50 μL . The cellular viability was 90–95% in these experiments, as determined by vital staining with Evans blue (Sigma, St. Louis, MO, USA).

Intratracheal infection

To mimic the most common infection in the chronic form of PCM, BALB/c mice were inoculated intratracheally with *P. brasiliensis* Pb18, and after 30 days of infection, the animals were subjected to a combined therapy of sulfamethoxazole/trimethoprim and a range of doses of P10 (1–40 μg) within

PLGA. The animals were anaesthetized by intraperitoneal injection of 200 μL of a solution of 80 $\text{mg}\cdot\text{kg}^{-1}$ ketamine and 10 $\text{mg}\cdot\text{kg}^{-1}$ xylazine (União Química, Brazil). After 10 min, their necks were hyperextended and incised to barely expose the trachea. Each animal received 3×10^5 viable fungi in 50 μL PBS using a 26.5-gauge needle. The incisions were sutured immediately after the delivery of the fungi.

Antifungal treatment

The infected mice were randomly divided into nine groups of six animals each and subjected to antifungal treatment with sulfamethoxazole/trimethoprim (15 $\text{mg}\cdot\text{kg}^{-1}$ and 3 $\text{mg}\cdot\text{kg}^{-1}$ respectively) in PBS, pH 7.4, with or without 20 μg P10 solubilized in 50 μL Freund's adjuvant named 'free' or P10 entrapped within PLGA at 1 μg , 5 μg , 10 μg , 20 μg or 40 $\mu\text{g}\cdot 50\text{ }\mu\text{L}^{-1}$. Group (i) was the control group treated only with PBS, group (ii) was treated with sulfamethoxazole/trimethoprim alone, group (iii) was treated with sulfamethoxazole/trimethoprim and 50 μL of 'empty' PLGA nanoparticles, group (iv) was treated with 20 $\mu\text{g}\cdot 50\text{ }\mu\text{L}^{-1}$ of 'free' P10, and groups (v) (vi) (vii) (viii) and (ix) were treated with 1 μg , 5 μg , 10 μg , 20 μg and 40 $\mu\text{g}\cdot 50\text{ }\mu\text{L}^{-1}$, respectively, of P10 entrapped within PLGA. The treatment regimens started 30 days after fungal challenge and were continued for 30 days. The empty PLGA nanoparticles given to group (iii) were prepared using the same procedure, methodology and ratio amount of PLA : PGA (50:50) that was used to prepare the nanoparticles associated with P10, which gave the same final concentration in both preparations with or without P10. The particle size and size distribution were measured by laser light scattering using a particle size analyser (Zetasizer, Malvern, UK). Size distribution was analysed over the range of 1–1000 nm, and the mean diameter was calculated for each sample. The PLGA particles with and without peptide P10 at various concentrations were measured, and the average diameter found was 430 ± 5.1 nm for the loaded particle and 410 ± 4.9 nm for the empty one.

Animals from groups (ii) to (ix) received daily injections of sulfamethoxazole/trimethoprim (15 $\text{mg}\cdot\text{kg}^{-1}$ and 3 $\text{mg}\cdot\text{kg}^{-1}$ respectively). Mice from groups (iv) to (ix) received four doses of peptide P10 administered once a week during 4 weeks as follows: 20 $\mu\text{g}\cdot 50\text{ }\mu\text{L}^{-1}$ of 'free' or 1 μg , 5 μg , 10 μg , 20 μg and 40 $\mu\text{g}\cdot 50\text{ }\mu\text{L}^{-1}$ entrapped within PLGA. 'Free' P10 was administered with complete Freund's adjuvant in the hind paw, and P10 entrapped within PLGA at various concentrations was administered i.p. without adjuvant.

Fungal burden assay

The combined treatment effects were assessed by the residual lung, liver and spleen fungal burden. The animals from all experimental groups were killed by cervical rupture 30 and 90 days after the beginning of therapy, and the lungs, liver and spleen were aseptically removed and weighed. The organs were rinsed and homogenized in sterile PBS, pH 7.4, and 100 μL of the homogenates was cultured in BHI agar supplemented with 4% horse serum, 5% *P. brasiliensis* 192 culture filtrate, 10 000 IU penicillin (Cultilab, Brazil) and 10 $\text{mg}\cdot\text{L}^{-1}$ streptomycin (Cultilab, Brazil) in duplicate. The Pb192

culture filtrate was prepared according to a previously published method (Singer-Vermes *et al.*, 1992). The plates were incubated at 37°C and colony-forming units (CFUs) were counted 10 days post-plating to determine the $\text{CFU}\cdot\text{g}^{-1}$ of tissue.

Cytokine assays

To assess cytokine production, we assayed IFN- γ and interleukins-4 (IL-4), -10 (IL-10) and -12 (IL-12) in lung homogenates. Lung fragments were aseptically removed, weighed and homogenized in sterile PBS with protease inhibitor (Roche, USA). The cytokines were determined using commercial ELISA kits (BD Biosciences – Pharmingen, San Diego, CA, USA).

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 15 was used to analyse our data. All results are expressed as means $\pm$ standard error. A one-way analysis of variance (ANOVA) with Tukey's post-test was applied to test inter-group differences. Differences between paired groups were analysed by the Mann-Whitney test. *P*-values less than 0.05 were considered significant.

Materials

Sulfamethoxazole was purchased from Sigma (St. Louis, MO, USA) and trimethoprim from Ducto (Bac-sulfitrin, Ducto). The poly-lactic acid (PLA) and poly-glycolic acid (PGA) used to prepare the nanoparticles were purchased from Sigma (St. Louis, MO, USA). The P10 peptide was synthesized by the 9-fluorenylmethoxy-carbonyl technique (Huang *et al.*, 1993) and provided by Dr Maria A. Juliano from the Department of Biophysics of the Federal University of São Paulo. Drug and molecular target nomenclature conforms to the British Journal of Pharmacology Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Fungal burden in treated animals

To evaluate the efficacy of the combined therapy using the P10 peptide entrapped within PLGA in association with sulfamethoxazole/trimethoprim, we carried out *in vivo* antifungal drug experiments in BALB/c mice infected with the fungus *P. brasiliensis*, isolate Pb18. The treatment regimens started after 30 days of infection and were evaluated 30 and 90 days after the beginning of treatment. The fungal load was investigated in the lungs, liver and spleen of the animals. No significant number of fungi in the liver and a very low number in the spleen were detected 30 days after intratracheal (i.t.) infection (data not shown). This result was expected as the liver or/spleen infection is only detectable in the early stages of this animal model of mycosis. In contrast, the main focus of the infection was the lungs. A high load of fungal cells was recovered in the lungs of the control animals that received only PBS, both 30 and 90 days after beginning

Figure 1 Fungal burden recovery assessed by colony-forming units [CFU (g-lung-tissue)⁻¹] in mice infected with *P. brasiliensis* Pb18 and subjected to a combined therapy of sulfamethoxazole/trimethoprim (Sulfa + Trim; 15 mg·kg⁻¹ and 3 mg·kg⁻¹ respectively) and either 20 µg·50 µL⁻¹ P10 peptide solubilized in Freund's adjuvant ('free') or P10 peptide (1, 5 or 10 µg·50 µL⁻¹) entrapped within PLGA. Each bar represents the average CFU (g-tissue)⁻¹ with standard deviations. After 30 days of treatment, 1 µg·50 µL⁻¹ of P10-PLGA/Sulfa + Trim yielded the best response (lowest fungal CFU recovery) of all groups (+*P* < 0.05). After 90 days of treatment, no significant differences were found between the responses of the 'free' P10 (20 µg·50 µL⁻¹) and the P10-PLGA (5–10 µg·50 µL⁻¹)-treated groups (marked with #). At day 90, a significantly lower number of fungal cells were recovered from mice treated with 1 µg·50 µL⁻¹ P10-PLGA compared with the PLGA alone treated group (**P* < 0.05). PLGA, poly(lactic acid-glycolic acid).

the treatment (Figure 1). In the group treated only with sulfamethoxazole/trimethoprim, the infection was controlled during the first 30 days of treatment followed by a high number of fungal cells after 90 days, comparable to the untreated group (Figure 1).

A reduction of CFUs in comparison with untreated animals was confirmed using 20 µg of 'free' P10 emulsified in Freund's adjuvant (Marques *et al.*, 2006), reversing the relapse observed in the group treated only with sulfamethoxazole/trimethoprim. The dose of 20 µg used for the 'free' form of P10 peptide was chosen on the basis of earlier results (Taborda *et al.*, 1998; Taborda *et al.*, 2004). Under this condition, lymphoproliferative and cell-mediated immune responses protecting mice from the *P. brasiliensis* infection were observed. In the groups subjected to the combined therapy of sulfamethoxazole/trimethoprim and P10 entrapped within PLGA, there was always a significant protection. After 30 days of treatment, amounts in the range of 1–10 µg·50 µL⁻¹ of P10 encapsulated with PLGA were efficient as an adjuvant to chemotherapy, reducing the dose of the peptide necessary to decrease the fungal load and to avoid disease relapse by at least 20-fold. The groups that received 20 or 40 µg·50 µL⁻¹ of P10 entrapped within PLGA, did not present a marked additive action as adjuvants to chemotherapy (data not shown), although they responded better than the mice given sulfamethoxazole/trimethoprim alone. The PLGA nanoparticles without P10 showed a protective effect after 30 days, proving to be an adjuvant to chemotherapy by themselves, but these 'empty' nanoparticles were unable to prevent the relapse observed by sulfamethoxazole/trimethoprim treatment after 90 days of treatment. Thus, the fungal burden recovery was greater after treatment with empty nanoparticles than after any of the loaded nanoparticles of PLGA (containing 1, 5 or 10 µg·50 µL⁻¹ of P10 peptide; Figure 1).

Cytokine production induced by P10-PLGA

Cytokine production was examined by monitoring the levels of IFN-γ, IL-4, IL-10 and IL-12 levels in lung tissue homoge-

nates from the animals given the combined therapy of sulfamethoxazole/trimethoprim and peptide P10.

The data presented in Table 1 show the production of type 1 cytokines IL-12 and IFN-γ. The treatment with 20 µg of P10 emulsified in Freund's adjuvant or 1 µg of P10 entrapped within PLGA induced higher levels of IFN-γ than in the respective controls after 30 and 90 days of the treatment. IFN-γ is an important Th1 cytokine, which elicits protection against *P. brasiliensis* infection. The levels of IL-12 were not similarly affected. Table 1 also shows changes in the cytokines more characteristic of Th2 responses (IL-4 and IL-10). After 30 and 90 days of starting treatment, the group that received sulfamethoxazole/trimethoprim and 20 µg·50 µL⁻¹ of 'free' P10 emulsified in Freund's adjuvant showed a significant decrease of IL-10, thus confirming earlier results. P10 entrapped within PLGA at 1 µg also reduced IL-10 after 30 days and less after 90 days. Decrease of IL-10 with increasing P10 amounts in PLGA was less evident, and no decrease was observed in the levels of IL-4.

Discussion

In the present work, we examined the antifungal effects and the protection elicited by a therapy using sulfamethoxazole/trimethoprim combined with the immunostimulatory peptide P10 encapsulated into PLGA-dimercaptosuccinic acid (DMSA) for treatment of experimental PCM. The therapeutic efficacy and cytokine production were examined in a murine model of chronic PCM. Sulfamethoxazole/trimethoprim is a low-cost sulphonamide treatment that has been used in the initial therapy of non-disseminated cases of PCM (Lortholary *et al.*, 1999; Travassos *et al.*, 2008b) and it is recommended for long-term treatments, but several cases of fungal resistance and relapsing disease have been reported (Travassos *et al.*, 2008a).

The development of immunotherapies using peptides as adjuvant molecules is a promising procedure that could be

Table 1 Determination of cytokine production (pg·mL⁻¹) on days 30 and 90 after starting treatment with sulfamethoxazole/trimethoprim and P10

Treatment Groups	Th1				Th2			
	IL-12		IFN- γ		IL-4		IL-10	
	30	90	30	90	30	90	30	90
PBS	11.2 $\pm$ 2.5	6.2 $\pm$ 2.4	3.2 $\pm$ 1.1	1.8 $\pm$ 0.6	13.9 $\pm$ 0.5	11.4 $\pm$ 6.0	2.5 $\pm$ 0.7	3.7 $\pm$ 0.4 [#]
Sulfa + Trim	7.3 $\pm$ 0.7	6.4 $\pm$ 0.1	2.3 $\pm$ 0.3	2.5 $\pm$ 0.1	11.4 $\pm$ 2.6	11.2 $\pm$ 0.3	4.1 $\pm$ 0.2	3.7 $\pm$ 0.2 [#]
PLGA/Sulfa + Trim	6.7 $\pm$ 0.7	6.8 $\pm$ 2.0	1.3 $\pm$ 0.4	1.8 $\pm$ 0.2	10.7 $\pm$ 0.4	8.6 $\pm$ 0.2	2.8 $\pm$ 0.9	3.2 $\pm$ 0.4
Sulfa + Trim/Freund/P10: 20 μ g	10.9 $\pm$ 1.0	6.1 $\pm$ 1.9	7.5 $\pm$ 1.0	10.6 $\pm$ 2.4*	12.7 $\pm$ 1.2	9.7 $\pm$ 0.9	0.6 $\pm$ 0.2	0.6 $\pm$ 0.1
Sulfa + Trim/PLGA/P10: 1 μ g	7.0 $\pm$ 1.9	5.5 $\pm$ 2.7	5.1 $\pm$ 0.8	7.7 $\pm$ 1.1*	11.6 $\pm$ 0.5	15.1 $\pm$ 1.2	0.8 $\pm$ 0.3	2.1 $\pm$ 0.3
Sulfa + Trim/PLGA/P10: 5 μ g	8.4 $\pm$ 2.5	8.7 $\pm$ 3.5	1.0 $\pm$ 0.1	2.9 $\pm$ 1.2	17.9 $\pm$ 6.7	12.1 $\pm$ 2.2	2.0 $\pm$ 0.8	2.0 $\pm$ 0.5
Sulfa + Trim/PLGA/P10: 10 μ g	7.6 $\pm$ 3.4	13.4 $\pm$ 7.6	2.9 $\pm$ 0.5	3.0 $\pm$ 0.3	14.8 $\pm$ 0.5	17.8 $\pm$ 0.3	2.5 $\pm$ 0.4	1.9 $\pm$ 0.3 [#]

*Not significant between groups, #different for 10 μ g P10-PLGA compared with PBS and sulfamethoxazole/trimethoprim groups ($P < 0.05$).

Values are means $\pm$ standard deviations of measurements from each group.

Bold: show high levels of IFN- γ compared with the group treated with sulfamethoxazole/trimethoprim. These two groups also showed lower CFUs (see Figure 1). Italic: decrease in the production of IL-10 in the groups treated with free P10 and encapsulated P10 (1 μ g) compared with the group treated with sulfamethoxazole/trimethoprim.

CFUs, colony-forming units; IFN-gamma, interferon- γ ; IL, interleukin; PBS, phosphate-buffered saline; PLGA, poly(lactic acid-glycolic acid); Sulfa + Trim, sulfamethoxazole/trimethoprim.

used to improve chemotherapy of fungal diseases. The use of P10 peptide has been effective in reducing the fungal burden in animals infected with *P. brasiliensis*, thus eliciting an efficient cellular immune response to combat the fungus (Taborda *et al.*, 1998). The association of P10 with sulfamethoxazole/trimethoprim was effective in treating PCM and in avoiding the time-dependent relapse of the mycosis in the murine model (Marques *et al.*, 2006). The disadvantage of the use of the free peptide *in vivo* could be its lack of metabolic stability and the requirement for adjuvants not allowed for human use. A multiple antigenic peptide (MAP) construct carrying four truncated P10 branches has been synthesized and shown to be protective without additional adjuvants. However, the chemical synthesis of MAPs is a complex procedure, and it is subject to errors during the process, making their purification and characterization difficult (Taborda *et al.*, 2004). Thus, efforts to prepare simpler and more efficient delivery systems for immunostimulating peptides are still needed.

In the present work, PLGA-DMSA acid nanoparticles containing P10 in amounts ranging from 1 μ g:50 μ L⁻¹ to 40 μ g:50 μ L⁻¹ were used combined with sulfamethoxazole/trimethoprim. The use of PLGA-based formulations for the encapsulation of these molecules aimed at reducing the frequency of injections and also protecting the peptide against *in vivo* degradation while helping drug administration (Amaral *et al.*, 2009). Nanoparticles composed of PLGA are appropriate devices for delivering the peptide due to the low rate of co-polymer degradation and a constant release of the peptide, which would be better at stimulating the immune cells. The proportion of PLA and PGA (50:50) used in this type of drug delivery system presented a sustained release of the active component over an average time of 72 h, as observed in other studies (Mittal *et al.*, 2007; Amaral *et al.*, 2009). Nanoparticulated PLGA as a drug delivery system has been used for some time based on its high stability, ready uptake into the cell by endocytosis and targeting of specific organs, with many different mixtures of PLA and PGA (Dhiman *et al.*,

2000). There is no toxicity associated with this drug delivery system by itself and its use has been approved by drug regulatory agencies, such as the Food and Drug Administration (Khang *et al.*, 2003; Gabler *et al.*, 2007). *In vitro* tests using different cell lines showed no toxicity for nanoparticulated PLGA (Khandare and Minko, 2006; Gomes *et al.*, 2008). PLGA by itself does have the advantage of eliciting cytotoxic T-cell responses and also of inducing a mild inflammatory response, which could be involved in its adjuvant characteristics (Jiang *et al.*, 2005).

In our study, incorporating peptide P10 into PLGA reduced the amount of this peptide necessary to decrease the fungal load in the infected animals and avoid disease relapse. A small amount (1 μ g:50 μ L⁻¹) of P10 in nanoparticles had the same immunotherapeutic effect as 20 μ g:50 μ L⁻¹ of 'free' P10 emulsified in Freund's adjuvant over the first 30 days of treatment. However, the best protective effect of the PLGA-encapsulated peptide after 90 days of treatment was shown by the groups that received 5 or 10 μ g:50 μ L⁻¹ of P10 in PLGA, making it possible to reduce the amount of the peptide by at least fourfold and preserving the protective capacity to avoid relapse of the infection. Based on a previous report (Johansen *et al.*, 2000), the entrapment of P10 within PLGA nanoparticles probably effectively protected it from *in vivo* enzymatic destruction, which could explain the effectiveness of the lower doses of P10-PLGA.

Interaction of PLGA with antigen-presenting cells could underlie the improvement of immunomodulatory effects (Jaganathan *et al.*, 2005). Further, the use of PLGA for controlled release of the peptide eliminates the need for an adjuvant, which becomes an advantage as only a limited number of adjuvants are accepted for human administration (Jiang *et al.*, 2005). The protection elicited by 20 μ g:50 μ L⁻¹ of 'free' P10 in Freund's adjuvant as well as P10-PLGA at 1 μ g:50 μ L⁻¹ most probably depended on the induction of the high levels of IFN- γ , as noted in previous studies (Marques *et al.*, 2006). Although the 10 μ g P10-PLGA treatment showed low IFN- γ and high IL-10 cytokine values after 90 days of therapy, it still

managed to have a good protective effect. This may be explained by the maintenance of high levels of IL-12 and low production of IL-10 at 90 days, almost twofold lower than that produced in PBS and Sulfa + Trim-treated groups (Table 1), which could compensate for the low IFN- γ level noted for this group. Most patients with the severe form of PCM present a polarized Th2 response, with production of IL-4 and IL-10 (Oliveira *et al.*, 2002). In contrast, individuals exhibiting a polarized Th1 response, with production of IFN- γ , tend to resolve *P. brasiliensis* infections (Oliveira *et al.*, 2002; Calich *et al.*, 2008).

In conclusion, the use of PLGA as a carrier for peptide P10 in combination with sulfamethoxazole/trimethoprim represents a promising alternative to treat mycosis, as P10 elicits a Th1-like immune response able to control fungal infection. Sulfamethoxazole/trimethoprim is an inexpensive medicine, and its therapeutic efficacy can be increased by the cellular immunity-stimulating P10 peptide. Encapsulation of peptide P10 within PLGA reduced the amount of this peptide needed for a therapeutic response, up to 20-fold and avoided the use of an adjuvant. Additionally, in terms of application to therapy in humans, use of the PLGA nanoparticles should lead to improved antifungal protection and, at the same time, to a reduction in costs.

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Conflicts of interest

None to declare.

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Artigo 6

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Correlation of fungi and endotoxin with PM_{2.5} and meteorological parameters in atmosphere of Sao Paulo, Brazil

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ABSTRACT

Particulate matter, especially PM_{2.5}, is associated with increased morbidity and mortality from respiratory diseases. Studies that focus on the chemical composition of the material are frequent in the literature, but those that characterize the biological fraction are rare. The objectives of this study were to characterize samples collected in Sao Paulo, Brazil on the quantity of fungi and endotoxins associated with PM_{2.5}, correlating with the mass of particulate matter, chemical composition and meteorological parameters. We did that by Principal Component Analysis (PCA) and multiple linear regressions. The results have shown that fungi and endotoxins represent significant portion of PM_{2.5}, reaching average concentrations of 772.23 spores μg^{-1} of PM_{2.5} (SD: 400.37) and 5.52 EU mg^{-1} of PM_{2.5} (SD: 4.51 EU mg^{-1}), respectively. Hyaline basidiospores, *Cladosporium* and total spore counts were correlated to factor Ba/Ca/Fe/Zn/K/Si of PM_{2.5} ($p < 0.05$). Genera Pen/Asp were correlated to the total mass of PM_{2.5} ($p < 0.05$) and colorless ascospores were correlated to humidity ($p < 0.05$). Endotoxin was positively correlated with the atmospheric temperature ($p < 0.05$). This study has shown that bioaerosol is present in considerable amounts in PM_{2.5} in the atmosphere of Sao Paulo, Brazil. Some fungi were correlated with soil particle resuspension and mass of particulate matter. Therefore, the relative contribution of bioaerosol in PM_{2.5} should be considered in future studies aimed at evaluating the clinical impact of exposure to air pollution.

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1. Introduction

Particulate matter (PM) refers to a complex mixture of pollutants consisting of smoke, dust and all kinds of solid and liquid material that is in suspension in the atmosphere and it includes bioaerosols. There is a positive correlation between acute (Rivero et al., 2005) and chronic exposure (Churg et al., 2003; Pope et al., 2002) to atmospheric particulate matter and risk of effects on health as airway inflammation (Fujii et al., 2001; Peden, 2002), remodeling (Churg et al., 2003) and exacerbation of asthma (Peden, 2001; Peden, 2002), increase in cardiopulmonary diseases and cancer deaths, being the last two factors related primarily to PM_{2.5} (Klemm et al., 2000; Pope et al., 2009, 2002; Schwartz et al., 1996). Previous studies conducted in Sao Paulo also disclosed significant effects of ambient particles on

human health (Braga et al., 1999; Cendon et al., 2006) as well as in animal models (Camargo Pires-Neto et al., 2006; Rivero et al., 2005).

There are many studies that characterize the chemical elements which account for such effects (Aust et al., 2002; Edgerton et al., 2006; Ostro et al., 2007; Turnbull and Harrison, 2000; Vallius et al., 2005; Wang et al., 2006), but fewer studies characterize the biological components, such as fungi (Adhikari et al., 2006; Glikson et al., 1995; Mastalerz et al., 1998) and bacteria components present in the PM (Adhikari et al., 2006; Alexis et al., 2006; Mueller-Anneling et al., 2004), which are agents also related to respiratory illnesses, such as allergies and toxic responses (Alexis et al., 2003; Burge and Rogers, 2000). The contribution of biogenic aerosols (bioaerosol) to the health end-points related to ambient particle inhalation has not been fully understood. Bioaerosol is composed by particles originated by microbes, plant or animals, including living or dead organisms and their by products (Douwes et al., 2003). Fungal spores represent a relevant part of the bioaerosol and are known risk factor for adverse health effects, such as inflammatory responses associated to allergies and asthma (Burge and Rogers, 2000; Bush and Portnoy, 2001; Dales et al., 2003).

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Endotoxin, a component of the outer cell membrane of Gram-negative bacteria, may also be identified in ambient aerosols, mostly the fraction resulting from road dust (Salonen et al., 2004). Depending on inhaled doses and individual sensitivity, endotoxin may elicit symptoms such as airway obstruction, asthma exacerbation and pro-inflammatory cytokines (Kline et al., 1999). Bioaerosol can be adsorbed to particulate matter of other sources (traffic, industry, soil, for instance), having its aerodynamic and antigenic properties modified, enabling the penetration in deeper regions of the lung (D'Amato, 2002; Glikson et al., 1995; Knox et al., 1997). This topic is very important due to the fact that the particles of biological origin may represent around 15% of the total particulate (Matthias-Maser and Jaenicke, 1995) and from 5 to 10% of the resuspended particulate matter (Glikson et al., 1995).

Thus, the objectives of this study were to determine possible correlations of these components of bioaerosol with meteorological parameters, mass and chemical composition of PM_{2.5} and to estimate the relative contribution of endotoxins (components of the cell wall of gram-negative bacteria) and fungi in the PM_{2.5} in the city of Sao Paulo, Brazil.

2. Methods

2.1. Collection and location of samples

The PM_{2.5} was collected from High-Vol sampler operating at 1130 L min⁻¹ (AVG-Energética Ltda.) coupled to an inlet (Tisch Environmental Inc, USA) that allows the separation of particles below 2.5 µm (PM_{2.5}). Some adjustments were made for the collection of three samples simultaneously for a 24 h period and the filters were used for the characterization of fungi, endotoxins, and PM_{2.5}. The samples were collected on the campus of the University of Sao Paulo, Brazil, located in an area of heavy traffic. The particles were sampled between April and July of 2008, totalizing 39 samples.

2.2. Characterization of fungi

Samples of fungi were collected at 1.2 L min⁻¹ in MCE filters (Mixed cellulose ester filters, 0.8 µm, 25 mm diameter, Millipore Brazil) placed in cassettes of 25 mm (Millipore Brazil). This low flow rate allowed fungal spore identification under the microscope without visual interference of particle debris. After the collection, the filters were immediately removed from the cassettes and dissolved in Triacetin (C2: 0 – Cibraquim, Brazil) as described by Yang and Heinsohn (2007). The slides were air dried, or quickly heated until the filter was transparent. After this procedure, acid fucsin with lactic acid (Merck, Brazil) and slide covers were added for identification and counting on direct microscopy based on morphology of the spores.

2.3. Endotoxins

Samples for analysis of endotoxin were collected at 10 L min⁻¹ (Carty et al., 2003) in polycarbonate filters (0.4 µm, 37 mm diameter, Millipore Brazil). The filters were placed in the filter cassettes of 37 mm (Millipore Brazil). The storage was performed by coupling a dissection chamber containing silica at the end of the filter medium. The filters were stored at 4 °C until analysis. The extraction was performed in 5 mL of Tween 20, 0.05% (Bioagency, Brazil) in pyrogen free water (Cambrex, Co) in endotoxin-free borosilicate tubes (Cambrex, Co). The tubes were placed in a sonicator for 60 min at room temperature and the samples were vortexed every 15 min. The suspensions were analyzed in duplicates using the kit Limulus Amebocyte Lysate (LAL) Pyrogen-5000 (Cambrex, Co). During plating, the Beta-blocker reagent (Cambrex, Co) was

added for inhibition of reaction with (1–3) β-D-glucan present in the material, in concentration of 1:1. The reading was held in ELx808LBS (Cambrex, Co) using the software Win KQCL. *Escherichia coli* 055: B5 was used as standard.

2.4. Particulate matter

Teflon filters were used to collect PM_{2.5} (Teflo™ W/Ring, PTFE Membrane W/PMP Ring, 2.0 µm, 37 mm, Pall Corporation, Michigan, USA). The filters were weighed (balance UMX 2, Micronal SA) in an acclimatized room before and after collection to verify the amount of particulate matter in micrograms. Analysis of chemical composition was done by the X-ray fluorescence EDX-700HS (Shimadzu Corporation, Analytical Instruments Division, Japan) so that the results were issued in comparison to the standard NIST 2783. The chemical elements: Al, As, Ba, Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Sb, Ti, V, Zn, S, Si were subject to quantification in ppm.

2.5. Meteorological data

The data of temperature and relative humidity were provided by the Department of Atmospheric Sciences USP/IAG/ACA, Campus of the University of Sao Paulo. Data were collected in the daily average at intervals of 5 min to be eventually converted to average daily data. The meteorological station was located approximately 2 km from the sampler.

2.6. Statistical analysis

It was used SPSS version 16.0. Kolmogorov–Smirnov's normality tests were performed for the use of parametric or non parametric tests. Chemical elements were subjected to principal components analysis (PCA) to be afterwards used in single and multiple linear regression involving fungi, endotoxins, PM_{2.5} and meteorological parameters. PCA was chosen as initial procedure because it allows clustering of a high number of variables into small groups called components, which will explain the variability observed. The tests were considered significant to $\alpha < 0.05$.

3. Results

3.1. Characterization of fungi

The fungal spores showed average values of 20,814 spores m⁻³ (SD: 11,768 spores m⁻³) and 772.23 spores µg⁻¹ of PM_{2.5} (SD: 400.37 spores µg⁻¹ of PM_{2.5}). It was possible to identify 22 types, the main ones listed in Table 1.

3.2. Principal components analysis

The PCA performed for chemical elements showed that three factors were responsible for explaining 75% of the results:

Table 1

Main types of fungal spores found from MCE filters. Mean values followed by standard deviation.

Types of fungi (diameter < 2.5 µm)	Concentration (Spores m ⁻³) ^a
Hyaline Basidiospores	14,153.83 ± 7,577.29
<i>Cladosporium</i> sp	2,648.81 ± 2,523.26
<i>Penicillium/Aspergillus</i>	727.75 ± 470.50
Colorless ascospores	366.21 ± 678.65
Total amount	20,814.00 ± 11,768.00

^a Mean and standard deviation.

Table 2
Elementary characterization of PM2.5.

Element (ng m ⁻³)	Concentration ^a
Al	407.15 ± 307.21
As	4.14 ± 0.85
Ba	8.15 ± 6.15
Ca	209.53 ± 161.05
Co	3.13 ± 0.03
Cr	14.52 ± 9.01
Cu	75.12 ± 34.46
Fe	443.94 ± 314.50
K	731.87 ± 326.19
Mg	70.87 ± 31.33
Mn	56.09 ± 20.75
Na	1287.96 ± 351.07
Ni	30.42 ± 6.46
Pb	35.46 ± 40.78
Sb	2.24 ± 1.48
Ti	26.63 ± 23.35
V	13.04 ± 7.89
Zn	198.25 ± 146.28
S	699.32 ± 463.01
Si	649.58 ± 412.13

^a Mean and standard deviation.

Factor 1 was composed of Ba, Ca, Fe, Zn, K and Si,
Factor 2 was composed of Cr and Ni,
Factor 3 was composed of Cu and S.

Concentration for each of the elements is shown in Table 2.

3.3. Fungi, PM2.5 and meteorological data – multiple regression models

During the collection, it was found average concentrations of PM2.5 of 32.85 μm^{-3} (SD: 14.87). The Multiple Linear Regression model showed that hyaline basidiospores had positive correlation with factor 1 of PM2.5 ($R^2 = 0.184$, $p < 0.05$) – Fig. 1a as well *Cladosporium sp* ($R^2 = 0.406$, $p < 0.05$) – Fig. 1b and total amount of fungi ($R^2 = 0.255$, $p < 0.05$) – Fig. 1c. Neither of other parameters (i.e. temperature, humidity, PM2.5 mass and components 2 and 3) showed significance when adjusted in the model.

The genera *Penicillium/Aspergillus* (Pen/Asp) showed significant positive correlation with the mass of the PM2.5 air ($R^2 = 0.186$, $p < 0.05$) – Fig. 2a. Neither of other parameters (i.e. temperature, humidity, and components 1, 2 and 3) showed significance when adjusted in the model.

Colorless ascospores showed significance when correlated to humidity ($R^2: 0.16$, $p < 0.05$) – Fig. 2b. Neither of other parameters (i.e. temperature, PM2.5 mass, and components 1, 2 and 3) showed significance when adjusted in the model.

3.4. Endotoxins

It was possible to obtain results in twenty one samples without interferences due to artifacts. The results for endotoxins ranged from 0.03 EU m⁻³ to 0.29 EU m⁻³ (mean 0.10 and SD: 0.07). The data for association with PM2.5 showed that the variation was from 0.46 EU mg⁻¹ PM2.5 to 15.81 EU mg⁻¹ PM2.5 (mean 5.52 and SD: 4.51). Multiple linear regression models showed that endotoxins were correlated only to the measures of temperature, with $R^2: 0.229$, $p < 0.05$ (Fig. 2c). Neither of other parameters (i.e. humidity, PM2.5 mass and components 1, 2 and 3) showed significance when adjusted in the model.

4. Discussion

Traditionally in the literature, there are efforts to quantify the maximum concentration of particulate matter to be inhaled daily or annually (CETESB, 2008; EPA, 1990). However, such an approach shows limitations since the elemental composition of the material can lead to different responses in the respiratory and cardiovascular systems. Thus, recent studies aimed to characterize the particulate matter composition (Edgerton et al., 2006; Ostro et al., 2007; Wang et al., 2006), but surprisingly, few have focused in chemical and biological composition, especially involving fungi characterization, even though these agents are considered major sources of pollution, including in urban areas (Di Giorgio et al., 1996). Chemical elements may have a key role in the release of spores into the air, possibly causing deleterious effects on metabolism, or, in contrast, favoring the preferential adsorption of fungi to the particulate matter (Matthias-Maser et al., 1998). Furthermore, fungi may present resistance to compounds such as heavy metals due to the different phenotypes and genotypes between species (Gingell et al., 1976).

Although a lot of fungal spores have aerodynamics sizes bigger than 2.5 μm (Burge and Rogers, 2000) and some studies have found endotoxin in higher concentrations associated to PM10 (Heinrich et al., 2003), this particle diameter has been chosen because studies have demonstrated PM2.5 to be able of penetrating deep into the lungs and it is more related to inflammatory responses and changes in life expectancy than PM10 (Klemm et al., 2000; Pope et al., 2009; Schwartz et al., 1996). It is a matter of discussion if biological contaminants may act in synergy to contribute to such effects (Ning et al., 2000). To the best of our knowledge, there is no record about main genera provided by spore counts in the atmosphere of Sao Paulo, Brazil. Our study intent to supply initial data in this field, because it is known that different genera have different allergens content and therefore, may trigger distinct health responses after inhaled (Bush and Portnoy, 2001).

Endotoxin content has been chosen in detriment of culture-based methods or other methodology, due to decrease in viability that would occur after sampling for period as long as 24 h, media selectivity of some species and because endotoxin is recognized as a potent inflammatory agent present in live or dead cells (Douwes et al., 2003).

Our estimations have shown that the fungi are a significant portion of the PM2.5. Still, the chemical composition seems to be correlated to the increase of spores in the atmosphere. The spore counts of hyaline basidiospores and *Cladosporium spp* were correlated to factor 1 of PM2.5, which corresponds to elements that signalize traffic of vehicles and crustal resuspension (Amato et al., 2009; Schauer et al., 2006). The low humidity associated with heavy traffic may be responsible for a high aerolization of the crustal elements due to resuspension of road dust, thus increasing the release of bioaerosol from soil, vegetation (Salonen et al., 2004). The fact that bioaerosol is incorporated in the same factor as elements markers of traffic poses a new challenge when interpreting the role of automotive emissions on health. Silicon was identified as an element associated to increased pulmonary inflammation in rodents inhaling concentrated ambient particles (Saldiva et al., 2002). The inflammatory reaction associated to silicon inhalation could not be explained by changes in their toxic potential by the particle concentrator (Savage et al., 2003). Since fungal spores may account for these kinds of health effects (Cooley et al., 2000; Kauffman et al., 2000; Young et al., 2001) and were not measured in both studies, it is plausible to speculate that bioaerosol combined with crustal elements resuspension may play a role in the aforementioned findings. Indeed, in places near the streets of heavy traffic it may be found a higher concentration of spores in suspension (Lugauskas et al., 2003). A study made in Brisbane, Australia took measurements of bioaerosols (ie fungi and pollen) in the atmosphere

Fig. 1. Multiple Linear Regression for fungi and Factor 1 ($p < 0.05$). a. Hyaline basidiospores. b. *Cladosporium* sp. c. Total amount of fungi.

associated with the particulate matter. It was verified the cytoplasmatic material of fungi adsorbed in the material from vehicular sources. The authors have not discarded the possibility of mechanisms of synergy between both (Glikson et al., 1995). Later, the same group found that the fungi were the most abundant bioaerosols in the atmosphere and again, cytoplasmatic material associated with particles from vehicle exhaust and resuspension in the crust (Mastalerz et al., 1998). These results are in accordance with our results, suggesting that the particulate matter can affect fungi, which are then subject to be more abundant in the atmosphere due to the turbulence mechanisms (resuspension of elements in the crust) and composition (elements of combustion or heavy traffic).

Cladosporium spp. is a typical fungus from organic and surfaces of leaves material (Awad, 2005; Levetin and Dorsey, 2006) and mechanisms of resuspension of the crust may be responsible for increasing the amount of such spores in the air. Studies which compare rural areas with urban ones have correlated this fungus to urban development (Awad, 2005). Other data show that this fungus may be relatively tolerant to heavy metals such as Zinc, Cadmium and Lead, being Zinc one of the elements of Factor 1 (Gingell et al., 1976).

In the case of genera *Pen/Asp*, the models have shown that the variation of these fungi may be better explained by the amount of particulate matter in the atmosphere. These results agree with those found in the atmosphere of Taipei in which it was also found a moderate correlation between PM₁₀ and the genus *Penicillium* (Lin and Li, 2000). A study made in three regions of Egypt (two rural and two urban areas) found greater number of genera *Aspergillus* and *Penicillium* in urbanized regions (Awad, 2005). Although the authors have suggested that these results could have been due to transport from rural areas, these data help to sustain the results of this study. Nevertheless, colorless ascospores show positive correlation with humidity. These results were expected, since these spores are protected in asci during periods of drought, only to be airborne during periods of moisture (Troutt and Levetin, 2001). Typically, samples collected after heavy rains show a tendency to have a high amount of these elements in the air (Haines et al., 2000).

4.1. Endotoxins

The results have shown that endotoxins ranged from 0.03 to 0.29 EU m⁻³ (mean 0.10 and SD: 0.07). These results are superior

Fig. 2. Multiple Linear Regression for fungi and endotoxin $p < 0.05$. a. *Penicillium/Aspergillus* and mass of PM2.5. b. Colorless ascospores and relative humidity. c. Endotoxin and atmospheric temperature.

to those found by other authors, in the atmosphere of Munich, Germany, with average values of 0.015 EU m^{-3} (Carty et al., 2003). One comparative study among Danish cities have shown that traffic jammed streets may have higher concentrations of endotoxins than in rural areas, with median values of 4.4 and 2.9 EU m^{-3} , respectively. In town areas, the values found are close to those found in this study, with median values of 0.33 EU m^{-3} (Madsen, 2006).

In relation to the amount of endotoxin associated with the PM2.5 the concentrations ranged from 0.46 to 15.81 EU mg^{-1} of PM2.5 (average of 5.52 EU mg^{-1} of PM2.5). For the study conducted in Munich by Carty and collaborators (2003), the average concentration was 1.07 EU mg^{-1} of PM2.5. Another German study found an average of 1.2 EU mg^{-1} of PM2.5 (Heinrich et al., 2003). In some U.S. cities, it was demonstrated an average concentration in the external environment of 2.0 EU mg^{-1} of PM2.5 (Long et al., 2001). In other regions such as Boston, MA, it was found an average of 2.3 EU mg^{-1} of PM2.5 in ambient of ambient particle concentrator (Imrich et al., 2000). Our results are shown to be below those collected in the southeast of Mexico City, with an average of 12.22 EU mg^{-1} of PM2.5 (Osornio-Vargas et al., 2003). Although our results have shown similarity with others in literature, it is quite difficult to

make comparisons, due to diversity of sampling and analytical methods.

The regression analysis have shown that endotoxin was correlated only with increasing temperature, partly agreeing with other studies in which the greatest amount of endotoxin was found during the warm seasons and correlated positively with increasing temperature and decreased moisture (Carty et al., 2003). In other European cities, larger amounts of endotoxin were also found in the warmer seasons of the year (Madsen, 2006). Further studies on characterization of the culturable bacteria have also correlated the concentration of bacteria in the air to temperature increases (Di Giorgio et al., 1996).

5. Conclusions

Our results have shown that bioaerosol (i.e. fungal spores and endotoxin) is present in considerable amounts in the fine particle mode in the atmosphere of Sao Paulo. Some of bioaerosol components, mainly fungi, are associated to soil resuspension and mass of particulate matter. Endotoxin seems to be more influenced by changes in temperature. Future studies regarding clinical analysis

of the impacts of concurrent exposure to these agents are needed to verify possible modulatory or synergistic effects between biological and chemical constituents of PM_{2.5}.

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Artigo 7

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RESEARCH ARTICLE

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Therapeutic use of a cationic antimicrobial peptide from the spider *Acanthoscurria gomesiana* in the control of experimental candidiasis

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Abstract

Background: Antimicrobial peptides are present in animals, plants and microorganisms and play a fundamental role in the innate immune response. Gomesin is a cationic antimicrobial peptide purified from haemocytes of the spider *Acanthoscurria gomesiana*. It has a broad-spectrum of activity against bacteria, fungi, protozoa and tumour cells. *Candida albicans* is a commensal yeast that is part of the human microbiota. However, in immunocompromised patients, this fungus may cause skin, mucosal or systemic infections. The typical treatment for this mycosis comprises three major categories of antifungal drugs: polyenes, azoles and echinocandins; however cases of resistance to these drugs are frequently reported. With the emergence of microorganisms that are resistant to conventional antibiotics, the development of alternative treatments for candidiasis is important. In this study, we evaluate the efficacy of gomesin treatment on disseminated and vaginal candidiasis as well as its toxicity and biodistribution.

Results: Treatment with gomesin effectively reduced *Candida albicans* in the kidneys, spleen, liver and vagina of infected mice. The biodistribution of gomesin labelled with technetium-99 m showed that the peptide is captured in the kidneys, spleen and liver. Enhanced production of TNF- α , IFN- γ and IL-6 was detected in infected mice treated with gomesin, suggesting an immunomodulatory activity. Moreover, immunosuppressed and *C. albicans*-infected mice showed an increase in survival after treatment with gomesin and fluconazole. Systemic administration of gomesin was also not toxic to the mice.

Conclusions: Gomesin proved to be effective against experimental *Candida albicans* infection. It can be used as an alternative therapy for candidiasis, either alone or in combination with fluconazole. Gomesin's mechanism is not fully understood, but we hypothesise that the peptide acts through the permeabilisation of the yeast membrane leading to death and/or releasing the yeast antigens that trigger the host immune response against infection. Therefore, data presented in this study reinforces the potential of gomesin as a therapeutic antifungal agent in both humans and animals.

Background

Antimicrobial peptides (AMPs) are components of the innate immune system of vertebrates and invertebrates, having a broad-spectrum activity against bacteria, fungi, viruses and protozoa [1]. In general, AMPs are small

molecules with 1 to 10 kDa of molecular mass and exhibit a high content of basic amino acids, which results in an overall positive net charge. AMPs also usually have an amphipathic structure. Thus, while the positive charges of basic amino acids facilitate interaction with the negative charges of the phospholipids of biological membranes, the hydrophobic amino acids facilitate the insertion of AMPs into the membrane, which will eventually lead to lysis of the microorganisms. Some AMPs

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can act on internal targets, such as the inhibition of nucleic acid and/or protein synthesis [1,2]. Alternatively, some AMPs selectively boost the host immune response through the regulation of the production of proinflammatory cytokines and chemokines and by promoting the chemotaxis of T cells, monocytes, neutrophils and eosinophils. They can also effect dendritic cell differentiation and stimulate angiogenesis [3].

Gomesin is a cationic AMP isolated from haemocytes of the tarantula spider *Acanthoscurria gomesiana* [4]. This peptide contains 18 amino acids and two disulphide bridges and adopts a β -hairpin structure [5]. The disulphide bridges provide stability in mammalian serum and resistance to proteolysis [6]. Gomesin exerts a strong microbicidal activity against Gram-positive and Gram-negative bacteria, filamentous fungi, yeast, parasites and tumour cells through a mechanism of pore formation or "detergent like" action [4,7-9].

Candidiasis is an infection caused by fungi from the genus *Candida* and can affect the skin, eyes, oral cavity, oesophagus, gastrointestinal tract, vagina and vascular system of humans. Most infections occur in patients who are immunocompromised or debilitated [10]. Vulvovaginal candidiasis is the most common form of mucosal disease, affecting up to 75% of women (review by [11]). In Brazil, candidiasis has become a public health problem. It is the 3rd leading cause of death from systemic mycosis in AIDS-negative patients. Records indicate an increase in mortality from an annual average of 39 deaths between 1996 and 1998 to 54 between 2005 and 2006. Taking in account the deaths of AIDS patients with underlying cases of candidiasis, the disease is the 2nd leading cause of death from systemic mycosis, with 1,780 deaths in Brazil from 1996 to 2006 [12]. Nosocomial candidiasis is also a public problem in Brazil [13]. In the USA, *Candida* species are the fourth leading cause of nosocomial bloodstream infections in several hospitals and the mortality from 1997 to 2003 was approximately 0.4 deaths per 100,000 population per year (review by [14,15]). The leading treatment of *Candida* infections is done with polyenes (amphotericin and liposomal amphotericin), azoles (fluconazole and voriconazole) and echinocandins (caspofungine) [16]. Regardless of which antifungal drug is used, there is frequent treatment failure [16]. In this paper, we show the potential therapeutic use of gomesin in an experimental infection of *C. albicans*.

Results

Evaluation of the antifungal activity of gomesin *in vitro*

The minimum inhibitory concentration (MIC) of gomesin in the isolate 78 and strain ATCC 90028 was 5.5 μ M and 11 μ M, respectively, while the MIC of Fluconazole in the isolate 78 and strain ATCC 90028 was 186

μ M and > 1.5 mM, respectively. In addition, we observed growth inhibition of the isolate 78 with the combined treatment of 0.6 μ M gomesin and 3.5 μ M fluconazole. Growth inhibition of strain ATCC 90028 was observed with the combined concentration of 1.3 μ M gomesin and 14.3 μ M fluconazole (Table 1). Furthermore, the fractional inhibitory concentration index (FICI) of the combination of gomesin and fluconazole was 0.11 in isolate 78 and 0.19 in strain ATCC 90028 (Table 1).

Evaluation of the antifungal activity of gomesin in mice with disseminated and vaginal candidiasis

Treatment with 5 mg/kg and 15 mg/kg of gomesin in mice with disseminated candidiasis effectively reduced the fungal burden of the kidneys, spleen and liver when compared with the control group (PBS-treated mice) (Figure 1A-C). Treatment with 10 mg/kg and 20 mg/kg of fluconazole also effectively controlled the infection (Figure 1A-C). Moreover, treatment of vaginal candidiasis with 0.2% and 0.5% gomesin and 2% miconazole showed a significant decrease in colony forming units (CFUs) when compared with vehicle treatment (control group) (Figure 1D). The combination of gomesin and fluconazole or miconazole did not result in a synergistic effect.

Cytokine levels in kidneys of gomesin-treated mice

Treatment with gomesin and fluconazole significantly increased the concentration of TNF- α , IFN- γ and IL-6 in the kidneys compared to controls that were not infected and not treated as well as controls that were infected and treated with PBS (Figure 2).

Evaluation of the effect of antifungal drugs in immunosuppressed mice with disseminated candidiasis

The group of infected animals that received PBS (control) reached 100% mortality on the fifteenth day after infection. No statistically significant difference was observed between the group treated with gomesin (5 mg/kg) and the group treated with fluconazole (20 mg/kg), although there was an increase in survival during the last treatment. Nonetheless, the combined treatment of 5 mg/kg of gomesin and 20 mg/kg of fluconazole produced a survival rate of 23% within 30 days after infection, which was statistically significant. The control groups that were not infected or those that received PBS or 5 mg/kg of gomesin remained alive until the end of the experiment (Figure 3).

In vivo toxicity

Gomesin administration did not alter the number of leukocytes in the non-infected mice. However, when specific cell populations were analysed, the number of

Table 1 Minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FICI) of gomesin and fluconazole

	MIC (μM)		FICI	
	<i>C. albicans</i> (78)	<i>C. albicans</i> (ATCC 90028)	<i>C. albicans</i> (78)	<i>C. albicans</i> (ATCC 90028)
Gomesin	5.5	11	-	-
Fluconazole	*	186	-	-
Gomesin + Fluconazole	0.6 + 3.5	1.3 + 14.3	0.11	0.19

* = not detected in up to 1.5 mM

neutrophils and eosinophils were increased, whereas the number of lymphocytes was decreased. The administration of gomesin did not alter the haemoglobin levels. Nevertheless, treatment with gomesin resulted in an increase in the percentage of circulating reticulocytes. Moreover, the administration of gomesin showed no change in the levels of total bilirubin, direct and indirect, as well as creatinine and gamma-GT (Table 2).

Biodistribution of radiolabeled gomesin

The biodistribution of gomesin labelled with technetium-99 m was evaluated in the kidneys, spleen and liver (Figure 4). The liver had the highest percentage of radiolabeled peptide detected (60%), which persisted for up to 24 h post-injection, whereas the kidneys showed a radioactive peak at 120 min followed by a gradual decrease during the following hours. The spleen was the

Figure 1 Gomesin treatment of mice infected with *C. albicans*. Evaluation of the number of colony forming units (CFU) per gram of tissue of the kidneys (A), spleen (B), liver (C) and vagina (D). The disseminated candidiasis was performed by intravenous injection of 3×10^5 yeasts suspended in 100 μ L of PBS and vaginal candidiasis was performed by inoculating 3×10^6 yeasts suspended in 20 μ L of PBS. The treatment was done one, three and six days after infection with *C. albicans* (strain 78). Animals were treated with different doses of gomesin (GOM), fluconazole (FLUCO) and miconazole (MICO). As a control, infected animals received only PBS or cream (CREAM). * Indicates statistical significance (ANOVA with post-Tukey test, $P < 0.05$).

Figure 2 Cytokine levels in kidneys. Cytokine levels were evaluated in the kidneys of mice treated with gomesin (5 mg/kg) and fluconazole (20 mg/kg). Non-infected and untreated animals (NINF), as well as infected animals that received PBS, were used as controls. * Indicates statistical significance (t-test, $P < 0.05$) compared to the control INF.

Figure 3 Survival of immunosuppressed mice with disseminated candidiasis treated with antifungal drugs. Animals were treated with 100 mg/kg of cyclophosphamide and infected with 10^3 yeasts of *C. albicans* (INF). The animals were treated with 5 mg/kg of gomesin (GOM), 20 mg/kg of fluconazole (FLUCO) or the combination of 5 mg/kg gomesin and 20 mg/kg of fluconazole. As controls, infected animals (NINF) received PBS and uninfected animals received PBS and gomesin 5 mg/kg. * Indicates statistical significance (Long-rank test, $P < 0.05$).

lowest of the organs tested (less than 5% detected) and was stable for only 60 min after administration of technetium-99 m-labelled gomesin, dropping to undetectable levels after 120 min.

Discussion

Gomesin is an antimicrobial peptide isolated from haemocytes of the spider *Acanthoscurria gomesiana* and has a broad-spectrum of activity against bacteria, fungi, protozoa and tumour cells [4,7,9,17,18]. The antifungal activity of gomesin *in vitro* has previously been reported [4,7]. However, the antifungal activity against clinical

Table 2 Evaluation of the toxicity of the gomesin treatment

	NINF*	NINF + GOM**
Leukocytes (mm ³)	4637 ± 1114	4462 ± 1580
Neutrophils (mm ³)	846 ± 288	1208 ± 388***
Eosinophils (mm ³)	46 ± 46	135 ± 72***
Lymphocytes (mm ³)	3744 ± 981	2660 ± 437***
Hemoglobin (g/dL)	13 ± 0.9	13 ± 0.5
Reticulocytes (%)	5.5 ± 0.7	9.3 ± 2.8***
Total Bilirubin (mg/dL)	0.48 ± 0.23	0.3 ± 0.1
Direct bilirubin (mg/dL)	0.35 ± 0.19	0.2 ± 0.1
Indirect bilirubin (mg/dL)	0.13 ± 0.13	0.09 ± 0.009
Creatinine (mg/dL)	0.32 ± 0.09	0.34 ± 0.05
Gamma-GT (mg/dL)	< 1 U/L	< 1 U/L

* Non-infected mice

** Non-infected mice treated with gomesin (GOM)

*** $p < 0.05$

isolates of *Candida albicans* resistant to antifungal drugs has not been studied. In this paper, we analysed the antifungal activity of gomesin *in vitro* and *in vivo* against a clinical strain of *C. albicans* (isolate 78), as well as its biodistribution and toxicity in mice.

Our data showed that *C. albicans* (isolate 78) is resistant to fluconazole up to 1.5 mM, but gomesin is effective against this strain at a lower concentration (MIC = 5.5 μ M). This resistance to fluconazole is a common cause of treatment failure [19]. A synergism between gomesin and fluconazole against two isolates of *Candida albicans* (78 and ATCC 90028) was demonstrated using the FICI calculation method. The synergistic mechanism of gomesin and fluconazole is not completely understood, but studies with *Cryptococcus neoformans* suggested that gomesin, through membrane permeabilisation, promotes an increased entry of fluconazole into the fungal cytoplasm, which results in a better inhibition of the ergosterol synthesis. In this way, fluconazole is effective against *C. neoformans* at lower doses when applied in combination with gomesin [7]. A similar phenomenon was observed in murine melanoma cells (B16F10-Nex2) treated with gomesin and the monoclonal Mab A4M *in vitro*. The cytotoxicity of Mab A4M was only detected in the presence of gomesin, after permeabilisation of the cell membrane allowed the entry and action of the monoclonal antibody [9]. From these studies, we hypothesised that gomesin facilitates the entry of fluconazole in *Candida albicans* through membrane permeabilisation.

The literature on the use of antimicrobial peptides in the treatment of disseminated candidiasis is rather scarce. A study of the HLF peptide (1-11) originated from lactoferrin in immunosuppressed mice with

disseminated candidiasis showed that a single dose of 0.4 ng/kg, 24 h after infection, was able to significantly reduce CFU in the kidneys [20]. ETD-151, an analogue of heliomicin also has been shown to be particularly effective against systemic candidiasis in comparison with amphotericin B and several azoles [21]. Likewise, treatment with gomesin proved to be effective against disseminated candidiasis. The peptide effectively reduced the fungal burden in the kidneys, which is the highest tropism organ for *Candida*. A similar effect was observed with fluconazole; however, this drug has some toxic effects and has selected resistance in *Candida albicans* [19]. Therefore, the use of gomesin as a therapeutic may be an alternative treatment for candidiasis because our results show that it is non-toxic in mice. Unlike *in vitro* treatment with gomesin and fluconazole, we have not detected any the synergistic effect of treatment with both drugs *in vivo*.

The treatment and prevention of recurrent vaginal candidiasis includes the use of imidazoles and triazoles as a first-line treatment, unless it is caused by a confirmed or suspected azole-resistant *Candida* strain. The efficacy of both oral and local therapy is similar, but, the local treatment presents several advantages, including a reduction of adverse effects; however, local treatment is contraindicated during pregnancy and breast feeding [22]. In recent years, there has been a focus on both understanding drug resistance to antifungal agents and optimising therapy of *Candida* infections [23]. There are no reports of topical treatment with antimicrobial peptides against vaginal candidiasis. In this paper, we are the first to describe an effective topical formulation of an antimicrobial peptide that is able to reduce CFUs count in an experimental vaginal candidiasis model. We found that 0.2% and 0.5% gomesin cream reduced the CFU on vaginas of the animals by 10 fold when compared to control animals. Minor changes in the treatment protocol with gomesin, either by increasing the frequency or changing the doses, may potentially produce better results. Treatment with 2% miconazole cream was also effective in controlling the CFUs of the vaginas of the animals. However, it was necessary to use a dose of miconazole that was at least four times higher than the dose of gomesin to produce a similar effect. No synergistic effect was observed after treatment with a combination of gomesin and miconazole.

In addition to the direct action of AMPs on microorganisms, either through membrane permeabilisation or internal target interference [2], it has been reported that some AMPs may possess an immunomodulatory function [3]. In order to verify if gomesin has such activity, the concentrations of IFN- γ , TNF- α and IL-6 were evaluated in the kidneys of mice that had been infected with *C. albicans* and treated with this peptide. These

cytokines, especially IL-6, activate neutrophils, which play an essential role in the defence mechanism against *Candida* [24]. We observed that treatment with 5 mg/kg gomesin significantly increased the concentration of the three cytokines analysed. A similar effect was also observed with fluconazole treatment. The increase of cytokine levels in the kidneys might help to control candidiasis through the activation of the host immune system. This action appears to be similar to that observed with another AMP, murine β defensin-2, which acts via TLR4 and leads to the production of various cytokines, such as IL-12 and IL-6, as well as chemokines [25]. However, we cannot dismiss the hypothesis that the direct action of gomesin can trigger the release of pathogen-associated molecular patterns, or PAMPs, which would exacerbate the immune response of animals. This has been previously reported for the antimicrobial peptide human β defensin-2 [26]. The use of antimicrobial peptides as immunomodulatory agents for therapeutic application is an effervescent field in progress [27].

After verifying that the gomesin treatment was effective against disseminated candidiasis in healthy mice, we decided to evaluate the activity of gomesin in immunosuppressed animals, as candidiasis is typically observed in immunocompromised hosts [10]. Treatment with gomesin (5 mg/kg) showed no significant increase in survival compared to control animals. This suggests that the direct action of gomesin was not sufficient to control the infection and that immunomodulatory action is required to suppress the candidiasis. Treatment with fluconazole (20 mg/kg) also did not result in a significant increase in the survival of treated animals as compared to control animals. However, the combined treatment of 5 mg/kg gomesin and 20 mg/kg of fluconazole resulted in 23% survival of mice 30 days after infection. This could be due to gomesin facilitating the entry of fluconazole into the yeast, thus leading to the survival of animals. Another hypothesis is that treatment with fluconazole, being fungistatic, would allow time for gomesin to act.

To evaluate whether gomesin could be used as a therapeutic treatment for *C. albicans* infection, we performed blood analyses to determine the toxicity of gomesin in mice. No difference in the total number of leukocytes was observed in animals treated with gomesin. However, the number of eosinophils in mice not infected with *Candida albicans* but treated with gomesin was higher than the control group. The eosinophilia caused by gomesin may be due to the induction of an allergic response. Further experiments are needed in order to evaluate this effect. We have also noticed that gomesin treatment leads to a higher number of

neutrophils. This effect might be a consequence of the induction of the pro-inflammatory response by gomesin, which would stimulate the bone marrow to recruit neutrophils. However it is not currently known if these cells are being recruited to the site of infection.

In addition, gomesin did not change the haemoglobin levels, which suggests that this peptide was not toxic to erythrocytes. However, the quantity of reticulocytes is greater in treated animals, suggesting that the peptide provokes an erythropoiesis compared to control animals (non-gomesin treated). Perhaps treatment with gomesin causes hypoxia in animals, thus increasing erythropoietin [28]. Furthermore, gomesin was not nephrotoxic or hepatotoxic, as the bilirubin, creatinine, and Gamma GT levels from treated animals are similar to the control group. Therefore, gomesin seems to be non-toxic to mice.

In addition to the evaluation of toxicity, the biodistribution of gomesin was performed to understand its pharmacokinetics and therefore its therapeutic potential. The biodistribution data revealed that the peptide mainly accumulates in the liver, although it also accumulates in the kidneys and spleen, within the first several minutes after administration. This suggests a rapid clearance from the circulation. The presence of gomesin in the sites of infection might explain the reduction of *Candida albicans* observed in our experiments. However, other studies are needed to monitor the excretion of the peptide.

Conclusions

Gomesin was effective against *Candida albicans* infection *in vitro* and *in vivo*. Gomesin can be used as an alternative treatment for candidiasis, either alone or in combination with fluconazole. Although the mechanism of action of gomesin is not fully understood, it has been suggested that it directly acts on the fungal membrane and/or stimulates the immune response against yeast infection. Data presented in this study reinforces the potential of gomesin as a therapeutic antifungal agent in both humans and animals.

Methods

Antimicrobial compounds

The chemically synthesised gomesin was obtained from GENEPEP (France) with 97% purity analysed by liquid chromatography - mass spectrometry. Fluconazole was obtained from Pfizer (Pfizer Inc., New York) and miconazole from Janssen Pharmaceutica (Janssen-Pharmaceutica, Beerse). Gomesin and fluconazole were dissolved in PBS for the *in vivo* assays and water for *in vitro* tests. Miconazole was dissolved in PBS with 20% dimethyl sulfoxide (DMSO) for incorporation into the vaginal cream.

Candida albicans strains

Two strains of *Candida albicans* were used: isolate 78 [29] and the isolate ATCC 90028. Periodically, isolate 78 was inoculated into mice in order to maintain its virulence.

In vitro studies

The antifungal activity of antimicrobial compounds was evaluated by using the protocol M-27A2, according to the Clinical and Laboratory Standards Institute (CLSI) [30]. Briefly, 80 μ l of RPMI 1640 with 1.6 M MOPS pH 7 containing 10^4 yeast/mL of *C. albicans* in logarithmic growth phase, were added to the wells of a polypropylene 96-well plate containing 20 μ l of serial two-fold dilution of gomesin (starting at 44 μ M), fluconazole (starting at 1,488 μ M) or the combination of gomesin (starting at 11 μ M) and fluconazole (starting at 115 μ M). After 48 h of incubation at 37°C fungal growth was evaluated by determining the absorbance at 595 nm. The lowest concentration that inhibited 100% growth was considered the minimum inhibitory concentration (MIC). The fractional inhibitory concentration index (FICI) was determined following the methodology described previously [31].

Animals

BALB/c mice (6- to 8-week-old males or females) were bred at the Animal Facility at the Institute of Biomedical Science of University of São Paulo, Department of Immunology under specific pathogen-free conditions. Food and water were given ad libitum. All animals were handled in accordance with good animal practice as defined by the relevant national animal welfare bodies and all *in vivo* testing was approved by the Institutional Animal Care and Use Committee of the University of São Paulo, reference number: 87/42. For immunosuppression of animals, doses of 100 mg/kg cyclophosphamide were administered intraperitoneally 4 days and 1 day before infection with *C. albicans*, the third day after infection and, from this point on, every 4 days until the end of treatment [32]. The animals were kept in cages lined with wood shavings and closed with autoclaved filter, and served autoclaved food and water in order to maintain a sterile environment. Cages were exchanged twice a week in laminar flow hoods. The animals were considered anergic when the number of leukocytes was found to be less than 100 cells/mm³ [33]. The vaginal candidiasis model was developed by inducing the pseudo oestrus phase by the subcutaneous administration of 0.5 mg of 17 beta-estradiol valerate (Sigma Chemicals, St Louis), dissolved in sesame oil (Sigma Chemicals, St Louis) 3 days before the vagina's infection [34]. Swiss mice were provided by the Animal Facility of IPEN-CNEN for the biodistribution studies.

Infections

One colony of *C. albicans* (isolate 78) was selected from the plate dishes and incubated in brain heart infusion (Oxoid, England) at 37°C for 24 h with 200 rpm agitation. The sediment obtained by centrifugation at 1500 g for 5 min was washed three times in PBS and resuspended in 5 mL of PBS. The number of yeast per mL of this suspension was determined with a Neubauer chamber.

The disseminated candidiasis was induced by intravenous injection of 3×10^5 /100 μ L of PBS and the immunosuppressed model was induced by intravenous injection of 10^3 yeasts suspended in 100 μ L of PBS. Vaginal candidiasis was induced by inoculating 3×10^6 yeasts suspended in 20 μ L of PBS.

In vivo treatments

Mice with disseminated candidiasis were treated with gomesin and fluconazole. The drugs were administered intraperitoneally in a final volume of 500 μ l at the following concentrations: gomesin (2.5 mg/kg, 5 mg/kg and 15 mg/kg), fluconazole (10 mg/kg and 20 mg/kg) and a combination of both (2.5 mg/kg to 5 mg/kg gomesin and 10 mg/kg to 20 mg/kg fluconazole). For mice with vaginal candidiasis, gomesin (0.02%, 0.2% and 0.5%), miconazole (2%) and a combination of both (0.2% gomesin and 2% miconazole) were incorporated into a vaginal cream (10% Wax self-nonionic emulsifier, 2% mineral oil, 5% propylene glycol and 84% distilled water, pH 4.5) for topical application. For all treatments, drugs were administered 1, 3 and 6 days after infection with *C. albicans*. An equivalent volume of PBS or cream was administered to the control animals. To evaluate the fungal burden, the kidneys, spleen, liver and vagina of the mice were dissected aseptically on the seventh day after infection, weighed and homogenised in 1 mL of PBS. Aliquots of the homogenate (100 μ l) were inoculated on brain and heart infusion (Oxoid, England) containing 2% agar. After incubation for 18 h at 37°C, the number of CFUs was determined. The effectiveness of treatment was determined by comparing the number of CFUs per gram of tissue of treated animals with the number of CFUs per gram of tissue of control animals (untreated). For the survival curves, the animals were monitored daily for 30 days.

Measurement of cytokines

The kidneys of animals infected with *C. albicans* and treated with gomesin or fluconazole were dissected, washed in PBS and homogenised with an electric tissue homogeniser in 1 mL of PBS containing a cocktail of protease inhibitors (Sigma Chemicals, St Louis). This cocktail of protease inhibitors was composed of 1 μ g/ml of pepstatin A (aspartyl protease inhibitor), 4 mM

benzamidine (serine protease inhibitor), 1 mM ethylenediaminetetraacetic acid acetic (metallo-protease inhibitor) and 1 mM N-Ethylmaleimide (cysteine protease inhibitor). Non-infected animals or animals infected and not treated were used as controls. The concentrations of IFN- γ , TNF- α and IL-6 were evaluated on a flow cytometer (BD FACSCalibur™, San José) using the kit Cytometric Bead Array and Mouse Inflammation™ (BD, San José) and the methodology described by the manufacturer.

Blood analysis

Blood was collected by puncturing the brachial plexus of anaesthetised mice using EDTA (1%) as an anticoagulant after 7 days of gomesin administration (15 mg/kg). Reticulocytes cells and leukocytes were counted by standard methods. The haemoglobin concentration was determined using the modified Drabkin method. Blood samples were prepared on microscopic glasses, dried and stained with May-Grünwald reagents for morphological examination of the blood. The number of reticulocytes was determined in blood smears stained with Supra Vital New Methylene Blue. We also determined the levels of bilirubin, creatinine and gamma GT biochemically using the Sims-Horm, Enzyme and Alkaline picrate methods, respectively.

Evaluation of the biodistribution of radiolabelled gomesin with technetium-99 m in mice

HYNIC-gomesin was manually synthesised by solid phase methodology as described previously, except that pyroglutamic acid was substituted for 6-hydrazino nicotinamide (HYNIC) [6]. The HYNIC-gomesin conjugate was labelled with the radioisotope technetium-99 m obtained from an alumina-based $^{99}\text{Mo}/^{99}\text{Tc}$ generator, supplied by the Radiopharmacy Centre of the Institute of Energetic and Nuclear Research (IPEN/CNEN). Briefly, 20 mg of tricine and 5 mg of ethylenediamine N,N'-diacetic acid (EDDA) were dissolved in 0.5 ml of 0.1 M PBS, previously nitrogenated. Ten micrograms of HYNIC-gomesin, 5 μl of 8.9 mM $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution in 0.1 N HCl (nitrogen-purged) and 500 μl of $\text{Na}^{99\text{m}}\text{TcO}_4$ was added to the vial. The reaction was conducted by heating the solution at 100°C for 20 min in a water bath and then allowing it to cool to room temperature. The pH of the reaction mixture was 7 [35]. The product $^{99\text{m}}\text{Tc}$ -HYNIC-gomesin (0.1 mL), with an approximate activity of 74 MBq (2 mCi), was administered to the tail vein of the mice. The animals were sacrificed in a CO_2 chamber at 5, 30, 60, 120, 240, 360, and 1,440 min after injection of the radiolabeled gomesin. Six animals were used for each time point. The kidneys, spleen and liver of each animal was dissected and

transferred to tubes to measure radioactivity. The radioactivity count was performed in the Gamma Counter Shaft type NaI, using the same standard dose in injected animals minus the radioactivity retained in the site injection (tail). The uptake of radiolabeled gomesin by each organ was calculated using the following equation: $\text{DI}\% = (\text{CPM organ}/\text{standard CPM}) \times 100$, where %DI = percentage of the injected dose and CPM = count per min [35].

Statistical analysis

ANOVA, with the post-Tukey test, was used to evaluate the statistical significance of results obtained in all experiments except the blood and survival analysis, where the Students *t*-test and Log-rank test were used, respectively. The differences between the results obtained with treatment compared to the controls were considered statistically significant when the *p* value was less than 0.05.

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Authors' contributions

DR carry out *in vitro*, *in vivo* studies and measurement of cytokines, participated in biodistribution experiments, blood analysis, experimental design and helped to write the manuscript. JEM participated in the *in vivo*, *in vitro* studies and measurement of cytokines. RB participated in *in vivo* and *in vitro* studies. AM synthesised the HYNIC-gomesin. BF was responsible for the biodistribution experiments. PB carries out the blood analysis. DDC was in charge of the statistical analysis. CPT and SD conceived of the study and manuscript preparation. All authors read and approved the final manuscript.

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Therapeutic DNA Vaccine Encoding Peptide P10 against Experimental Paracoccidioidomycosis

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Abstract

Paracoccidioidomycosis (PCM), caused by *Paracoccidioides brasiliensis*, is the most prevalent invasive fungal disease in South America. Systemic mycoses are the 10th most common cause of death among infectious diseases in Brazil and PCM is responsible for more than 50% of deaths due to fungal infections. PCM is typically treated with sulfonamides, amphotericin B or azoles, although complete eradication of the fungus may not occur and relapsing disease is frequently reported. A 15-mer peptide from the major diagnostic antigen gp43, named P10, can induce a strong T-CD4+ helper-1 immune response in mice. The TEPITOPE algorithm and experimental data have confirmed that most HLA-DR molecules can present P10, which suggests that P10 is a candidate antigen for a PCM vaccine. In the current work, the therapeutic efficacy of plasmid immunization with P10 and/or IL-12 inserts was tested in murine models of PCM. When given prior to or after infection with *P. brasiliensis* virulent Pb 18 isolate, plasmid-vaccination with P10 and/or IL-12 inserts successfully reduced the fungal burden in lungs of infected mice. In fact, intramuscular administration of a combination of plasmids expressing P10 and IL-12 given weekly for one month, followed by single injections every month for 3 months restored normal lung architecture and eradicated the fungus in mice that were infected one month prior to treatment. The data indicate that immunization with these plasmids is a powerful procedure for prevention and treatment of experimental PCM, with the perspective of being also effective in human patients.

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Introduction

Paracoccidioides brasiliensis is a thermally dimorphic fungus that causes a systemic granulomatous disease known as paracoccidioidomycosis (PCM). PCM is widespread in Latin America, mainly affecting rural workers, and its incidence has increased in recently deforested areas associated with soil churning [1]. Acquisition of *P. brasiliensis* may arise from inhalation of aerosolized conidia.

Recently we reviewed the death rates by systemic mycoses in Brazil [2]. PCM was the principal cause of death identified for 3,583 patients in the 1996–2006 decade and representing 51.2% of total deaths due to systemic mycoses. It ranks as the 10th most common cause of death among infectious diseases in Brazil [2].

There are distinct forms of PCM. The acute and sub-acute forms affect both genders with primary involvement of the reticuloendothelial/lymphatic system. The chronic form affects mainly adult males and predominantly causes pulmonary and/or mucocutaneous disease [3]. Antifungal chemotherapy is required for treatment, though treatment may not assure complete eradication of the fungus, with frequent relapses. Treatment with itraconazole usually takes 6–9 months in the low and 12–18

months in the moderately severe cases. Frequently, a combination of trimethoprim and sulfamethoxazole (TMP/SMZ) is used, held for 12 months in the low severity forms and 18–24 months in the moderately severe forms. Patients with severe PCM forms require endovenous treatment with amphotericin B or the TMP/SMZ association for long periods, monitored by clinical, radiological and serological tests [4].

The 43 kDa glycoprotein was characterized as the major diagnostic antigen of *P. brasiliensis* [5,6,7]. Immunization with gp43 elicited delayed hypersensitivity reactions in guinea pigs [8] and humans [9], implying the presence of T-CD4+ reacting epitopes. Based on the sequence of gp43 [6], which encodes a polypeptide of 416 amino acids with a single high mannose *N*-glycosylated chain [10], the T-cell epitope was mapped to a 15-mer peptide called P10 [11]. The hexapeptide HTLAIR comprises the essential core of P10 that induces proliferation of lymph node cells from mice sensitized to gp43 or infected with *P. brasiliensis* [11]. Type 1-T helper lymphocytes producing IL-2 and IFN- γ are induced by P10 [11,12,13]. Intratracheally infected mice previously immunized with P10 in the presence of complete Freund's adjuvant (CFA) had >200-fold reduction of lung *P. brasiliensis* colony-forming units (CFUs). In many cases the immunization rendered preserved lung

Author Summary

Paracoccidioidomycosis (PCM) is the predominant systemic mycosis in Latin America causing half of the total deaths among systemic fungal infectious diseases in Brazil. Chemotherapy is the standard treatment, but the long time required, severe cases of immunosuppression and frequent relapses indicate that additional methods should be introduced such as immunotherapy combined with antifungal drugs. Previously, the protective activity of P10, a peptide derived from the major diagnostic antigen gp43, was demonstrated, alone or combined with chemotherapy. P10 elicited a vigorous IFN- γ mediated Th-1 immune response. Presently, the reduction of fungal load, and even sterilization, was attempted using a specific DNA vaccine encoding P10. Plasmid pcDNA3 expression vector with P10 insert was tested as a vaccine in intratracheally infected BALB/c and B10.A mice. Our results showed that vaccination with pP10 induced a significant reduction of the fungal burden in the lung. Co-vaccination of pP10 with a plasmid encoding mouse IL-12 proved to be even more effective in the elimination of the fungus with virtual sterilization in a long term infection and treatment assay system. The data suggest that immunization with these plasmids, without the need of an adjuvant, could be used in the prevention and treatment of PCM in human patients.

architecture with few or no yeasts, whereas the infected, unimmunized mice displayed dense pulmonary inflammation characterized by epithelioid granulomas with numerous yeast cells [11,12].

The immunoprotection by P10 depends on the IFN- γ -producing Th-1 response since mice deficient in IFN- γ , IFN- γ -R or IRF-1, but not IFN- α -R/IFN- β -R, were not protected by P10 immunization [12]. The essential role of IFN- γ in organizing granulomas that contain *P. brasiliensis* yeasts has also been recognized by other investigators [13,14,15].

Several experimental avenues have been pursued to validate P10 as a vaccine candidate. These studies have included: a) the presentation of P10 by MHC molecules from different murine haplotypes [11]; b) its conservation in nature, confirmed by examining gp43 molecules from different isolates [16]; c) its immunogenicity and effective immunoprotection in formulations that do not require complete Freund's adjuvant [17]; d) its presentation by most human HLA-DR molecules as well as that of neighbor peptides to P10, based on the sequence of gp43 [18]; and e) the effectiveness of P10 as an adjuvant to chemotherapy in normal [19] and anergic [20] mice challenged intratracheally with virulent *P. brasiliensis*.

The immunoprotective properties of P10 emulsified in Freund's adjuvant have been well documented in an established murine model of PCM [11]. Since CFA is not allowed in human vaccines and a tetramer of truncated P10 although immunogenic, involves laborious chemical methods [17], we have explored alternative approaches for P10 delivery. In the present work we have investigated the effectiveness of plasmid immunization with P10 and/or IL-12 inserts given prior to or after challenge with a virulent Pb18 isolate of *P. brasiliensis* using a murine pulmonary PCM disease model. Our results demonstrate that plasmid immunization with P10 with or without IL-12 inserts is highly therapeutic in mice intratracheally infected with this fungus. Most importantly, immunization was effective either prior to, or after infection suggesting that these plasmids are candidates for use in human PCM.

Materials and Methods

Plasmid constructions

Yeast cells of *P. brasiliensis* isolate 18 (Pb18) were grown in Sabouraud Dextrose Broth (BD, MD, USA) at 37°C for 7 days. Cells were washed and frozen in liquid nitrogen then disrupted by grinding on a mortar. Total RNA was isolated with Trizol according to manufacturer's instruction (Invitrogen, CA, USA). Complementary DNA was synthesized from 1 μ g of total RNA in the presence of oligo(dT)₁₈ (Fermentas, MD, USA) and Revertaid M-MuLV (Fermentas, MD, USA).

The P10 nucleotide sequence was obtained using the sense PCR primer derived from the gp43 [6] 5' nucleotide sequence: [5'-AAT AAG CTT CAA ACC CTG ATC GCC-3'], and the antisense primer derived from the 3' end of the gp43 gene: [5'-AAT GAA TTC ATT GGC GTA ACG GAT TGC-3']. A HINDIII site and an EcoRI site were added to the sense and antisense primers, respectively, for cloning into plasmid pcDNA3 (Invitrogen, CA, USA). PCR reactions (50 μ l) were carried out following the protocol provided by Fermentas, using 100 ng of cDNA and 100 ng of each primer. The P10 PCR reaction started with one cycle at 94°C (2 min), followed by 40 cycles at 94°C (30 sec), 55°C (1 min) and 72°C (1 min), and a final 7-min extension at 72°C. PCR products were purified using Wisard SV gel and PCR Clean-UP system (Promega, Brazil) and each PCR product was digested with the appropriate restriction enzyme (Fermentas) and cloned into the pcDNA3 by directional insertion in the HINDIII/EcoRI sites. The resulting plasmid was called pP10.

Plasmid pORF-mIL-12 was acquired from InvivoGen (CA, USA). The confirmation of the insert was done using the primers: sense [5'-CGG GTT TGC CGC CAG AAC ACA-3'] and antisense [5'-GGC CAC CAG CAT GCC CTT GT-3']. The IL-12 PCR started with one cycle at 94°C (2 min), followed by 40 cycles at 94°C (1 min), 45°C (1 min) and 72°C (2 min), and a final 7-min extension at 72°C.

Preparation of plasmid DNA

To prepare plasmid DNA for immunization, *Escherichia coli* XL1Blue and DH5 α cells were transformed by electroporation using Cellject Duo according to the manufacturer's directions (Hybaid, Middlesbrough, UK) with the DNA constructs or the vector plasmid alone and then cultured at 37°C in Luria broth supplemented with ampicillin (50 μ g/ml).

The positive clones were confirmed by automatic sequencing carried out following the protocol provided by Applied Biosystems (CA, USA) and analyzed by BioEdit and Blast. The parental vectors, pcDNA3 and pORF were used as negative controls. DNA for immunization was purified using the EndoFree Giga Kit (Qiagen, CA, USA) and was diluted in TE buffer to the final concentration of 1 μ g/ μ l.

Plasmid gene expression in mammalian cells

For the expression of pORF-mIL-12 in mammalian cells, a transient-transfection assay was performed using Lipofectin (Invitrogen) and 1 or 2 μ g plasmid transfected into HeLa cells (2×10^5 cells/well). The cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) (Cultilab, SP, Brazil). After 24 h incubation, the cells were harvested, and total RNA was isolated with Trizol for reverse transcription (RT)-PCR. IL-12 PCR was used as described above. IL-12p70 was detected (80 ng/ml) by ELISA, in the supernatant of transfected HeLa cells.

P10 expression from vector pcDNA3, followed by IFN- γ production

DNA immunization was performed by injecting groups of 5 six-week-old male BALB/c mice intramuscularly in both quadriceps with three doses of 100 μ g of plasmid encoding P10 (pP10), 50 μ g of either pP10 and pcDNA3 vector alone, or 50 μ g of the pcDNA3 vector alone, each in 50 μ l of TE buffer. A total of three immunizations were given at weekly intervals in alternating sites on the left and right hind legs. The mice were euthanized one week after the last immunization, their spleens were isolated and single-cell suspensions were prepared by gentle homogenization in

RPMI medium supplemented with 1% FCS. Cells were suspended and treated with isotonic ammonium chloride to lyse erythrocytes. The splenocytes were washed by centrifugation, suspended in RPMI containing 10% FCS, and dispensed into wells on a microtitering plate (5×10^5 mononuclear cells per well). The cultures were stimulated with 20 μ g/ml of synthetic P10. After 24 and 48-h incubation at 37°C with 5% CO₂, supernatants were collected and IFN- γ was assayed by a sandwich enzyme-linked immunoassay (ELISA) (BD Pharmingen, CA, USA). Splenocytes from animals immunized with pP10 and stimulated with synthetic P10 produced 10 and 15 ng/ml IFN- γ after 24 and 48 h,

Immunoprophylactic treatment of PCM

Therapeutic treatment of PCM

Long term therapeutic treatment of experimental PCM

Figure 1. Summary of the treatment protocols used. **First protocol:** BALB/c mice received 4 weekly injections. Animals were infected and sacrificed 30 or 60 days later. **Second protocol:** BALB/c and B10.A mice were infected intratracheally. Mice received 4 weekly vaccine doses and animals were sacrificed 1 week after the last injection. **Third protocol:** B10.A mice were infected i.t. and one month after infection, they were immunized with the DNA vaccine. The animals were sacrificed one month after the last injection, six months after infection. doi:10.1371/journal.pntd.0001519.g001

respectively. When 50 µg of pcDNA3 was used for immunization, 9 and 11 ng/ml IFN-γ was released by splenocytes at the two examined times.

Ethics statement

This study was carried out as recommended by the Brazilian college of animal experimentation (COBEA). The protocol has been approved by the Ethical Committee on Animal Experimentation of University of São Paulo (Permit number: 039).

Animals

BALB/c and B10.A mice were bred at the Institute of Biomedical Science of University of São Paulo, Department of Immunology animal facility under specific-pathogen-free conditions.

Fungal strain

Yeast cells of the virulent isolate Pb 18 of *P. brasiliensis* were maintained by weekly subculturing on Sabouraud Dextrose Agar and incubation at 37°C. Before experimental infection, 7–10 day-old cells were inoculated into Sabouraud Dextrose Broth and incubated at 37°C for 5–7 days with rotary shaking. Fungal cells were washed three times in phosphate-buffered saline pH 7.2 (PBS) and counted in a haemocytometer. The viability of fungal cells in the inoculum was determined by staining with Janus B (Merck, Darmstadt, Germany) and was greater than 90%.

Intratracheal infection of BALB/c and B10.A mice

BALB/c and B10.A mice (6- to 8-week-old males) were inoculated intratracheally (IT) with 50 µl suspension of 3×10^5 Pb18 yeast cells in sterile saline (0.85% NaCl). Mice were anesthetized i.p. with 200 µL of a solution containing 80 mg/kg ketamine and 10 mg/kg of xylazine (both from União Química Farmacêutica, Brazil). After approximately 10 min, their necks were extended to expose the trachea at the thyroid level and cell suspensions were injected with a 26-gauge needle. The incisions were sutured with 5-0 silk.

Immunization of mice with plasmid DNA

Three different protocols were used (**Fig. 1**). Injections of 50 µg plasmid were given in the quadriceps muscle. **First protocol:** Groups of 10 BALB/c mice were injected with PBS (control), pcDNA3 (50 µg; control), pORF (50 µg; control), plasmid encoding P10 (pP10, 50 µg), plasmid encoding IL-12 (pIL-12, 50 µg) or with both pP10 and pIL-12 (50 µg each). A total of 4 injections were given weekly on alternating sites, on the left and right hind legs. One week after the last injection, mice were infected intratracheally then sacrificed 30 or 60 days later. **Second protocol:** BALB/c and B10.A mice (10 mice per group) were infected intratracheally. One month after infection, the mice received 4 weekly injections of either PBS (control), pcDNA3 (50 µg; control), pP10 (50 µg), pIL-12 (50 µg) or both pP10 and pIL-12 (50 µg each). Mice were sacrificed 1 week after the last

Figure 2. Immunoprophylactic treatment of PCM. Gene immunization was initiated 30 days before fungal challenge. CFUs are from lungs of BALB/c mice infected intratracheally with 3×10^5 yeast cells and subjected to immunization with vectors containing P10 (pP10) and/or IL-12 (pIL-12) DNA insert. Control mice were inoculated with PBS or with vectors without insert. Mice were sacrificed 30 (■) and 60 (□) days after infection. Each bar represents the average counts and standard deviations of CFUs in lungs from 10 animals in each group. Experiments were carried out in triplicate with similar results. ** $p \leq 0.0001$, comparing vector with and without insert; ## $p \leq 0.0001$, comparing untreated and other groups. doi:10.1371/journal.pntd.0001519.g002

injection. **Third protocol:** B10.A mice (10 mice per group) were infected intratracheally. One month after infection, they were treated with 4 weekly injections followed by a monthly booster for 3 additional months (total of 7 injections). The injections were with either PBS (control), pcDNA3, pP10, pIL-12 or both pP10 and pIL-12. The mice were sacrificed one month after the last injection, six months after infection.

Fungal burden in organs of infected mice

Mice were sacrificed and the lungs, liver and spleen were removed. Weighed tissue sections were homogenized and then washed 3 times with PBS and suspended in 1 ml PBS. Suspensions (100 μ l) were inoculated on brain-heart infusion (BHI) agar medium supplemented with 4% FCS and 5% spent culture medium of *P. brasiliensis* (strain-192), streptomycin/penicillin 10 IU/ml (Cultilab) and cycloheximide 500 mg/ml (Sigma, MO, USA). Colonies were counted after 10 days of incubation at 37°C.

Histopathology

Lung sections from sacrificed mice were fixed in 10% buffered formalin for 24 h and embedded in paraffin. Four-micra sections

were stained with haematoxylin-eosin (HE) or silver nitrate (Gomori) and examined microscopically (Optiphot-2; Nikon, Tokyo, Japan).

Cytokine detection

Sections of excised lungs were homogenized in 2 ml of PBS in the presence of protease inhibitors: benzamidine HCl (4 mM), EDTA disodium salt (1 mM), N-ethylmaleimide (1 mM) and Pepstatin (1.5 mM) (Sigma, St Louis, MO). The supernatants were assayed for IL-4, IL-10, IL-12, and IFN- γ using ELISA kits (BD OpTeia, San Diego, CA). The detection limits of the assays were as follows: 7.8 pg/ml for IL-4, 31.3 pg/ml for IFN- γ and IL-10, 62.5 pg/ml for IL-12, as previously determined by the manufacturer.

Statistical analysis

Statistical analyses were performed using GraphPad Prism5 software. The results are expressed as means and standard deviations (SD). The nonparametric Kruskal-Wallis honestly significant difference test was used. *p* values are shown in the Figure legends.

Figure 3. Therapeutic treatment of PCM. Gene immunization started 30 days after infection. CFUs are from lungs of BALB/c (□) and B10.A (■) mice infected intratracheally with 3×10^5 yeast cells and subjected to immunization with vectors containing P10 (pP10) or IL-12 (pIL-12) DNA inserts. Control mice were inoculated with PBS or with vectors without insert. Mice were sacrificed 60 days after infection. Each bar represents the average counts and standard deviations of CFU in lungs from 10 animals in each group. Experiments were performed three times and similar results were achieved. * $p \leq 0.05$, ** $p \leq 0.005$, comparing vector with and without insert; ## $p \leq 0.005$, comparing untreated and other groups. doi:10.1371/journal.pntd.0001519.g003

Results

Colony-forming units (CFU) in mice immunized prior to infection (prophylactic immunization). First protocol

To explore the effects of the plasmid with the P10 insert (pP10) with or without the murine IL-12 gene insert (pIL-12), BALB/c mice were immunized and then infected intratracheally with virulent *P. brasiliensis* Pb 18. Animals were sacrificed after 30 or 60 days, and the fungal burden in the lungs, spleens and livers was determined. The number of lung CFU per gram of tissue was significantly reduced in animals immunized with pP10 and/or pIL-12 compared to controls at both time intervals (**Fig. 2**). Notably, we observed that the empty plasmids (pcDNA3 and pORF) also induced a significant reduction in CFU relative to mice that received PBS alone, which is presumably a result of dendritic cell activation through Toll-like receptor 9 binding of plasmid unmethylated CpG motifs. Immunostimulation by DNA from *P. brasiliensis* also attributed to CpG motifs showed protective effects in susceptible mice [21,22]. Nevertheless, the fungal load measured in CFUs in mice receiving pP10 and/or pIL-12 was significantly lower than that in mice treated with control pcDNA3 and pORF. Livers and spleens from all animals had no detectable fungal cells.

Organ CFUs in mice immunized 1-month after infection (therapeutic immunization). Second protocol

The therapeutic protocol attempts to reproduce the clinical reality of patients presenting to medical attention after developing symptomatic PCM. We studied two mouse strains with different susceptibilities to PCM, BALB/c (susceptible) and B10.A (highly susceptible) [23]. The data showed that immunization with pP10 and/or pIL-12 was therapeutic in mice infected with *P. brasiliensis* for 1 month prior to receiving plasmid immunizations (**Fig. 3**). CFU reductions were significant in infected mice receiving pP10

and/or pIL-12 compared to mice injected with PBS or pcDNA3. In contrast to the first protocol, injection of pcDNA3 after installing PCM was not sufficient to reduce the fungal burden. The most significant reduction in the lung CFUs from B10.A mice was achieved when pP10 and pIL-12 were combined. The CFUs from the livers and spleens were barely detectable in all groups.

Organ CFUs in a long-term infection model of B10.A mice submitted to gene immunization. Third protocol

This protocol allowed us to analyze the efficacy of therapeutic plasmid treatment during long-term infection (six months) of the highly susceptible mouse strain, B10.A. Treatment of mice with PCM using pP10 and/or pIL-12 significantly reduced lung CFUs (**Fig. 4**). However, the impact of pIL-12 alone was not as dramatic as either pP10 alone or pP10 with pIL-12. Notably, treatment with the combination of pP10 and pIL-12 virtually eradicated the infection in all organs examined.

Lung histopathology

The lungs of control animals in each experimental protocol group showed intense inflammation and large numbers of yeast cells, whereas mice receiving pP10 with or without pIL-12 had significantly reduced inflammation, and lower or undetectable fungal cells. Analysis of the lungs of animals from the third protocol (6 months infection) that received control plasmids revealed dense infiltration of inflammatory cells, mainly of macrophages, lymphocytes and epithelioid cells, and numerous fungal cells (**Fig. 5A**). Around the foci of epithelioid granulomas, giant cells were observed. In contrast, there were large areas of normal lung architecture in pP10-immunized mice and a global reduction in the number of granulomas with few yeast cells (**Fig. 5B**). Treatment with pIL-12 resulted in histological findings that were more similar to controls than to pP10-immunized mice (**Fig. 5C**). Importantly, the lungs of mice treated with the

Figure 4. Long term therapeutic treatment of experimental PCM. Gene immunization started 30 days after infection and mice were sacrificed 6 months after infection. CFUs were counted in lungs of B10.A mice infected intratracheally with 3×10^5 yeast cells and immunized with vectors containing the insert encoding P10 (pP10) or IL-12 (pIL-12). Control mice were inoculated with PBS or with vector without insert. Each bar represents the average counts and standard deviations of CFU in lungs from 5 to 10 animals in each group. Experiments were carried out in triplicate, with similar results. ** $p \leq 0.001$, comparing vector with and without insert; ## $p \leq 0.001$, comparing untreated and other groups. doi:10.1371/journal.pntd.0001519.g004

Figure 5. Histopathology of lungs from intratracheally infected B10.A mice. Animals were infected with *P. brasiliensis* for one month, treated with or without vectors carrying P10 or IL-12 DNA inserts according to protocol 3, and sacrificed 6 months after the initial infection. Infected mice treated with (A) control pcDNA3, (B) pP10, (C) pIL-12 DNA, and (D) P10 and IL-12 DNA. Gomori staining; original magnification, 40 $\times$. doi:10.1371/journal.pntd.0001519.g005

combination of pP10 and pIL-12 were mostly histologically normal and no yeast cells were identified (Fig. 5D).

Cytokine assays

Previous studies with BALB/c mice have established that P10 elicits a protective Th-1 immune response [11]. BALB/c and B10.A mice have different genetic backgrounds that strongly influence their response to infection by *P. brasiliensis*. Their different susceptibility to fungal infection depends in part on their capacity to produce pro-inflammatory cytokines, which are often reduced in B10.A relative to BALB/c. IL-4, IL-10, IL-12 and IFN- γ were measured in the lungs of infected B10.A mice and BALB/c. In mice subjected to the second protocol, BALB/c mice responded to pcDNA3 and pP10 gene immunization with significant increase in IFN- γ in the lung homogenate compared to untreated or pcDNA3 treated animals (data not shown). In contrast, B10.A mice produced significantly less IFN- γ after immunization with pP10 and pcDNA3 in comparison to treatment with pcDNA3 gene alone, which suggests that the increase in IFN- γ in both of these groups relative to untreated mice could be due to dendritic cell activation by plasmid CpGs. The cytokine production in the group of animals submitted to the third protocol, in which B10.A mice treated with pP10 with or without pIL-12 had undetectable

yeasts in the lung tissue, is shown in Table 1. After 6 months post-infection, cytokine analyses in these mice showed a persistent IFN- γ production regulated by an IL-10-rich immune response, which is compatible with a protective therapeutic effect in B10.A mice.

Discussion

A vaccine against *P. brasiliensis* using plasmid DNA was first tested in 2000 [24,25]. BALB/c mice were immunized with a

Table 1. Cytokines in lung homogenates of B10.A mice infected i.t. with *P. brasiliensis* Pb 18 yeasts and submitted to gene immunization for 5 months.

Cytokines pg/ml	IL-10	IFN- γ
Untreated	12.18 $\pm$ 4.61	4.71 $\pm$ 1.75
pcDNA3	10.38 $\pm$ 6.53	4.73 $\pm$ 2.45
pP10+pcDNA3	18.71 $\pm$ 6.10	7.64 $\pm$ 2.46
pP10+pIL-12	12.00 $\pm$ 2.06	7.31 $\pm$ 1.37

Mice were sacrificed 1 month after the last dose.
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mammalian expression vector carrying the full gene of the gp43 under the control of CMV promoter with Freund's adjuvant resulting in the induction of both B and T cell-mediated immune responses characterized by a mixed Th-1/Th-2 long-lasting cellular immune response, chiefly modulated by IFN- γ . This immunization method was protective when performed in mice prior to challenge with virulent *P. brasiliensis*. When tested for immunoprotection, P10 in Freund's adjuvant was also active in the murine model of PCM, eliciting an IFN- γ -dependent Th-1 immune response [11,20]. The combined treatment of P10-vaccine in Freund's adjuvant and chemotherapy, either an azole, amphotericin B, or sulfamethoxazole, stimulated a protective Th-1 response, rich in IL-12 and IFN- γ , that was therapeutically beneficial if initiated 2 or 30 days after intratracheal infection [19]. The combined treatment was also effective in anergic animals challenged with the fungus [20].

Presently we used a DNA vaccine encoding P10 with or without a plasmid encoding IL-12 that is administered without adjuvant. We found that the pP10 vaccine, either given prior to or 1 month after intratracheal infection, induced a significant reduction in the fungal burden in the lungs of mice. Co-vaccination with murine pIL-12 significantly enhanced vaccine effectiveness, particularly in a long-term infection model in B10.A mice. The combined DNA vaccine (Protocol 3) achieved virtual sterilization after 6 months with histologically normal lungs and undetectable fungal burden. Full protection was mediated by IFN- γ production and the pro-inflammatory effect of pP10 and pIL-12 was regulated by IL-10 in these susceptible mice.

The mechanism of fungal killing by gene immunization is not solely mediated by cytokines since the empty plasmid pcDNA3 is a strong stimulator of the immune system. However, a significant protection is only achieved with pP10 or pP10+pIL-12 administration. P10 is not protective in IFN- γ -KO mice [12], indicating that this cytokine is essential for fungal killing through macrophage activation. T-CD4 $^{+}$ lymphocytes recognizing P10 and other cells induced by fungal infection are the main producers of IFN- γ . A role for a simultaneous induction of protective antibodies against fungal antigens [26] is also recognized.

IL-12 administration has been previously studied in experimental PCM [27]. Our current results show that IL-12 protected mice against disseminated infection. In the long term infection protocol, pIL-12 alone was only partially effective in the protection of infected mice, but the cytokine facilitated the elimination of *P. brasiliensis* when combined with pP10. This is a very encouraging result and

strongly suggests that a pP10-based vaccine associated with pIL-12 could be used as a powerful adjuvant to chemotherapy.

Despite the effectiveness of chemotherapy, fatalities from invasive or systemic fungal diseases are not uncommon. Vaccines against fungal diseases are gaining increasing attention, owing to their capacity to effectively modulate the immune response (reviewed in [28]. The frequent occurrence of clinical relapses and sequellae, such as pulmonary fibrosis, following antifungal chemotherapy suggest that immunoprotective vaccines could also reduce the incidence of these complications [29].

In addition to our work with P10, there have been other notable attempts to develop vaccine strategies for the treatment of PCM. They included a cDNA encoding the antigenic protein rPb27 [30], the recombinant heat shock protein 60 emulsified in adjuvant [31], radioattenuated *P. brasiliensis* yeast cells [32] and *Mycobacterium leprae* DNAhsp65 plasmid in infected BALB/c mice [33]. Braga *et al.* [34] immunized BALB/c mice either with recombinant purified flagellins (FliC) genetically fused with P10 or with the synthetic P10 peptide mixed with purified FliC. A prevailing Th1-type immune response was obtained that reduced *P. brasiliensis* growth and lung damage in infected mice.

From a practical standpoint, the broad use of antifungal vaccines is not realistic when considering the perspective of a large number of infected people relative to the number of individuals who develop the disease. Mycoses caused by dimorphic fungi, such as PCM, coccidioidomycosis, histoplasmosis and blastomycosis, have low incidence as a deep-seated disease. Certain fungal diseases such as cryptococcosis, aspergillosis and candidiasis typically occur in immunocompromised hosts. Hence, targeted prophylactic vaccination may be a more practical approach to control disease. In the case of PCM, immunization of those at highest risk, such as farmers in highly endemic regions would be reasonable. However, we have also demonstrated that the pP10/pIL-12 combination is highly efficacious after PCM has developed. Therefore, immunization could be most useful in combination with standard therapy in PCM patients in order to enhance treatment efficacy, reduce treatment duration and, perhaps, prevent relapses.

Author Contributions

Conceived and designed the experiments: CPT LRT. Performed the experiments: GMGR JEM AFM. Analyzed the data: JDN CPT LRT. Contributed reagents/materials/analysis tools: CPT. Wrote the paper: CPT LRT JDN.

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Artigo 9

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The role of adjuvants in therapeutic protection against paracoccidioidomycosis after immunization with the P10 peptide

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Paracoccidioidomycosis (PCM), a common chronic mycosis in Latin America, is a granulomatous systemic disease caused by the thermo-dimorphic fungus *Paracoccidioides brasiliensis*. The glycoprotein gp43 is the main antigen target of *P. brasiliensis* and a 15-mer internal peptide (QTLIAHTLAIRYAN), known as P10, defines a major CD4⁺-specific T cell epitope. Previous results have indicated that, besides having a preventive role in conventional immunizations prior to challenge with the fungus, protective anti-fungal effects can be induced in *P. brasiliensis*-infected mice treated with P10 administered with complete Freund's adjuvant (CFA). The peptide elicits an IFN- γ -dependent Th1 immune response and is the main candidate for effective immunotherapy of patients with PCM, as an adjunctive approach to conventional chemotherapy. In the present study we tested the therapeutic effects of P10 combined with different adjuvants [aluminum hydroxide, CFA, flagellin, and the cationic lipid dioctadecyl-dimethylammonium bromide (DODAB)] in BALB/c mice previously infected with the *P. brasiliensis* Pb18 strain. Significant reductions in the number of colony forming units of the fungus were detected in lungs of mice immunized with P10 associated with the different adjuvants 52 days after infection. Mice treated with DODAB and P10, followed by mice treated with P10 and flagellin, showed the most prominent effects as demonstrated by the lowest numbers of viable yeast cells as well as reductions in granuloma formation and fibrosis. Concomitantly, secretion of IFN- γ and TNF- α , in contrast to interleukin (IL)-4 and IL-10, was enhanced in the lungs of mice immunized with P10 in combination with the tested adjuvants, with the best results observed in mice treated with P10 and DODAB. In conclusion, the present results demonstrate that the co-administration of the synthetic P10 peptide with several adjuvants, particularly DODAB, have significant therapeutic effects in experimental PCM.

Keywords: *Paracoccidioides brasiliensis*, paracoccidioidomycosis, P10, adjuvants, dioctadecyl-dimethylammonium bromide, FlC flagellin, aluminum hydroxide, complete Freund's adjuvant

INTRODUCTION

Paracoccidioidomycosis (PCM) is a systemic mycosis that typically starts as a granulomatous pulmonary disease subsequent to the inhalation of conidia of the dimorphic fungus *Paracoccidioides brasiliensis*. When it is not diagnosed and treated properly, *P. brasiliensis* yeast cells can spread rapidly to lymph nodes, tegument, spleen, liver, and lymphoid organs of the digestive tract (Shikanai-Yasuda et al., 2006). PCM is endemic in Latin America, mostly affecting rural workers in Brazil, Colombia, and Venezuela (Wanke and Londero, 1994), and the majorities are involved in agricultural activities (Blotta et al., 1999; Restrepo et al., 2008). In Brazil, approximately 1,853 (~51.2%) of 3,583 confirmed deaths due to systemic mycoses from 1996 to 2006 were caused by PCM (Prado et al., 2009).

gp43 is a glycoprotein of 416 amino acids (Puccia et al., 1986; Cisalpino et al., 1996). A specific T-CD4⁺ cell epitope was mapped to a 15-amino acid sequence designated P10, which is recognized by T cells from mice infected with *P. brasiliensis*. Immunization of previously intratracheally infected BALB/c mice with P10 reduces the fungal load in the lungs more than 200-fold as compared to non-immunized animals (Taborda et al., 1998). P10 immunized animals produced greater amounts of IFN- γ and interleukin (IL)-12. These mice also had significantly reduced damage to lung tissue. In fact, the immune response elicited by P10 prevents the rapid spread of *P. brasiliensis*, and we have hypothesized that P10 administered as with newer adjuvants might enhance the immunoprotection by the peptide. Hence, we have begun to investigate the efficacy of different adjuvants co-administered with

P10. Along this line, we have found FliC flagellin, derived from *Salmonella enterica*, can significantly modify the Th-1 immune response associated with P10 (Braga et al., 2009).

Aluminum hydroxide (Alum) is another adjuvant widely used in human and veterinary vaccines, which is highly effective in eliciting primary immune responses to target antigens. Concerning secondary responses, the adjuvant stimulates a Th-2 immune response that mainly stimulates the production of antibodies. In this sense it is perhaps less suitable for vaccines against intracellular microorganisms (HogenEsch, 2002). Alum has been associated with severe local reactions such as erythema, subcutaneous nodules, and contact hypersensitivity (Baylor et al., 2002).

Promising results have been observed with complete Freund's adjuvant (CFA) and P10 (Marques et al., 2008). However, this adjuvant causes a variety of side effects such as localized injection-site granulomas, hepatic and renal granuloma formation, and necrotizing dermatitis. Therefore, the use of CFA has been limited to experimental immunizations in animal studies. Due to the severity of adverse reactions, this adjuvant has been banned for use in humans as well as for non-experimental veterinary administration (Stills Jr., 2005).

The idea of using cationic lipids (dioctadecyl-dimethylammonium bromide, DODAB) as adjuvants arose from the effective uptake of microparticles by both dendritic cells and macrophages (Lincopan et al., 2009). Cationic polymer particles carry antigen to these phagocytes and can efficiently stimulate antibody production and activate cytotoxic T cells at low antigen dose (Singh et al., 2000; Lincopan et al., 2009). DODAB also induces maturation of dendritic cells (Thiele et al., 2001; Little et al., 2004) with high levels of IL-12 and IFN- γ production, which may be an important benefit in the design of an anti-*Paracoccidioides* vaccine.

In the present work, a comparative appraisal of the various adjuvants is presented aiming to identify which compound produces the most effective immune response to P10 using murine models of PCM.

MATERIALS AND METHODS

ANIMALS

Six male BALB/c mice per group (6- to 8-week old) were housed in polypropylene cages under specific pathogen free conditions. Animals used in this study were bred at University of São Paulo animal facility. All experiments involving animals were conducted and approved by the Ethics Committee of University of São Paulo and conducted in accordance with international recommendations.

FUNGAL STRAIN

Virulent *P. brasiliensis* Pb18 yeast cells were used to infect the animals. The strain was maintained by weekly passage on solid Sabouraud medium at 37°C and yeast cells were used after 7–10 days of growth. Before the experimental infection, the fungus was grown in modified McVeigh–Morton medium at 37°C for 5–7 days (Restrepo and Arango, 1980). Fungal cells were washed in phosphate-buffered saline (PBS, pH 7.2) and counted in a hemocytometer. The viability of fungal suspensions was determined by

staining with trypan blue (Sigma, St. Louis, MO, USA) and was always higher than 90%. The virulence of the Pb18 strain was checked in each experiment by infecting BALB/c mice i.t. and recovering the yeast cells from the infected organs.

INTRATRACHEAL INFECTION OF BALB/c MICE

BALB/c mice were inoculated i.t. with 3×10^5 virulent Pb18 yeast cells/animal, grown on Sabouraud agar and suspended in sterile saline (0.85% NaCl). A maximum volume of 50 μ l was inoculated per mouse. Briefly, mice were anesthetized i.p. with 200 μ l of a solution containing 80 mg/kg ketamine and 10 mg/kg of xylazine (both from União Química Farmacêutica, Brazil). After approximately 5 min, their necks were hyperextended, and the tracheas were exposed at the level of the thyroid and injected with 3×10^5 yeast cells.

PEPTIDE SYNTHESIS AND PURIFICATION

Peptide synthesis and purification was carried out at the Department of Biophysics, UNIFESP as described previously (Taborda et al., 1998). HPLC analysis showed that the synthetic P10 in the amidated form was >90% pure.

IMMUNIZATION OF MICE

Immunization of BALB/c mice (6- to 8-week old males) was initiated 30 days after infection and repeated on days 37 and 44, by the subcutaneous route, with 20 μ g of P10 in presence of the respective adjuvant. The adjuvants used were CFA with subsequent immunizations with incomplete Freund's adjuvant (IFA); Alum 100 μ g/ml; FliC flagellin 5 μ g/animal, and cationic lipid at 0.1 mM/animal. All adjuvants were vortexed with the peptide before immunization. The animals were sacrificed 7 days after the last immunization, at day 52 of infection.

COLONY FORMING UNITS

For each mouse, the lungs, spleen, and liver were excised and weighed immediately after sacrifice. Tissues were individually homogenized in PBS and 100 μ l of this suspension was plated on brain heart infusion medium (BHI; Difco Laboratories, Detroit, MI, USA), supplemented with 4% fetal bovine serum (Gibco, NY, USA) and 5% of the spent culture medium of *P. brasiliensis* 192 isolate (Castañeda et al., 1988), streptomycin/penicillin 10 IU/ml (Cultilab, Brazil), and cycloheximide 500 μ g/ml (Sigma, St Louis, MO, USA). The plates were incubated at 37°C for a period of 10 days. The numbers of colonies were counted and results expressed in colony forming unit (CFU) per gram of tissue.

CYTOKINE ANALYSIS

Lungs of each mouse were macerated with protease inhibitor (Sigma, St Louis, MO, USA) and centrifuged; supernatants of these samples were used for cytokine detection. IL-4, IL-10, TNF- α , and IFN- γ were measured using ELISA kits (BD Biosciences, San Diego, CA, USA). The detection limits of the assays were as follows: 7.8 pg/ml for IL-4, 31.25 pg/ml for IL-10 and IFN- γ , and 15.6 pg/ml for TNF- α , as previously determined by the manufacturer. Cytokine levels present in the supernatant preparations were analyzed using GraphPad Prism 5.

HISTOPATHOLOGY

The lungs were excised, fixed in 10% buffered formalin (Merck, Germany) and submitted to histopathological analysis [(hematoxylin and eosin (H&E) and Masson's trichrome].

STATISTICAL ANALYSIS

Statistics was done using GraphPad Prism 5 software (San Diego, CA, USA). The results were expressed as mean values and standard deviations (SDs) of the indicated values. The non-parametric Tukey's honestly significant difference test was employed. *p*-Values of ≤ 0.05 indicated statistical significance.

RESULTS

COLONY FORMING UNITS IN INFECTED BALB/c MICE IMMUNIZED WITH PEPTIDE 10 (P10)

After 30 days of infection, immunizations were initiated with three weekly doses of P10 with or without the different adjuvants. After 52 days of infection, the fungal load was evaluated by enumeration of CFUs from recovered organs (lung, spleen, and liver). Lungs of mice immunized with P10 along with each of the different adjuvants had a significantly reduced number of CFU compared to controls (**Figure 1**). The order of efficacy for CFU reduction was cationic lipid and P10 $\gg$ CFA/IFA $>$ Alum $>$ FliC. In this model, we did not recover any fungal cells from the spleens or livers of mice that received any of the immunizations with P10 (data not shown), demonstrating the effectiveness of the peptide in the control of experimental PCM. In contrast, the fungal burdens were 625 ± 60 CFU/g of tissue in the spleens and 296 ± 59 CFU/g of tissue in the livers of unimmunized mice.

CYTOKINE PATTERN INDUCED BY IMMUNIZATION WITH P10 ASSOCIATED WITH ADJUVANTS

In comparison with control infected animals, IFN- γ was significantly increased in mice that received P10 with either cationic lipid or FliC (**Figure 2A**). TNF- α levels were significantly increased only in mice treated with P10 and cationic lipid (**Figure 2B**). P10 immunization with either cationic lipid or FliC significantly reduced

levels of IL-4 (**Figure 2C**) and IL-10 (**Figure 2D**). Immunization with P10 and Alum also reduced IL-10 levels, but did not significantly alter the levels of the other cytokines analyzed.

LUNG HISTOPATHOLOGY IN BALB/c MICE INFECTED (i.t.) WITH Pb18 AND IMMUNIZED WITH P10

Lung tissues from mice immunized with cationic lipid with or without P10 were stained with H&E and compared with unimmunized infected tissues as well as uninfected lungs (**Figure 3**). As expected, the non-infected group (**Figure 3A**) showed normal lung tissue, and the unimmunized infected lungs (**Figure 3B**) showed dense cell infiltrates with high numbers of fungal cells disseminated throughout the lung parenchyma. In the case of mice immunized only with the cationic lipid, we observed the formation of loose granulomas with many fungal cells (**Figure 3C**). In contrast, lungs of mice immunized with cationic lipid and P10 showed significantly preserved lung parenchyma without fungal cells (**Figure 3D**). The histological appearances of lungs from mice immunized with either FliC (**Figure 3E**) or Alum (**Figure 3G**) alone were similar to that with cationic lipid alone (**Figure 3C**). Immunization of mice with P10 and FliC resulted in improved granuloma formation in the lungs, which prevents the spread of the fungus, and increased preservation of normal lung parenchyma (**Figure 3F**). Although there was a slight increase in normal lung parenchyma in mice immunized with P10 and Alum, there was poor granuloma formation and large numbers of yeast cells within areas of inflammation (**Figure 3H**).

The amount of pulmonary collagen type I in mice immunized with P10 and FliC, Alum, or cationic lipid were compared with unimmunized infected controls using Masson's trichrome staining. Tissues from control mice revealed abundant collagen I fibers within cellular infiltrates containing large numbers of fungal cells (**Figure 4A**). Although no fungal cells were visualized, lungs of mice immunized with FliC flagellin and P10 nevertheless diffusely displayed increased amounts of collagen (**Figure 4B**). Mice immunized with Alum and P10 showed large granulomas containing fungal cells and the formation of collagen fibers on the granuloma's periphery (**Figure 4C**). In contrast, mice immunized with cationic lipid and P10 displayed preserved lung tissue without increased collagen (**Figure 4D**).

DISCUSSION

The P10 peptide (QTLIAHTLAIRYAN) has important immunoprotective properties that make it a leading candidate for the development of a therapeutic vaccine (Taborda et al., 1998). We have also shown that P10 immunization can be utilized concomitantly with standard antifungal drugs in the treatment of PCM and that co-administration may also prevent disease recurrence (Marques et al., 2008).

The protective effect of P10 is related to the induction of an INF- γ dependent Th-1 immune response (Taborda et al., 1998; Travassos et al., 2007), so it may be stimulated by adjuvants or nanoparticle encapsulation that increase the efficiency of P10 uptake by dendritic cells resulting in the enhanced presentation of the peptide for cellular immune responses (Amaral et al., 2010; Magalhães et al., 2012). In the present work, we show that the association of P10 with cationic lipids led to a significant reduction of

CFU in the lungs of 52-day infected animals (Figure 1). In contrast, animals immunized only with P10 (without adjuvants), had minimal reductions in fungal burden (not shown data). Hence, combining adjuvants, such as cationic lipids, with P10 can generate augmented immune responses mediated by Th-1 cells, directly related to the secretion of IFN- γ (Figure 2A), which leads to peritoneal and lung macrophage activation as well as enhancing their fungicidal effect on yeasts and conidia of *P. brasiliensis* (Buissa-Filho et al., 2008). Immunization performed with P10 associated with antifungal drugs in animals infected with Pb18, has been shown to induce a significant reduction in IL-4 (Marques et al., 2008), which we now show also occurs in the setting of immunizations with cationic lipid and P10 (Figure 2C). Moreover, P10 administration with cationic lipid also significantly reduces levels of IL-10 (Figure 2D). It is worth noting, however, that depending on the degree of inflammation generated by the Th-1 response, Th-2 cytokines are essential for balancing the immune response and reducing the risk of self-damage (Travassos et al., 2008).

Studies with cationic lipids associated with recombinant HSP of *Mycobacterium leprae* have demonstrated the ability of the adjuvant to promote antigen presentation in lymph node cells with production of high levels of IL-12 and IFN- γ , suggesting that cationic lipid can be useful in the formulation of vaccines against intracellular bacteria, as well as against protozoa (Lincofan et al., 2009). IFN- γ is required for the synthesis of TNF- α by macrophages and is essential for the accumulation of these cells and their subsequent differentiation into epithelioid cells. IFN- γ is also responsible for granuloma's formation and maintenance, and, therefore, it plays a vital role in the control of dissemination by fungi such as *P. brasiliensis* (Souto et al., 2000).

Although the cationic lipid/P10 association has demonstrated the best protective response in the setting of short-term infection in our murine model, it is relevant also to consider the protective effect of the association of P10 and FliC flagellin. A significant reduction in the number of CFU was observed and the cytokine profile was similar to that achieved with cationic lipid and P10

FIGURE 3 | Histological sections of murine lungs from i.t. infected BALB/c mice submitted to immunization with P10 associated to cationic lipid. Groups of mice included (A) uninfected controls, (B) unimmunized infected controls, (C) infected and immunized only with the cationic lipid, (D) infected and immunized with cationic lipid plus P10, (E) infected and immunized only with FlIC flagellin, (F) infected and immunized with FlIC flagellin plus P10, (G) infected and immunized only with aluminum hydroxide, (H) infected and immunized with aluminum hydroxide plus P10 after 52 days of infection. H&E staining, $\times 10$ magnification.

(Figures 1 and 2). We have previously shown that prophylactic experiments performed with FlIC flagellin have demonstrated the effectiveness of the association of this adjuvant with P10 allowing for the control of fungal infection in vaccinated mice (Braga et al., 2009). Intranasal immunization carried out with this formulation induced high levels of IFN- γ and IL-12 production by lung cells and suppressed the production of Th-2 cytokines. The formation of compact granulomas (Figure 3F) as well as cytokine levels obtained with FlIC Flagellin and P10, can be associated to the administration route, since present immunizations were

FIGURE 4 | Histological sections of murine lungs from i.t. infected BALB/c mice immunized after 30 days of infection with P10 associated with FlIC flagellin, aluminum hydroxide, or cationic lipid. (A) infected, unimmunized control mice, (B) infected and immunized with FlIC flagellin plus P10, (C) infected and immunized with aluminum hydroxide plus P10, and (D) infected and immunized with cationic lipid plus P10 after 52 days of infection. Masson's trichrome staining, $\times 40$ magnification. Blue staining and arrows indicate type I collagen fibers.

performed subcutaneously, according to Braga et al. (2009), better results are obtained with intranasal immunizations.

In the lung parenchyma, *P. brasiliensis* induces chronic damage leading to the development of pulmonary fibrosis, which is presumably due to persistent antigenic stimulation and an ongoing active immune response. Granulomatous inflammation can increase the formation of connective tissue rich in collagen type I and III, leading to functional changes and subsequent fibrosis in the lung (Naranjo et al., 2010). Since fibrosis is a well-known sequela of PCM, it is therefore important that the adjuvants used do not induce an exacerbated inflammatory response. In fact, the association of cationic lipid and P10 resulted in a significant reduction of pulmonary fibrosis in mice infected with Pb18 (Figure 4D).

In summary, we have shown that the examined regimens combining P10 with different adjuvants results in significantly different protective responses. Administration of P10 with cationic lipid provided the most effective response profile, with the greatest reduction in fungal burden and a Th-1 biased cytokine response that maintained pulmonary architecture without inducing fibrotic injury.

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