UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA

Débora de Fátima Almeida

Fibrose pulmonar na paracoccidioidomicose: influência do fungo e do hospedeiro na fibrogênese

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

Orientador: Prof. Dr. James Venturini

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2017

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Almeida, Débora de Fátima.
Fibrose pulmonar na paracoccidioidomicose : influência do fungo e do hospedeiro na fibrogênese / Débora de Fátima Almeida. - Botucatu, 2017

Dissertação (mestrado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina de Botucatu
Orientador: James Venturini
Capes: 21102007

1. Pulmões - Doenças. 2. Fibroblastos. 3. Fibrose pulmonar. 4. Paracoccidioidomicose. 5. *Paracoccidioides*.

Palavras-chave: Fibroblasto pulmonar; Fibrose pulmonar; *Paracoccidioides*.

"A verdadeira riqueza de um homem é o bem que ele faz ao seu semelhante."

(Mahatma Gandhi)

Dedicatória

Dedico este trabalho a todos os pacientes portadores da Paracoccidioidomicose. Espero que estes achados possam auxiliar futuros estudos.

Agradecimentos

Agradecimentos

À minha ***mãe Fátima*** *e ao* ***meu pai Luiz*** *por todo o amor e cuidado. Por sempre acreditarem na minha capacidade e me apoiarem nas dificuldades da vida.*

Ao meu grande amor ***Bruno Donanzam,*** *por estar do meu lado nos momentos bons e ruins sempre me dando colo nos momentos de fraqueza, puxão de orelha quando foi preciso e acima de tudo por sempre querer o meu bem. Obrigada por me fazer rir, por me dar esperança e por todo o amor.*

Ao meu orientador ***Dr. James Venturini*** *por tornar possível todas as minhas conquistas, me permitir ser sua primeira orientada na iniciação científica e me acompanhar também no mestrado. Foi uma honra fazer parte do grupo tão bonito que é o LIPE. Conviver com você me fez amadurecer tanto na vida profissional como na pessoal. Hoje eu percebo o quanto eu cresci convivendo com você e te desejo cada vez mais sucesso.*

À ***Dra. Tatiani Donato*** *por me ajudar na fase mais difícil do Mestrado e acima de tudo pela amizade. Você não só me ajudou a superar as dificuldades, você tornou esse trabalho possível. Saiba que me inspiro muito no seu jeito leve de viver e sou muito agradecida por toda ajuda.*

Às minhas amigas e companheiras de longa data ***Amanda Ribeiro*** *e* ***Angela Finato*** *que estão comigo desda iniciação científica. Somos pessoas tão diferentes e como foi bom conhecer vocês! Foram longos anos de amizade, aprendizado, superação de dificuldades e trabalho em equipe. Crescemos juntas nesse mundo acadêmico. Vocês marcaram a minha vida e quero que saibam que mesmo que sigamos caminhos diferentes no futuro, sempre guardarei vocês no meu coração.*

Aos meus companheiros do Lipe e todos os "agregados": ***Barbara Mello, Angela Liberali, Raul, Caio, Rodolfo Ferreira, Victor Resende, Karol, Adriely, Bia, Jonatas, Luiz Gustavo, Vitória, Silas, Thainá, Gaby, Anne, Laysla Leite e Pedro Hori,*** *vocês presenciaram todas as coisas boas e ruins, partilharam conselhos, ensinamentos e risadas. Estar do lado de vocês deixou com certeza as coisas mais divertidas e leves por mais que houvesse dificuldades. Muito obrigada por todo o carinho e*

amizade.

Um agradecimento especial para ***Babi Amorim e Karoline Reis*** *por toda a ajuda em todo o meu percurso e por dividir comigo as dificuldades da cultura celular. Vocês foram grandes amigas e me ajudaram até o último minuto, nunca me esquecerei de tudo que fizeram e sempre guardarei vocês no meu coração. Muito obrigada pelo amor, companheirismo e amizade acima de tudo.*

À ***Dr. Maria Sulei Parreira de Arruda*** *pela oportunidade de estagiar no LIPE, pelo incentivo a sempre fazer o melhor, e por todo carinho.*

Às minhas amigas ***Thais Fraga, Camila Marchetti*** *e* ***Luiza Mimura*** *por todos os ensinamentos, conselhos e apoio em toda a minha jornada e formação. Aprendi muito com vocês.*

À ***Dra. Priscilla Maria Ponce Vareda*** *e ao* ***Dr. José Roberto Bosqueiro*** *pelos ensinamentos de Western Blot e pelo carinho.*

Ao ***Prof. Dr. Fábio Porto-Forestí*** *e ao* ***LaGenPe*** *por abrir as portas sempre quando precisei.*

Ao ***Dr. Willian Fernando Zambuzzi*** *e ao* ***Labio*** *pelos ensinamentos e ajuda com o Western Blot no início do mestrado.*

À ***Heloísa*** *e* ***Dra. Elenice Deffune*** *por me acolherem no início do mestrado e ensinar as boas práticas da cultura celular.*

Ao ***Dr. Rinaldo Poncio Mendes*** *por disponibilizar o soro de pacientes para este estudo e toda a ajuda concedida. E também, ao grupo de micoses sistêmicas da Faculdade de Medicina de Botucatu.* ***Thaty, Drica, Lari, Bia e Camila*** *por toda a ajuda e risadas nas disciplinas do mestrado.*

Aos amigos do Instituto Lauro de Souza Lima ***Mariane, Keren, Giovanna, Elderson,*** *e* ***Rodrigo*** *pela parceria e em todas as fases do mestrado e pelas viagens à Botucatu. Às* ***Dra.Vania, Dra. Ana Carla e Dra. Maria Renata*** *pelo acolhimento e ajuda nos momentos de sufoco.*

Aos funcionários da seção técnica de pós-graduação e à Bruna Quirino Jorgetto pela atenção a nós destinada.

Ao programa de Pós-Graduação em Doenças Tropicais e a todos os

funcionários da Faculdade de Medicina de Botucatu por toda atenção e ajuda quando necessário.

Aos funcionários da Faculdade de Ciências da UNESP de Bauru.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de estudos.

Lista de abreviaturas e siglas

ATP: Trifosfato de adenosina
BSA: Albumina do soro bovino
CMX: Cotrimoxazole
COL I: Colágeno tipo I
COL III: Colágeno tipo III
COL IV: Colágeno tipo IV
CONCEA: National Council for the Control of Animal Experimentation
CXCL: C-X-C motif chemokine ligand
DMEM: Dulbecco's Modified Eagle's Medium
DMSO: Dimetilsulfóxido
EMT: Transição epitelial-mesenquimal
EndMT: Transição endotelial-mesenquimal
FA: Forma aguda
FC: Forma crônica
FGFb: Fator de crescimento de fibroblasto básico/ Basic fibroblast growth factor
FP: Fibroblastos pulmonar/ Pulmonary fibroblast
FR: Forma residual
gp43: Glicoproteína de 43KDa
GPY: Glucose-peptone-yeast extract agar
IFN-β: Interferon-beta
IFN-γ: Interferon-gamma
Ig: Imunoglobulina
IL: Interleucina
ITC- Itraconazole
MMP- Metaloproteinase de matriz
MRC-5- Linhagem cellular de fibroblastos pulmonar humano
NFkB: fator nuclear kappa B
PAMPS- Padrões moleculares associados a patógenos
PBS: Phosphate buffered saline
PbSAP: aspartil protease
PCM: Paracoccidioidomicose
PGE2: Prostaglandina E2
SBF: Soro bovino fetal
SDF-1: Stromal cell-derived factor 1
TBST: Tris Buffered Saline with Tween 20
TGF-β1: Fator de transformação do crescimento beta 1
Th: T helper
TIMP: Inibidor tecidual de metaloproteinase/ Tissue inhibitor of metalloproteinase
TLR: Receptor tipo Toll
VEGF: Fator de crescimento endotelial vascular
α-SMA: Alpha-actina de músculo liso / Alpha-Smooth Muscle Actin

Resumo

Resumo

ALMEIDA, D. F. **Fibrose pulmonar na paracoccidioidomicose: influência do fungo e do hospedeiro na fibrogênese.** 2017. Dissertação (Mestrado) – Faculdade de Medicina de Botucatu, Universidade Estadual Paulista, Botucatu, 2017.

Paracoccidioidomicose (PCM) é uma micose sistêmica causada por fungos do gênero *Paracoccidioides*; suas principais formas clínicas são aguda/subaguda e crônica (FC). Restrita à América Latina, a PCM apresenta alta incidência no Brasil, Colômbia e Venezuela, especialmente entre os trabalhadores rurais. A maioria dos pacientes com a forma crônica da doença, mesmo após tratamento eficaz, apresentam sequelas, incluindo fibrose pulmonar (FP). Os problemas sociais, econômicos e psicológicos desencadeados pela FP são subestimados. Apesar da fibrogênese na PCM ser reconhecida como um processo precoce, seus mecanismos não estão totalmente conhecidos. Assim, o presente estudo tem por objetivo avaliar a influência de componentes dos isolados fúngicos e do hospedeiro sobre fibroblastos pulmonares. Foram utilizados fibroblastos pulmonares humanos da linhagem MRC-5 e fibroblastos pulmonares murinos, isolados de camundongos BALB/c. Fibroblastos foram cultivados *in vitro* com exoantígenos de isolados das espécies *P. brasiliensis* (Pb18 e Pb326) e *P. lutzii* (Pb01, Pb8334 e Pb66); gp43 purificada, e soros de pacientes com FC em diferentes momentos do tratamento (antes do tratamento, cura clínica, cura sorológica e cura aparente). Após 24 horas de cultivo, foram avaliadas a proliferação celular e produção de citocinas e fatores de crescimento por fibroblastos murinos e humanos. Nossos resultados demonstraram que componentes do fungo interferem em fibroblastos pulmonares, pela indução da proliferação, aumento dos níveis de TGF-β1 e diminuição dos níveis de IL-6 e VEGF e FGFb. Além disso, nós observamos que exoantigenos induziram altas produções de pró-colágeno I por fibroblastos humanos. Gp43 induziu aumento nos níveis de TGF-β1 em fibroblastos humanos. Em conclusão, nossos resultados demonstram pela primeira vez que componentes de *P.brasiliensis* e *P.lutzzi* interferem na fibrogênese por agir diretamente na biologia de fibroblastos pulmonares. Além disso, evidenciamos que soros de pacientes com FC interferem diferentemente na proliferação e ativação de fibroblastos pulmonares de acordo com cada momento do tratamento.

Palavras-chave: paracoccidioidomicose, fibroblasto, fibrose pulmonar.

Abstract

ALMEIDA, D. F. **Pulmonary Fibrosis in Paracoccidioidomycosis: Influence of fungus and host in Fibrogenesis.** 2017. Thesis (Master) – Faculty of Medicine of Botucatu, Universidade Estadual Paulista, Botucatu, 2017.

Paracoccidioidomycosis (PCM) is an endemic mycosis caused by fungi of genus *Paracoccidioides;* its main clinical forms are acute/subacute form and the chronic form (CF). Endemic in Latin America, PCM presents high incidence in Brazil, Colombia and Venezuela, especially among rural workers. Most part of patients with FC exhibits sequelae even after an effective fungal treatment, presenting pulmonary fibrosis (PF). The social, economic and psychological issues caused by PF are still underestimated. In PCM, fibrogenesis is recognized as an early process; however, its mechanisms are unknown. Thus, the present study aimed to evaluate the modulatory and functional influence of fungal and host components on pulmonary fibroblast. For this, it was utilized human pulmonary fibroblasts of cell line MRC-5 and murine pulmonary fibroblasts isolated of BALB/c mice. Next, cells were cultivated with different concentrations of isolates of *P. brasiliensis* (Pb18 and Pb326) and *P. lutzii* (Pb01, Pb8334 and Pb66); purified gp43, and serum of patients with CF in different moments of treatment (before treatment, clinical cure, serological cure and apparent cure). After 24 hours, cellular proliferation and production of cytokines and grow factors by murine and human pulmonary fibroblasts were evaluated. Our results showed that the fungal components interfered in fibroblast by induce proliferation and increased levels of TGF-β1 and decreased IL-6, VEGF and bFGF. In addition we observed that exoantigens induced high production of pro-collagen I by human fibroblasts. Gp43 induced increased levels of TGF-β1 by human cells. In conclusion, our findings showed at first time that components of *P.brasiliensis* and *P.lutzii* interfere in the fibrogenesis by directly act in the biology of pulmonary fibroblast. Furthermore, we verified that serum of FC-patients interfere in proliferation and activation of pulmonary fibroblasts according to each moment of treatment.

Keywords: paracoccidioidomycosis, fibroblast, pulmonary fibrosis

Sumário

Introdução

1.INTRODUÇÂO

1.1. Paracoccidioidomicose

A Paracoccidioidomicose (PCM) é uma micose sistêmica granulomatosa causada por fungos termo-dimórficos do gênero *Paracoccidioides* [1,2]. A PCM foi descrita pela primeira vez, em 1908, por Adolfo Lutz. O termo paracoccidioidomicose foi instituído em 1971 na reunião de micologistas das Américas em Medellin (Colombia) e persiste, até hoje, como nomenclatura oficial.

O nicho e habitat deste gênero de fungos ainda não foram totalmente caracterizados, porém, sabe-se que colonizam solos úmidos na fase micelial e podem ter como hospedeiro natural o tatu *Dasypus novemcintus* [3,4]. A PCM representa grande impacto na saúde pública em países da América Latina, sendo Brazil, Venezuela e Colômbia os mais acometidos com prevalência de mais de 15.000 casos relatados entre 1930 e 2012 [5]. E no Brasil, foi considerada em 2009 a nona maior causa de mortalidade entre doenças parasitárias [6]. São considerados fatores de risco para a manifestação da doença a desnutrição, tabagismo e consumo de álcool [7]

A infecção ocorre através da inalação de conídeos e/ou fragmentos de hifas (forma infectante) que atingem os pulmões e se transformam em leveduras (forma patogênica) [8]. Uma vez dentro do hospedeiro pode ocorrer disseminação do parênquima pulmonar para os linfonodos regionais, podendo também se disseminar via corrente sanguínea e/ ou linfática [9].

As principais formas clínicas são a forma aguda/subaguda (FA) e forma crônica (FC). A FA é caracterizada por acometer principalmente crianças e adultos jovens, apresenta história clínica de curta duração (mediana de dois meses) e atinge órgãos do sistema fagocítico mononuclear como fígado, baço e linfonodos. A FC acomete em geral adultos com 30 anos ou mais, apresenta história clínica de longa duração (acima de 6 meses) e acomete principalmente pulmões e mucosa das vias aero-digestivas. A maioria dos pacientes acometidos é do sexo masculino, trabalhadores rurais, que assim se tornam impossibilitados de exercerem suas atividades devido aos problemas respiratórios decorridos da doença, levando a diversos problemas socioeconômicos e psicológicos ainda subestimados**.** Pacientes que apresentam sequelas após o tratamento são classificados como FR [10].

Para o tratamento da PCM são utilizadas drogas como os derivados azólicos cetoconazol e itraconazol (ITC) e também a associação sufametoxazol-trimetoprim, também chamada de cotrimoxazol (CMX) [11]. A sulfadiazina é pouco utilizada e a anfotericina B é

reservada para casos mais graves, devido à sua toxicidade. O ITC é atualmente, a melhor escolha para o tratamento da PCM, Cavalcante *et al*. evidenciaram que sua utilização propiciou cura clínica mais precoce e uma melhor tolerância [12].

1.2 Fungos do gênero *Paracoccidioides*

O gênero de fungos *Paracoccidioides* pertence à ordem Onygenales e a família Ajellomycetaceae [13] e é composto por duas espécies: *Paracoccidioides brasiliensis* e o *Paracoccidioides lutzii*. Sendo ainda a espécie *P.brasiliensis* divida em 5 espécies crípiticas: S1a e S1b que compreende isolados distribuídos por toda a América Latina, principalmente Brasil, Argentina e Paraguai [14,15], PS2 composta por isolados venezuelanos e brasileiros; PS3 restrita na Colômbia e PS4 restrita na Venezuela. O isolado Pb01 é filogeneticamente distinto das outras cepas, definido como *Paracoccidioides lutzii* e principalmente isolado em pacientes da região Centro-Oeste [2,14] e região Norte do Brasil, mais precisamente no Estado do Pará [16].

Este gênero ainda possui a característica do termo-dimorfismo, ou seja, possui a capacidade de mudar morfologicamente dependendo da temperatura. O fungo se encontra na forma micelial (forma infectante) e a 37°C no interior do hospedeiro transforma-se na forma leveduriforme (forma patogênica) [8].

Em relação ao metabolismo, são capazes de secretar de 30 à 35 proteases como ubiquitinil hidrolase (família C19) e metaloendopeptidase dependente de ATP (família M41) que são exclusivas desses fungos. Além disso, secretam enzimas que degradam açúcares abundantes em paredes celulares de plantas, indicando que esse grupo pode ser capaz de se alimentar de celulose, enquanto crescem no solo [17].

Em cultura, estes fungos na fase micelial apresentam o crescimento lento (20 – 30 dias), a colônia apresenta micélio aéreo curto de cor branca ou creme e, muitas vezes, apresenta pigmento castanho na parte reversa, quando aderidas ao ágar, apresentam rachaduras e irregularidades no contorno. À temperatura entre 35-37°C, em meio de cultura, a colônia se desenvolve mais rapidamente (8-12 dias), apresenta um aspecto cerebriforme e consistência macia, cor creme e pode ser facilmente removida do substrato. [18].

Sua parede celular é composta principalmente por polissacarídeos como quitina, polímeros de glicose, proteínas e lipídeos podendo variar de acordo com sua morfologia. A quitina apresenta-se em maior quantidade na forma leveduriforme, podendo alcançar 43% do peso seco da parede celular e no máximo 13% da parede celular micelial. A camada externa da parede na fase leveduriforme é constituida por α-1-3glucana predominante e por β-1-3-

glucana, na fase de micélio [19]. Especula-se que essa dinâmica de composição de parede representa um mecanismo de escape do fungo, uma vez que fagócitos humanos são incapazes de digerir a α-1,3 glucana, por produzirem apenas a enzima β- glucanase, sendo capazes de digerir a parede somente quando o fungo se encontra na fase micelial [20].

Os vários isolados podem apresentar diferenças em sua composição e também podem produzir quantidades variáveis de antígenos. Entre as proteínas intracelulares estão: a glicoproteína de 43 kDa (gp43) principal antígeno secretado e o mais específico para o diagnóstico de infecção por *P. brasiliensis* [20–22]; aspartil protease (PbSAP) de 66-kDa [23]; chaperona mitocondrial de choque térmico Mdj1 [24] e proteína gp75 que tem atividade fosfatase [25].

1.3. Resposta Imune na PCM

A resposta imune do indivíduo à infecção pelo *Paracoccidioides* spp determina a evolução da infecção. A resposta pode variar de acordo com o estado nutricional, tamanho do inoculo, carga genética e gênero do indivíduo. A ação do peróxido de hidrogênio (H_2O_2) produzido pelos macrófagos [26], é a principal causa da morte do *Paracoccidioides* spp. A produção deste metabólito é potencializada pela resposta imune adaptativa do tipo Th_1, caracterizada pela produção de IFN-γ pelos linfócitos ativados, atraídos para o sítio da lesão. Qualquer descompasso, deficiência ou alteração nesse processo leva ao estabelecimento da doença e a sua progressão.

O comportamento imunológico é distinto entre as formas clínicas da doença [27–30]. Em pacientes com FA, observa-se produção de citocinas do perfil Th_2/Th_9 (IL-4, IL-5 e IL-9). A produção elevada dessas citocinas acentua a deficiência da resposta imune celular, levando à ausência de reatividade frente a antígenos de *Paracoccidioides* spp. Além disso, as citocinas de perfil Th_2/Th_9 induzem produção muito elevada de anticorpos do isotipo IgG4 [31] que apresentam reduzida capacidade de fixação de complemento e pequena afinidade pelos receptores FcR, fagocitose e, consequentemente, a eliminação do fungo, tornam-se comprometidas.

A FC, que ocorre devido à reativação de focos latentes e, portanto, após o hospedeiro ter organizado uma resposta imune adaptativa eficiente frente ao *Paracoccidioides* spp, apresenta instalação lenta e progressiva. À exceção dos que apresentam a forma grave, pacientes com a FC apresentam resposta Th_1 preservada, isto é, são reatores ao teste intradérmico com paracoccidioidina. Apresentam ainda, intensa produção de citocinas pró-inflamatórias como TNF-α, IL-1β, IL-17 e de peróxido de hidrogênio. Embora esses

mediadores sejam importantes para a eliminação do fungo, sua superprodução induz efeitos deletérios e não conferem proteção. A produção de anticorpos também pode estar elevada, e se caracteriza por imunoglobulinas dos isotipos IgG1 e IgG2 [31], que possuem maior capacidade de fixação de complemento e maior afinidade pelos receptores FcR (IgG1 > IgG2 > IgG4). Embora esse conjunto de elementos seja importante para a eliminação do fungo, a capacidade de lise microbiana não acompanha a multiplicação do fungo e o indivíduo adoece. Por se tratar de processo inflamatório crônico, esses pacientes, em geral, já apresentam fibrose logo no primeiro atendimento clínico, quando se observa produção mais acentuada de TGF-β_1 e do fator de crescimento de fibroblasto básico (FGFb) [32].

1.4. Fibrose Pulmonar na PCM

Após tratamento antifúngico eficaz, pacientes com FC podem apresentar fibrose nos diferentes órgãos comprometidos, os pulmões, particularmente, também exibem o enfisema. Essas sequelas levam ao comprometimento funcional e incapacitação do paciente. A função pulmonar poucas vezes é normal, revelando padrão obstrutivo em 85% dos casos, com frequências iguais de obstrução leve, moderada e intensa [33–35]. Os achados necroscópicos de pacientes com PCM revelam que a fibrose pulmonar é caracterizada por extensas áreas de depósito de colágeno próximas à região hilar, envolvendo outras estruturas como linfonodos, brônquios e artérias. As fibras colágenas se encontram na periferia dos granulomas e se estendem a brônquios e vasos sanguíneos próximos. Além disso, Tuder *et al.* [36] observaram proliferação de fibras reticulares em áreas distintas do processo granulomatoso, indicando que o próprio fungo ou seus componentes podem induzir a produção de colágeno.

Apesar da sua importância, poucos estudos têm focado especificamente a fibrogênese pulmonar que ocorre na PCM. Cock *et al.*[37] demonstraram em modelo experimental murino que esse processo é precoce. Araujo [38], em estudos necroscópicos demonstrou a presença de fibrose em pacientes que não receberam tratamento antifúngico, mais recentemente, Venturini *et al.* [32] demonstraram que a produção de TGFβ1 e FGF por monócitos de pacientes com FC já se encontra elevada no momento do diagnóstico, e em outro estudo, foi demonstrado que após a introdução do tratamento antifúngico, ocorre aumento da fibrose pulmonar [39]. Em geral, durante a evolução da PCM, a função pulmonar encontra-se alterada observando-se hipoxemia com predominância da perfusão sobre a ventilação pulmonar [40]. De acordo com os autores, é possível que o envolvimento pulmonar ocorra na fase precoce da doença, uma vez que pacientes com padrão obstrutivo e misto apresentam envolvimento precoce das vias respiratórias com alterações na difusão e ventilação. Mesmo com a regressão

das lesões radiológicas após o tratamento, não há recuperação da função pulmonar sendo comum a dispneia a grandes e pequenos esforços [34].

1.5. Mecanismos relacionados à fibrose pulmonar

Fibrose é uma patologia associada com um amplo espectro de injúrias que podem ser causadas por diversas razões, incluindo tratamento antibiótico, infecção e exposição ambiental [41–43].

Denomina-se fibrose a formação ou desenvolvimento de tecido conectivo em órgãos ou tecidos quando em processo de cicatrização. Ocorre em determinadas circunstâncias em que há aumento do estroma conjuntivo de um órgão decorrente de cicatrização ou de um processo reacional em que a produção de matriz extracelular pode não estar relacionada com o processo reparativo. Em consequência das modificações na arquitetura do órgão e das alterações na função das células parenquimatosas, podem surgir distúrbios funcionais [44].

Os mecanismos que induzem a fibrose ainda não foram elucidados, porém, acredita-se que seja resultado de uma desregulação durante o reparo tecidual [45]. O reparo tecidual ocorre em 4 estágios: 1- Coagulação, 2- Homeostase, 3-Inflamação, 4-Proliferação e remodelação [46]. Durante a proliferação e remodelação, os fibroblastos proliferam no local da injúria [47] e secretam citocinas incluindo o TGF-β1 que atua de forma autócrina e parácrina para induzir proliferação e/ou eventual diferenciação de fibroblastos em miofibroblastos [48–50]. Estas células secretam matriz extracelular e proteínas incluindo colágeno e fibronectina [45,47,51] que são entrelaçadas para proporcionar um substrato de re-epitelização do tecido [47]. Miofibroblastos também expressam α-SMA, proteína que integra filamentos de actina, proporcionando as células o fenótipo contrátil [52,53]. Embora inicialmente benéfico, o processo de regeneração tecidual pode se tornar patogênico se este continuar desenfreadamente, resultando em remodelamento da matriz extracelular e formação de cicatriz permanente. Em alguns casos, pode até evoluir para falha do órgão e morte.

No ápice da interação célula-célula durante a cura tecidual há duas classes chave de citocinas: mediadores pró-fibróticos e mediadores antifibróticos. O TGF-β1 é o mais bem caracterizado mediador pró-fibrótico na fibrose pulmonar este é um fator de crescimento secretado por um amplo espectro de células, incluindo fibroblastos, com efeitos que são específicos para o tipo celular e tecidual [49,54–58].

Em oposição ao TGF-β1, a prostaglandina E2 (PGE2) é um medidor lipídico anti-fibrótico predominantemente secretado por células epiteliais como indutor de quiêscencia de fibroblastos [59,60]. Ele ainda atua como regulador negativo de TGF-β1, inibindo a

proliferação de fibroblastos e diferenciação em miofibroblastos e secreção de matriz extracelular [48,50,60–62].

Acredita-se que o balanço de reguladores positivos e negativos (TGF-β1 e PGE2, por exemplo) seja necessário para o alcance da homeostase e assim evitar a ativação excessiva de fibroblastos [63]. A rede de colágeno depositada por fibroblastos é regulada continuamente pela síntese do colágeno e seu catabolismo. O "desligamento" do colágeno e outras proteínas da matriz extracelular são controlados por várias metaloproteinases (MMPS) e seus inibidores de metaloproteinases (TIMPs) que são produzidas por granulócitos, macrófagos, células epidermais e miofibroblastos.

Pacientes com fibrose pulmonar apresentam dificuldade ao respirar, obtêm um mau prognóstico e consequentemente uma redução da qualidade de vida [64–68]. Tratamentos para a fibrose pulmonar são limitados. Transplante de pulmão foi considerado a única intervenção até recentemente. Em outubro de 2015 duas drogas Nintedanib (lheim, Ingelheim am Rhein, Alemanha) e perferidone (Shionogi & Co., Ltd., Osaka, Japan; Marnac Inc., Dallas, TX, USA) foram aprovadas nos EUA para o tratamento para a fibrose pulmonar idiopática [69]. Nenhuma dessas terapias é curativa, ambos os tratamentos diminuem, mas, não param ou revertem o progresso da fibrose pulmonar idiopática marcada pela redução do declínio da capacidade vital dos pacientes [70–72]. Ambas as drogas atuam na dinâmica dos fibroblastos inibindo proliferação, diferenciação e produção de TGF-β1. Contudo, nenhuma destas drogas demonstrou promover a sobrevivência ou regeneração das células epiteliais em pulmões fibróticos.

1.6. Papel dos fibroblastos na inflamação

Os fibroblastos são células presentes em diversos tecidos e possuem a capacidade de se diferenciar em outras células, incluindo cartilagem, osso, adipócito e células de músculo liso. Fibroblastos são uma população heterogênea e se origina através de diferentes processos. Entre esses processos estão a migração e diferenciação de células da medula óssea, denominadas de fibrócitos; a diferenciação de células epiteliais através da transição epitelial-mesenquimal (EMT), e sendo recentemente sugerido a diferenciação de células endoteliais através da transição endotelial-mesenquimal (EndMT) [73]. Apesar dos fibroblastos serem uma das células mais abundante do estroma, estas células permanecem relativamente mal caracterizadas em termos moleculares [74]. Esta ausência de marcadores específicos dificulta a caracterização dos subconjuntos dessas células. Porém, algumas proteínas específicas

possibilitam a fenotipagem de fibroblastos como fibronectina, CD90, COL I, III e IV e pró-colágeno [75].

Em resposta a estímulo microbiano, injúria mecânica, sinais derivados de linfócitos e macrófagos, fatores autócrinos e padrões moleculares associados a patógenos (PAMPS) que interagem com receptores de reconhecimento (TLR), fibroblastos podem secretar citocinas, quimiocinas e prostaglandinas [76]. A interação entre fibroblastos e leucócitos através de receptores CD40/CD40L, resulta na ativação do fator nuclear NFkB com níveis elevados de IL-6, IL-8 e ciclooxigenase 2 [77]. Além disso, essas células são responsáveis por definir ativamente o microambiente dos tecidos e desempenharem papel importante na transição da inflamação, visto que a produção inapropriada de quimiocinas como SDF-1, IFN-β e componentes da matriz extracelular por fibroblastos pode afetar diretamente as células do sistema imune levando ao estabelecimento da inflamação crônica [78].

1.7. Racional científico do estudo

A PCM é uma doença endêmica da América latina, e apesar de representar problemas na saúde pública, é negligenciada. Mesmo após o tratamento antifúngico finalizado com sucesso, a maior parte dos pacientes portadores da forma crônica apresenta sequelas, dentre elas a fibrose pulmonar. Como consequência esses pacientes apresentam problemas respiratórios que acabam incapacitando-os muitas vezes de exercerem suas profissões e atividades. Em alguns casos, essa condição pode desencadear problemas psicológicos, e intensificar o quadro de alcoolismo já frequente nesses pacientes.

Até o momento, ainda não foram totalmente elucidados os mecanismos que levam a fibrose durante a PCM, e poucos são os estudos que focam no papel das células pulmonares durante esse processo. Na literatura estudos focam no mecanismo de adesão e infecção de células por parte do fungo [79,80]. Uma lacuna ainda não explorada é o papel do fungo e seus componentes no aspecto funcional e modulador nas células responsáveis pela instalação da fibrose, como fibroblastos e miofibroblastos. Esse racional tem por fundamento estudos patológicos que verificaram que áreas de deposição de colágenos em septo alveolar ocorrem à distância da lesão granulomantosa, sugerindo que componentes do fungo poderiam atuar nesse processo.

Objetivos

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar a influência do fungo *Paracoccidioides* spp. e componentes do hospedeiro sobre aspectos funcionais e moduladores de fibroblastos pulmonares.

2.2 Objetivos Específicos

- Isolar fibroblastos pulmonares murinos de camundongos BALB/c e obter cultura primária;
- Produzir exoantígenos dos isolados pertencentes às espécies *P.brasiliensis* Pb326, Pb18 e *P. lutzii* Pb66, Pb8334 e Pb01;
- Avaliar a proliferação de fibroblastos pulmonares murinos e humanos da linhagem MRC-5 frente a diferentes concentrações de exoantígenos, gp43 e soro de pacientes com FC da PCM nos momentos pré-tratamento, cura clínica, cura sorológica e cura aparente;
- Avaliar a produção de citocinas IL-6, IL-1β, VEGF e TGF-β1 por fibroblastos pulmonares murinos frente aos exoantígenos e gp43 e avaliar a produção de IL-1β, TGF-β1, FGFb e pró-colágeno I por fibroblastos pulmonares humanos frente aos exoantígenos, gp43 e soro de pacientes com FC da PCM nos momentos pré-tratamento, cura clínica, cura sorológica e cura aparente.

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Manuscrito I

4. MANUSCRITO I

An easy method for isolation and differentiation of murine pulmonary fibroblasts

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Key Words: pulmonary fibrosis; tissue dissociation; pulmonary fibroblasts; cell culture

Journal: Journal of Immunological Methods

Abbreviations: EMT, epithelial-mesenchymal transition; EndMT, endothelial-mesenchymal transition; TGF-β, transforming growth factor beta; α-SMA, alpha-smooth muscle actin; PBS,

phosphate-buffered saline; DMEM, Dulbecco's modified eagle medium; TBST, tris buffered saline with tween 20; BSA, bovine serum albumin.

ABSTRACT

Fibroblasts play a pivotal role in tissue repair by differentiation in myofibroblasts and production of extracellular matrix. Fibroblasts are abundant in many tissues and, in lungs, play a critical role in pulmonary fibrosis (PF). The studies of PF are usually restricted to *in vivo* models and, thus, the isolation and study of pulmonary fibroblasts could help in researches of cellular mechanisms as well as trials for new drugs. In the present study, we report an easy method to efficiently isolate and maturate fibroblasts in culture for subsequent functional analysis.

1. Introduction

Fibroblasts are the main cellular constituents of connective tissues that could be originated by different process, such as epithelial-mesenchymal transition (EMT) (Kalluri and Neilson, 2003), endothelial-mesenchymal transition (EndMT) (Frid et al., 2002), and by the differentiation from bone marrow cells, denominated fibrocytes (Schmidt et al., 2003).

Fibroblasts play a central role in tissue repair, proliferating in the injury area (Midwood et al., 2004) and producing cytokines, including the TGF-β1 that acts in paracrine and autocrine form to induce differentiation of fibroblasts into myofibroblasts (Thannickal et al., 2003). These mature cells secrete extracellular matrix and proteins including, collagen and fibronectin (Midwood et al., 2004; Witte and Barbul, 1997) that are interlaced to proportionate a substrate for re-epithelization (Midwood et al., 2004). Myofibroblasts are recognized by expresses α-SMA, a protein that integrates filaments of actin that provide a contractile phenotype (Hinz et al., 2001). Due to the central role in the remodeling and tissue repair, studies have focused on the biology of these cells in the context of fibrosis. Although the fibrosis may present a variety of etiologies, it is generally characterized by the abnormal deposition of extracellular matrix and alteration of architecture of the affected organ (Luzina et al., 2015). The studies of the process and establishment of fibrosis in deep tissues, such as the lungs, have some limitations due to invasive methodologies for collecting patient samples. Thus, the development and improvement of *in vitro* techniques that seek to mimic this environment or provide the development of functional, molecular and biochemical studies are important. There are, at least, two basic methods for establishing primary fibroblast cultures from tissues: the explant method and the tissue dissociation method that may require a prolonged time to promote efficient isolation and stability of culture.

Here, we describe a modified protocol of tissue dissociation based on protocols previoulsy reported in the literature and utilizing the association of type II collagenase and

trypsin, in order to increase the efficiency isolation of mice pulmonary fibroblast. In addition, we also validated the use of these cells in an assay for maturation.

2. MATERIALS AND METHODS

2.1. Mice. Male BALB/c mice were o purchased from Instituto Lauro de Souza Lima (Bauru, Brazil). All mice aged 21 to 45 days old and received a sterile balanced diet, water *ad libitum* and were kept in a ventilated shelf ALERKS-56 housing system (Alesco®, Monte Mor, Brazil). The experimental protocol was performed in accordance with the ethical principles for animal research adopted by the National Council for the Control of Animal Experimentation (CONCEA). This study was approved by the Ethical Committee of School of Sciences (Proc. #760/2016, UNESP, Bauru, Brazil).

2.2. Mouse lung fibroblast isolation and culture. *Naïve* mice were randomly selected and euthanized by intraperitoneal administration of ketamine and xylazine by overdose. After thoracotomy, in aseptic conditions, the lungs were perfused through inoculation of sterile PBS. The lungs were then removed and cut into small pieces and washed three times in PBS plus 1% penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA) during 5 minutes each. After, the pieces were washed in PBS plus 2% antibiotics and 9% DMEM during 5 minutes each. After, the fragments were underwent to two steps of enzymatic digestion. Each digestion consisted in the incubation of lung fragments with solution of 1mg/ml of type II collagenase (Gibco® Life Technologies, Paisley, UK) and Trypsin 0,25% (Gibco® Life Technologies,), under stirring for 45 minutes at 37°C. The result of digestion was centrifuged for 10 minutes at 1500 rpm, re-suspended in DMEM 20% heat-inactivated fetal calf serum (Sigma-Aldrich), penicillin (100 IU/ml), and streptomycin (100 mg/ml) (Gibco® Life

Technologies,). Afterwards, the material was centrifuged and re-suspended in 5 ml of DMEM and filtrated in 70 µm cell strainer. The viability was determined by 0.1% trypan blue staining. Cells were aliquoted into cell culture flasks with 25 cm^2 surface area (Corning Costar® New York, NY, USA) following a proportion of 1 lung/flask (1 ml/ flask) with 4.0 ml complete medium and incubated at 37°C, 5% CO_2 in a humidified chamber. After 24 hours, medium was changed to remove the non-adherent cells. When the cell culture reached 70% of confluence, cells were dispersed by adding trypsin-EDTA (Sigma-Aldrich) for 5 minutes and then transferred to new culture flasks.

2.3. Maturation of fibroblasts. After the 70% of confluence, cells were dispersed by trypsin-EDTA (Sigma-Aldrich) for 5 minutes and then adjusted to $2x10^4$ cells cultivated above round coverslips (13 mm^2) in 24-well plate. After 24 hours, the cells were washed with sterile PBS and stimulated with 10 ng/ml of recombinant TGF-β1 (R&D Systems, Inc. Minneapolis, MN, USA). The maturation process was evaluated 24 hours after the stimuli for α-SMA expression.

2.4. Immunofluorescence. After 24 days of treatment, cells were fixed with methanol at 20°C for 6 minutes and washed with PBS. After, cells were permeabilized with 0.3% of triton X-100, followed by blocking with 5% of skimmed milk for 30 minutes at room temperature. Cells were incubated with the primary antibodies anti CD90 (1:100) (Thy1.2) (Acris Antibodies, Inc, San Diego, CA, USA) and anti α-SMA (1:50) (Santa Cruz, Dallas, Texas, USA) overnight at 4°C in humidified chamber. After, cells were incubated with the secondary antibody anti-mouse biotinylated (1:50) (Vector Laboratories Inc, Burlingame, CA, USA) for 1 hour, and incubation with conjugated streptavidin with fluorescein (Vector Laboratories Inc) at room temperature for 30 minutes. Coverslips were mounted on glass slides using

Fluroshield™ with DAPI (4',6'-diamino-2-phenylindole, dihydrocloride) (Sigma-Aldrich). The observations and photomicrographs were performed under a fluorescence microscope (Olympus), with camera (Q color 5, Olympus).

2.5. Western Blotting. After treatment cells were lysed with RIPA buffer (Sigma-Aldrich) containing protease inhibitor (cOmplete ULTRA Tablets, Mini, EASYpack – Roche Applied Science, Mannheim, GER), sonicated and maintained on ice for 10 min. Then lysates were centrifuged at 14000 rpm at 4°C for 20 minute, the supernatant was collected and protein estimation was performed by Bradford kit (Bio-Rad Kit, Hercules, CA, USA). Next, samples containing 20µg protein were boiled at 100°C for 4 minutes in 30% of the volume in Laemmli buffer, separated by 12% SDS PAGE, transferred to a nitrocellulose membrane (Bio-Rad), blocked with 5% BSA in 1× TBST for 10 min at room temperature (RT) in SNAP i.d. 2.0 (Millipore, Billerica, MA, USA) and incubated with primary antibodies includes anti- α-SMA, (clone B4 - Santa Cruz Biotecnology, Dallas, Tx, EUA) at dilution 1:500 and anti-GAPDH (clone I-19 - Santa Cruz Biotecnology), at 1:200 dilution for 10 min at RT in SNAP i.d. 2.0 (Millipore). After, the samples were incubated with an appropriate secondary antibodies, anti-mouse (sc-2060 - Santa Cruz Biotecnology), conjugated with peroxidase-HRP and anti-goat (sc-2768 – Santa Cruz Biotrcnology) in 3% BSA in 1x TBST for 10 min in RT in SNAP i.d. 2.0 (Millipore). Then protein expressions were visualized with chemiluminescence substrate (SuperSignal™ West Pico Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, USA) and read in a fluorescence system (G:BOX, Syngene, Beacon House, Cambridge, UK). At last, the protein band intensity was quantified by using Image J software.

3. Results

3.1. Mains steps of fibroblast isolation

The main steps to pulmonary fibroblasts isolation are summarized in Figure 1. First step was the perfusion to eliminate the excess of blood cells from the lungs. We used at least the injection of 40 ml of sterile PBS in the heart to effectively remove erythrocytes. In the second step, lungs were fragmented in small pieces and put them in a solution of sterile PBS with antibiotics to remove all contaminants. In the third step, fragments were soaked in a solution of DMEM, antibiotics and PBS in order to allow tissue to slowly absorb culture medium.. The fourth step was the digestion promoted by trypsin and type II collagenase association. After this process, cells are dispersed at supernatant. In the fifth step, cellular and tissue debri were eliminated by filtration in cell strainer. The viability reached more than 95%.

After 24 hours of the isolation, the culture medium was replaced to eliminate the non-adherent cells. The DMEM medium will only support the growth of fibroblasts and other cell types will die or stop proliferating.

After three days of the isolation it was possible to observe cells with morphology similar to fibroblasts. (Figure 2A). The confluence of 70% was observed after 8-10 days. (Figure 2B).

3.2. Identification of fibroblasts and myofibroblats

To confirm the isolation of fibroblasts we determined the expression of CD90 (Thy 1.2) by immunofluorescence, as show in Figure 3A.

Next, we evaluated the ability of these cells to differentiate in myofibroblasts *in vitro.*

Fibroblasts were cultivated and stimulated with recombinant TGF-β1 for 24 hours. Differentiation was confirmed by α-SMA expression by immunofluorescence (Figure 3B) and western blotting (Figure 4). Immunofluorescence allowed to observe actin filaments in cell cytoskeleton.

4. Discussion

As above mentioned, there are two basic methods for establishing primary fibroblast cultures from tissues: the explant method and the tissue dissociation method. In the literature, we found two protocols used to isolate pulmonary fibroblasts. Seluanov et al. (2010) described a protocol using two steps: the first using Liberase™ (Sigma-Aldrich), highly purified type I and II collagenases trigger tissue digestion, and the second step in which the lung-digested fragments are cultivated without any dissociation process. According to the authors, the fibroblasts migrate out of the tissue with 3-5 days as expected to the explant methodology. In the present study, we isolated pulmonary fibroblast by dissociation method using the enzymatic digestion with type II collagenase and trypsin. Although digestion step is composed of two steps, in only 24 hours the fibroblast are adhered, and after 8-10 days it is possible to observe 70% of cell confluence. Trentin et al. (2015) utilized a classic dissociation method with type I collagenase; however, there is no detailed description of the used method.

In the present protocol, we utilized the association of type II collagenase, that degrade the triple-helical native collagen fibrils, and trypsin, that is composed by a mixture of proteases and has digestive strength, promoting the advantage of increased tissue dissociation. Therefore, we consider that the combination of collagenase and trypsin favors the dissociation of fibroblast from tissue without interfere in cell viability.

One of the purposes of using pulmonary fibroblast culture is the possibility to differentiate these cells in myofibroblasts. Myofibroblasts are cells usually involved in establishment of

fibrosis by the ability to produce of extracellular matrix, fibronectin and collagens, also presenting contractile phenotype (Hinz et al., 2001; Peyton et al., 2008). In the present study we confirmed that using this protocol, fresh isolated fibroblasts were able to differentiate in myofibroblasts. Thus, further *in vitro* studies may be performed using our protocol to evaluate, for instance, new therapeutic drugs for pulmonary fibrosis. One important advantage of primary culture is the heterogeneity of genetic background that favors, for instance, mechanistic investigations. Besides the alternative method to *in vivo* studies, primary culture provides better cost-benefit in comparison to cell lines.

Taken together, the use of an easy method to isolate a primary pulmonary fibroblast culture and the possibility to promote the differentiation in myofibroblasts phenotype *in vitro* may be important to better understand the biology of pulmonary environment affected by fibrosis. Moreover it may promote new strategies to test novel anti-fibrotic drugs and pathogen influence on these cells.

5. Acknowledgments

We thank the Laboratório de Genética de Peixes (LAGENPE), School of Sciences, São Paulo State University (UNESP), Bauru, SP, Brazil for the images acquired using the digital microscope camera.

6. Funding

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant #470221/2014-3).

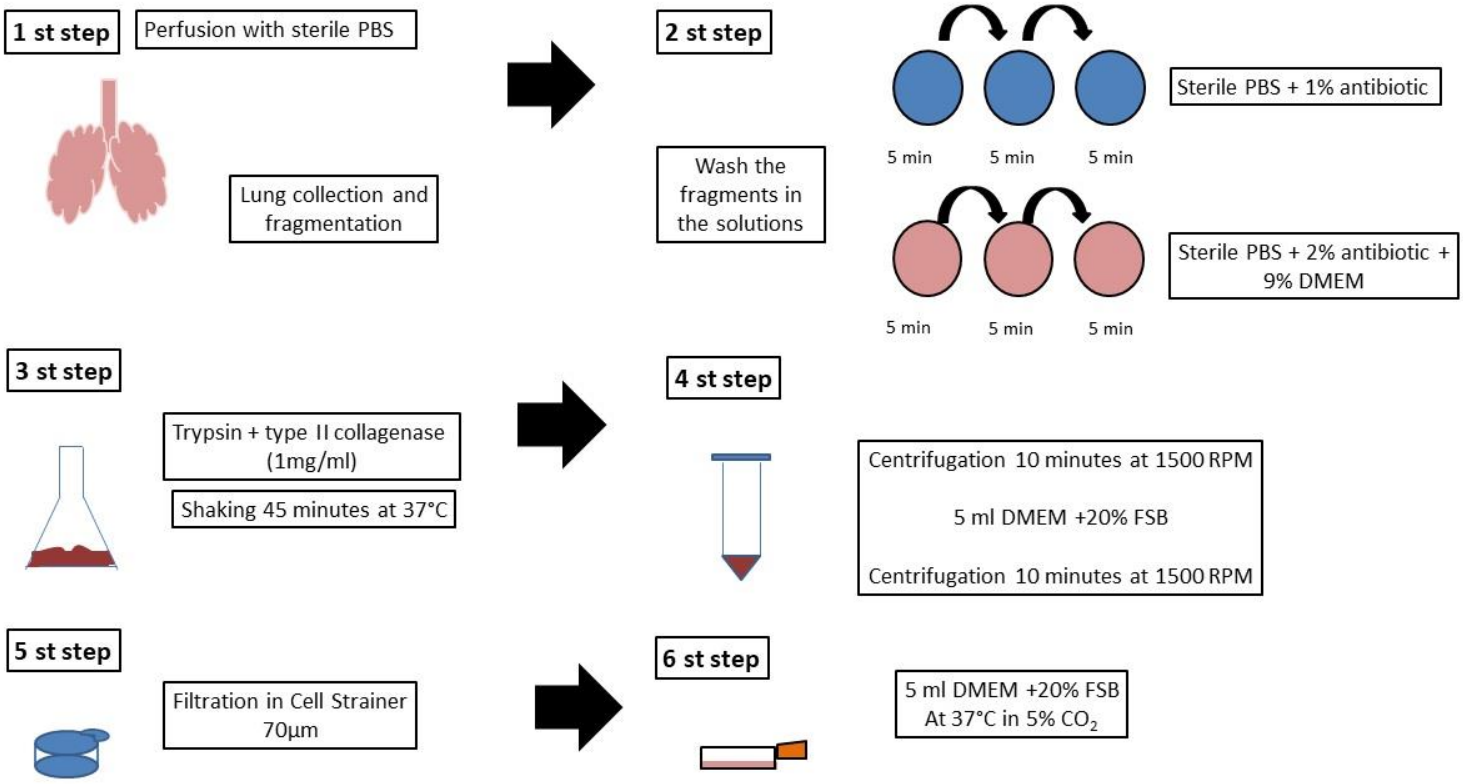

Figure 1. Schematic presentation of six steps for pulmonary fibroblasts isolation.

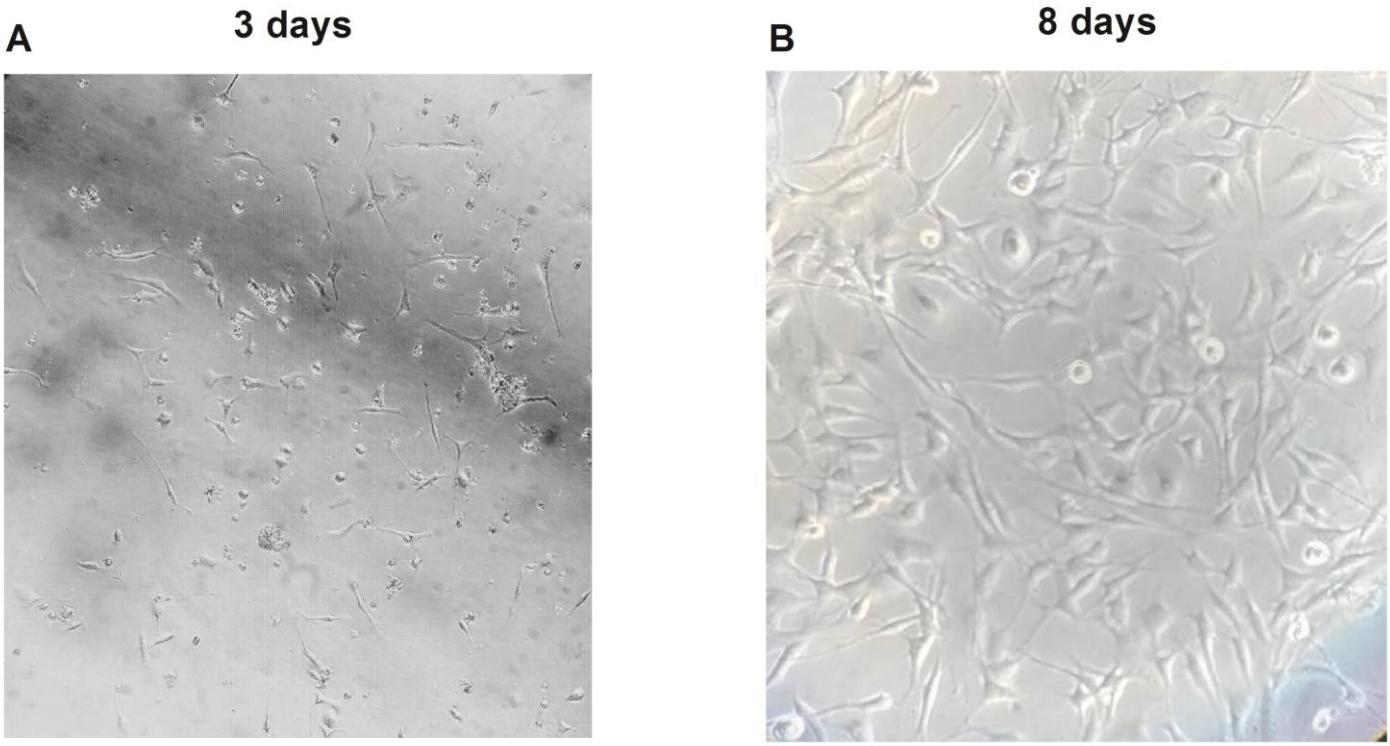

Figure 2. Fresh isolated pulmonary fibroblasts. (A) Third day after isolation, showing a large amount of cells (light microscopy, objective of 10X). (B) Eighth day after isolation, presenting 80% confluence (light microscopy, augment 40X).

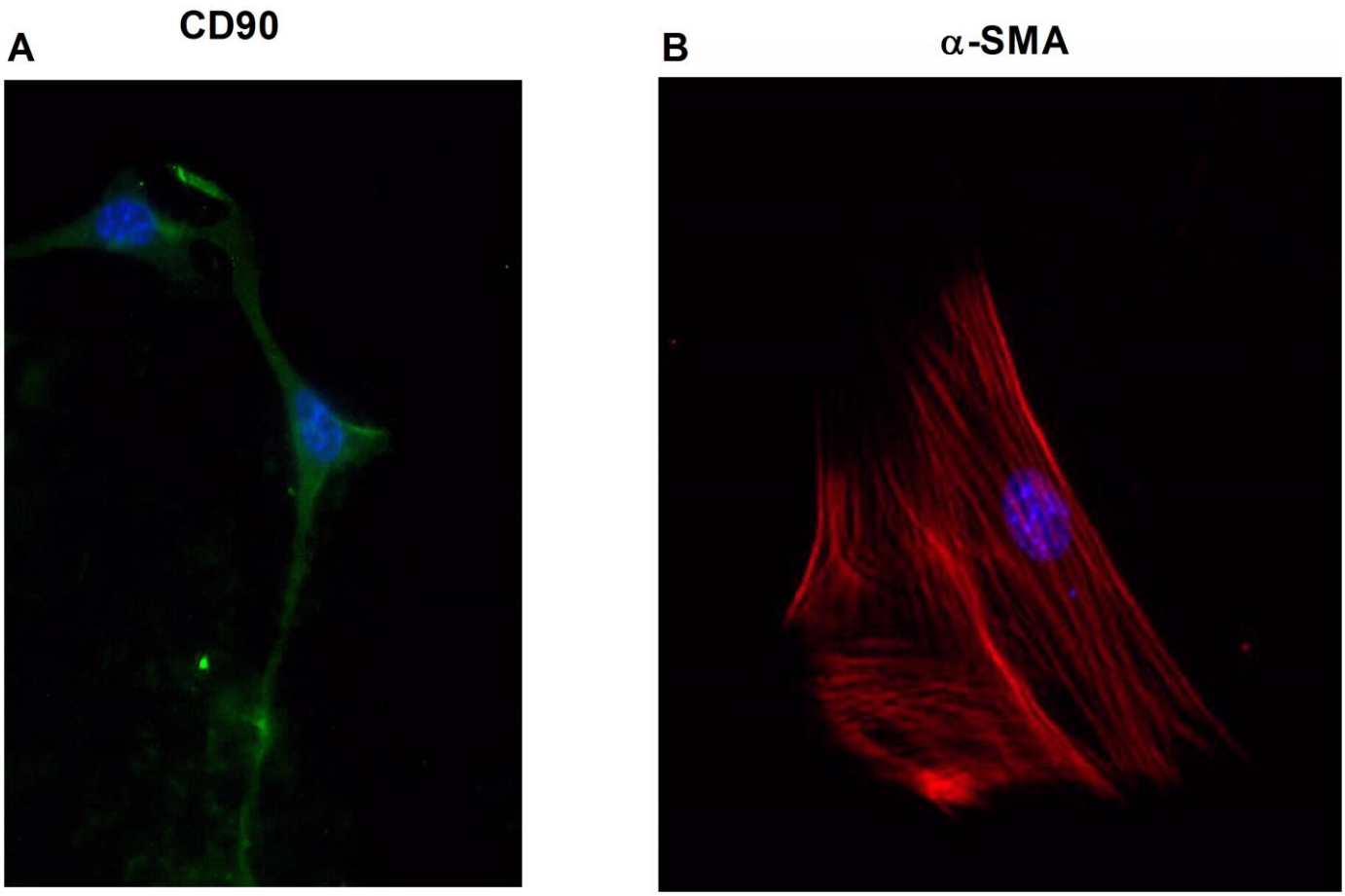

Figure 3. (A) Isolated pulmonary fibroblasts. Pulmonary fibroblasts were harvested after 8 days of culture and seeded in round coverslips for 24 hours and submitted to immunofluorescence determined CD90 expresssion (objective of 40X; green: CD90; blue: nuclei stained with DAPI). (B) Myofibroblast expressing α-SMA by immunofluorescence. Pulmonary fibroblasts were stimulated with 10ηg/ml of recombinant TGF-β1 for 24 hours (objective of 40X; red: α-SMA; blue: nuclei stained with DAPI).

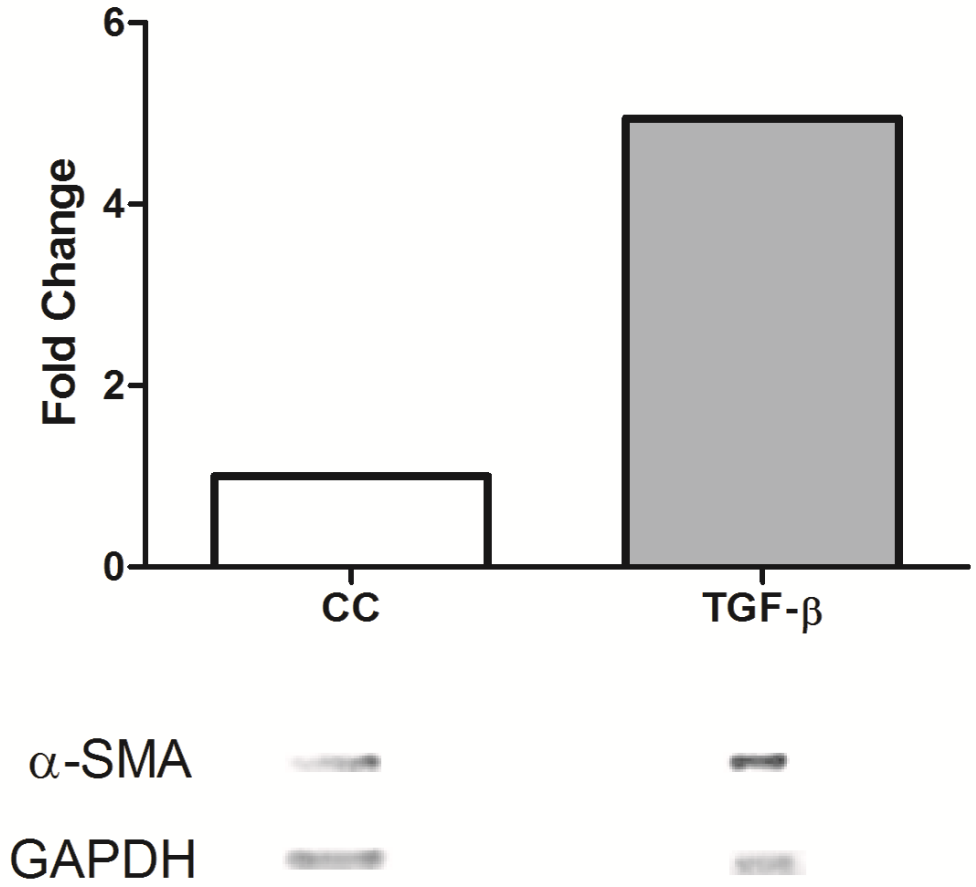

Figure 4. Expression of α-SMA by immunoblotting. Pulmonary fibroblasts were cultivated in the presence or absence of 10ηg/ml of recombinant TGF-β1 for 24 hours. Results are expressed as *fold change* represented by ratio between the tested culture and control culture (CC).

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Manuscrito II

5. MANUSCRITO II

Exoantigens of *Paracoccidioides* spp promote proliferation and modulation of human and mouse pulmonary fibroblasts

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Short title: *Paracoccidioide*s ssp. induce pulmonary fibroblasts proliferation

Key Words: Pulmonary Fibroblasts, Pulmonary Fibrosis, Paracoccidioidomycosis

Journal: PLoS Neglected Tropical Diseases

ABSTRACT

Paracoccidioidomycosis (PCM) is an endemic mycosis caused by fungi of genera *Paracoccidioides;* its main clinical forms are acute/subacute (AF) and the chronic form (CF). Endemic in Latin America, the PCM presents high incidence in Brazil, Colombia and Venezuela, especially among rural workes. Almost part of patients with CF demonstrated sequelae even after an effective antifungal treatment, such as pulmonary fibrosis. In PCM, fibrogenesis is recognized as an early process; however, its mechanisms are unknown. Thus, the present study aimed to evaluate the modulatory and functional influence of fungal and host components on pulmonary fibroblast. Human pulmonary fibroblasts (MRC-5 lineage) and murine pulmonary fibroblasts isolated from Balb/c mice were cultivated with different concentrations of *P. brasiliensis* (Pb18 and Pb326) and *P. lutzii* (Pb01, Pb8334 and Pb66) exoantigens; purified gp43, and serum of patients with CF in different moments of treatment (before treatment, clinical cure, serological cure and apparent cure). After 24 hours, cellular proliferation and production of cytokines and grow factors by murine and human pulmonary fibroblasts were evaluated. Our results showed that the fungal components interfered in fibroblast by induce proliferation ($p < 0.05$) and increased levels of TGF-β1 ($p < 0.05$) and decreased IL-6 ($p < 0.05$), VEGF ($p < 0.05$) and bFGF ($p < 0.05$). We also observed that exoantigens induced high production of pro-collagen I ($p < 0.05$) by human fibroblasts. Gp43 induced increased levels of TGF-β1 ($p = 0.02$) by human cells. In addition, serum of patients with serological cure induced proliferation ($p =0.01$) and production of pro-collagen I ($p = 0.007$) by human cells In conclusion, our findings showed at first time that components of *P.brasiliensis* and *P.lutzii* interfere in the fibrogenesis by directly act in the biology of pulmonary fibroblast. Furthermore, we verified that serum of CF-patients interfere in proliferation and activation of pulmonary fibroblasts according to each moment of treatment.

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INTRODUCTION

Paracoccidioidomycosis (PCM) is a systemic and granulomatous mycosis caused by thermally dimorphic fungi of the genus *Paracoccidioides* [1]. The infection occurs by inhalation of conidia or hypha fragments that reach the lungs and change morphologically to yeast form [2].

The mainly clinical forms are acute/subacute (AF), and chronic (CF) form. The AF is characterized by involvement of organs rich in the mononuclear phagocytic system such as bone marrow, liver, spleen and lymph nodes. CF usually affects male adults, with more than 30 years old and exhibit predominant pulmonary and mucocutaneous involvement. After treatment, patients have to live with pulmonary fibrosis (PF) [3] and emphysema due to smoke. A total of 85% of patients with CF show pulmonary impairment with obstructive pattern [4].

In general PF is associated with a wide spectrum of injuries such as infection, use of antibiotics, and ambient exposure. [5–7]. The establishment of this pathology is still not elucidated, but it is related to a deregulation of wound healing [8]. During the wound healing, fibroblasts proliferate and produce factors related to tissue repair such as, TGF-β1 that acts in paracrine and autocrine form to induce the differentiation of fibroblasts in myofibroblasts [9,10]. Myofibroblasts are cells involved in production of extracellular matrix, fibronectin and collagen, also are characterized by expression of alpha smooth actin (α-SMA), protein that integrates actin filaments and proportionate the contractile phenotype of these cells [11,12]. Although benefic in the beginning, tissue repair can become pathogenic if occur rampantly, resulting in extracellular matrix remodeling and a permanent scar.

The sequela in PCM patients is observed even before treatment since necroscopic findings of patients revel presence of PF, characterized by extensive areas of collagen

deposition, near the hilar region, involving other structures such as lymph nodes, bronchi and arteries, collagen fibers are found on the periphery of granulomas and extend to nearby bronchi and blood vessels [13]. The fibrotic sequelae alters respiratory function and incapacitates the patient [3]. Usually it is observed disproportion between ventilation and perfusion and also alveolar-capillary blockade causing dyspnea.[14]. The most common abnormalities are architectural distortion and interlobular septal thickening and reticulate [15] and residual lesions occurs in up to 53% of treated patients [3].

Cock et al [16] verified that in experimental model of PCM, the PF is an early process with establishment around 8 weeks after infection. Araujo et al [17] verified PF in patients that do not received treatment. Furthermore, Tuder et al [13] verified proliferation of reticular fibers in remote areas of granulomatous reaction, leading to the hypothesis that components of fungus could promote the collagen production. Despite this evidence, the interaction between *Paracoccidioides* ssp and host cells has not yet fully elucidated.

Considering, the possible influences of the fungus *Paracoccidioides* ssp on the development of pulmonary fibrosis and the influence of host immune response on activity of pulmonary fibroblasts, in the present study, we investigated at first time the influence of *Paracoccidioides* ssp components and serum of PCM patients in the proliferation and responsiveness of pulmonary fibroblasts.

MATERIALS AND METHODS

Patients. CF-PCM patients were categorized into four groups according the moment of antifungal therapy: M1) non-treated patients newly diagnosed (n=12). M2) patients under treatment that showed disappearance of the initial symptomatology (clinical cure) but with detectable titles of specific antibodies to *P. brasiliensis* antigen determined by double

immunodifusion test (n=5). M3) patients with clinical cure and undetectable titles of specific antibodies to *P. brasiliensis* antigen (serological cure) determined by double immunodifusion test and under antifungal therapy (n=8). M4) patients with clinical cure, serological cure and without antifungal therapy (n=4). The control group was composed of ten healthy individuals of age- and gender-matched, selected among blood donors from the same geographical area. This study was approved by the Research Ethics Committees of Botucatu Medical School – UNESP (CAEE # 62177516.3.0000.5411). After being informed of the study, written informed consent was obtained from all participating adults.

Paracoccidioides ssp. **isolates.** The isolates of *P.brasiliensis* (Pb18 and isolate Pb326, recently isolated of a patient from Botucatu, SP, Brazil) and *P. lutzii* (Pb01, Pb8334 and Pb66) were maintained by frequent subculture in semi-solid medium containing 2% glucose, 1% peptone, 0.5% yeast extract and 2% agar (GPY) at 36º C.

Exoantigen production. Total exoantigen was produced according to Camargo et al. [18] with modifications. Briefly, yeats forms of *Paracoccidioides* spp, were subcultured in Sabouraud containing 2% of dextrose (Sigma-Aldrich, St Louis, MO, USA) supplemented with 0.01% tiamine (Sigma-Aldrich) and 0.14% L-asparagine (Sigma-Aldrich) and maintained at 37°C, for 3 days. The fungi were cultivated in supplemented Sabouraud broth for three days shaking at 50 rpm at 37°C. Next, more supplemented Sabouraud broth was added and the inoculum was cultivated for more seven days shaking 50 rpm at 37°C. The fungi were killed by the addition of sodium merthiolate (0.2 g/L) for 24 hours at 4°C and filtered in Whatman™ filter paper #1. The filtrate was dialysate against distilled water with several exchanges during 24 hours at 4ºC. The dialysate was then filtered and concentrated by centrifugation with Amicon® Ultra 15 Filter (Millipore, Billerica, MA, USA) at 4000 rpm,

for 30 minutes, at 4ºC. The protein concentration was determined by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Human Lung Fibroblasts. Human lung fibroblast cell line MRC5 cells (ATCC CCL 171) were purchased from Banco de Células do Rio de Janeiro, RJ, Brazil. Fibroblasts were incubated at 37ºC in a humidified 5% CO_2 95% air atmosphere in DMEM (Sigma-Aldrich), containing 10% fetal bovine serum (FBS) (Sigma-Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich). When the cell culture reached 80% confluence, cells were dispersed by adding trypsin-EDTA (Sigma-Aldrich) for 5 minutes and then transferred to new culture flasks (Greiner BioOne, Frickenhausen, BW, GER).

Mice. Male BALB/c, 8-12 weeks old, mice were obtained from Instituto Lauro de Souza Lima, Bauru, SP, Brazil. All mice received a sterile balanced diet, water *ad libitum* and were kept in a ventilated shelf ALERKS-56 housing system (Alesco®, Monte Mor, SP, Brazil). The experimental protocol was performed in accordance with the ethical principles for animal research adopted by the National Council for the Control of Animal Experimentation (CONCEA). This study was approved by the Ethical Committee of School of Sciences (Proc. #760/2016, UNESP, Bauru, São Paulo, Brazil).

Murine pulmonary fibroblast culture. *Naïve* young mice were euthanized by intraperitoneal administration of ketamine and xylazine. After thoracotomy, in aseptic conditions, the lungs were perfused through inoculation of sterile PBS. The lungs were then removed and cut into small pieces and underwent to two steps of enzymatic digestion using collagenase type II (Gibco® Life Technologies, Paisley, UK) and Trypsin 0,25% (Gibco®). The viability was determined by 0.1% trypan blue staining. The cells were aliquoted into 25

cm^2 cell culture flasks (Corning Costar, New York, NY, USA) following a proportional of 1 lung/flask (1 mL/ flask) with 4.0 mL complete medium and incubated at 37°C, 5% CO_2 in a humidified chamber. After 24 hours, the medium was changed to remove the non-adherent cells. When the cell culture reached 70% of confluence, cells were dispersed by adding trypsin-EDTA (Sigma-Aldrich) for 5 minutes and then transferred to new culture flasks.

Experimental design. The study was performed using three protocols: **Protocol I:** Human and murine fibroblasts were incubated with exoantigens of *P.brasiliensis* and *P. lutzii* at concentrations of 2.5, 5, 10, 100 and 250µg/mL and gp43, the immunodominant antigen purified from exoantigen of *P. brasiliensis* [19] at concentrations of 5 and 10 µg/mL for 24 hours, after the cells were submitted to proliferation assay ; **Protocol II:** Human and murine fibroblasts were incubated with exoantigens of *P.brasiliensis* and *P. lutzii* at concentrations of of 2.5, 5, 10µg/mL and gp43 at concentrations of 5 and 10 µg/mL for 24 hours, after cell-free supernatants were submitted to cytokine dosages; **Protocol III:** Human fibroblasts were incubated 10% of serum of health individuals or 10% of serum of CF patients in different moments of treatment. After 24 hours of stimuli, cells were submitted to proliferation assay and the cell-free supernatants were submitted to cytokine dosages.

Proliferation Assay. Proliferation was measured by the MTT assay according to Mosmann (1983) [20]. Briefly, fibroblasts were seeded ($2x10^4$ murine pulmonary fibroblast/well and $1x10^4$ human pulmonary fibroblasts/well) into 96-well culture plates in octuplicate. After 24 hours, the cells were stimulated with 1) *Paracoccidioides* ssp exoantigens in the concentrations of 2.5, 5, 10, 100 and 250µg/mL, 2) gp43 at concentration of 5 and 10 µg/mL, 3) 10% of serum of health or 10% of serum of CF patients in different moments of treatment. After 24 hours of stimuli, the supernatant was collected and the cells incubated in complete

DMEM medium containing MTT (5 mg/mL). The plate was incubated for 2 h at 37 ◦C in 5% CO_2 and then centrifuged for 5 min at 1500 rpm. The supernatant was removed and the cells resuspended in 100 mL of dimethyl sulfoxide (DMSO) per well. After 5 min, the plate was read in a spectrophotometer reader at 540 nm. The percentage of proliferation was calculated by the ratio between the treated culture and control culture, times hundred.

Functional analyses. Human levels of bFGF, TGF-β1, IL-1β, TNF-α, and pro-collagen I or mouse TGF-β1, IL-1β, IL-6, and VEGF were measured in cell-free supernatants and human TNF-α and bFGF in serum of health individuals or CF-PCM patients using a the Duo-Set Kit (R&D Systems, Minneapolis, MI, USA), according to the manufacturer's instructions. The results were expressed in pg/mL, and determined from standard curves established for each assay.

Statistical analyses. The two matched groups were compared using the paired t-test. All statistical analyses were carried out using GraphPad Prism 5.0 software (GraphPad Software. Inc., San Diego, California, USA) at a significance level of 5 % ($p < 0.05$) [21].

RESULTS

Paracoccidioides ssp. exoantigens induced proliferation in murine pulmonary fibroblasts and discrete proliferation in human pulmonary fibroblast

In general, exoantigens of *Paracoccidioides* ssp. induced proliferation of murine pulmonary fibroblast in the lowest concentration (2.5 µg/mL). (Fig 1). The purified gp43 did not promote fibroblast proliferation (Fig 1F). We also challenged human pulmonary fibroblast

with exoantigens of *Paracoccidioides* ssp. Our results showed that fibroblasts slightly proliferate in the presence of 10 µg/mL of Pb18 (Fig 2A); 2.5, 5 and 10 µg/mL of Pb01 (Fig 2B); 5 µg/mL of Pb8334 (Fig 2C). Pb66 exoantigens induced decrease viability in all concentrations (Fig2D). Pb326 exoantigens promoted proliferation at 2.5 µg/mL (Fig 2E). Decrease of viability was also observed in the concentrations of 100 and 250 µg/mL of Pb18, Pb01, Pb8334 and MIC (Fig 2A-C, E); 2.5 and 10 µg/mL of Pb8334 (Fig 2C); 10 µg/mL of Pb326 (Fig2E); and 5 and 10 µg/mL of gp43.

Paracoccidioides ssp. exoantigens decreased the production of IL-6 and VEGF by murine pulmonary fibroblasts and induced intense production of pro-collagen I by human pulmonary fibroblast

To evaluate the effects of exoantigens in the activity of pulmonary fibroblast, we measured the levels of cytokines and grow factors involved in inflammation and wound healing. We verified that all exoantigens triggered decreased production of IL-6 in lowest concentrations in murine pulmonary fibroblasts (2.5, 5 and 10 µg/mL) (Fig 3A-E). We also observed decreased levels of VEGF by murine fibroblasts stimulated with 10 µg/mL of Pb18 (Fig 3A), 2.5 and 10 µg/mL of Pb01 (Fig 3B), 2.5, 5 and 10 µg/mL of Pb66 (Fig 3C), 10 µg/mL of Pb8334 (Fig 3D). We verified increased production of TGF-β1 by murine fibroblasts stimulated with 2.5 µg/mL of Pb66 (Fig 3C), and 10 µg/mL of Pb326 (Fig 3E). Decreased production of TGF-β1 was observed in murine cultures stimulated with 2.5 µg/mL of Pb18 (Fig 3A) and 2.5 µg/mL of Pb326 (Fig 3E). No differences were observed in the levels of IL-6, VEGF and TGF-β1 in murine fibroblasts stimulated with gp43 ($p > 0.05$) (Fig 3F). Levels of IL-1β were not detected in supernatants of murine fibroblast cultures.

The functional analyses revealed intense production of pro-collagen I in human fibroblasts stimulated with 2.5, 5 and 10 µg/mL of Pb18 (Fig 4A), 2.5 µg/mL of Pb01 (Fig

4B), 5 µg/mL of Pb66 (Fig 4C). No differences were observed in human fibroblasts stimulated with P8334 (Fig 4D) and increased production of pro-collagen I was also observed in 2.5 µg/mL of Pb326. We verified that exoantigen of Pb18 decreased the production of TGF-β1 in all concentrations (Fig 4A) and Pb01 do not interfere in the normal production compared to control culture (Fig 4B). However, related to Pb66, we verified a decrease in TGF-β1 levels at 5 µg/mL and an increase at 10 µg/mL (Fig 4C). We also observed an increase of TGF-β1 levels in human fibroblasts stimulated with 5 and 10 µg/mL of Pb8334; 5 µg/mL of Pb326 (Fig 4E).

Furthermore, we evaluated the production of bFGF by human pulmonary fibroblasts; we verified decreased production in cultures stimulated with 2.5, 5 and 10 µg/mL of Pb18 (Fig 4A) and 5 and 10 µg/mL of Pb326 (Fig 4E). No differences were observed in human fibroblasts stimulated with Pb66 (Fig 4C) and Pb8334 (Fig 4D). Gp43 did not interfere in the proliferation and production of pro-collagen I of human pulmonary fibroblast but increased the production of TGF-β1 at 5 µg/mL (Fig 4F). Levels of IL-1 β were not detected in supernatants of human pulmonary fibroblasts

Remarkable alterations in pulmonary fibroblast cell culture are observed after incubation with serum of PCM patients that achieved serological cure.

To evaluate the interference of PCM patient serum in proliferation and activity of pulmonary fibroblasts, we cultivated MCR-5 cells in the presence of serum from health individual and CF-PCM patients categorized into 4 groups according to moments of treatment. The division of the follow-up is useful in both clinical and immunological evaluations during the treatment since each moment is based on biological aspects of each patient, i.e., it reflects the time each patient required to meet the established criteria. Serum of patients staged into M1 induced no alterations in pulmonary fibroblast proliferation as well as

in functional activity (Fig. 5). Proliferation of MCR-5 cells (Fig. 5A) and higher production of bFGF (Fig. 5C) and pro-collagen I (Fig. 5B) was observed in cultures stimulated with serum of patients that reached serological cure (M3), a stage in which the patients show an improvement of specific immunity to the fungi. In M2, when the patients present absence of the inital symptoms, the serum stimulated the production of bFGF (Fig. 5C). We also observed that serum of patients after complete successfully treatment (M4) slightly induced decrease in viability of human fibroblasts ($p = 0.006$) (Fig. 5A).

DISCUSSION

The interaction of *P.brasiliensis* and host cells has been investigated in the mechanisms involved in adherence and mechanism of scape [22–24]. Vicentini et al. [22] verified that extracellular matrix protein laminin binds specifically to yeast forms of *P. brasiliensis* and enhances adhesion of the fungus to the surface of epithelial cells. Ywazaki et al [23] verified adhesion of *P. brasiliensis* in GM1 and GM3 gangliosides of human pulmonary fibroblasts, and this could be the pathway of bound/infection by the fungus. In other study, Mendes-Giannini et al [24] verified interactions between *P. brasiliensis* and epitelial cells of Vero/A549 cell line and these authors suggest that the adhesion and invasion of these cells could represent an escape mechanism and corroborate to spread of infection.

In the present study, we showed at the first time the interaction of *P.brasiliensis* and *P.lutzii* with human and murine pulmonary fibroblasts in the context of fibrogenisis. We verified in the murine cells that low concentrations of exoantigens downregulate production of IL-6 and VEGF and increased cell proliferation. We also observed that the isolates Pb01 and Pb66 of *P.lutzii* and Pb326 of *P. brasiliensis* caused a dose-dependent effect, while the isolates Pb18 of *P.brasilensis* and Pb8334 of *P.lutzii* promoted proliferation independently

exoantigen concentration. It is possible that the observed differences among exoantigens are associated with the variation in fungal composition between species and isolates, once the composition and production of secreted molecules varies among isolates. Machado et al [25] verified differences in composition of isolates from *P. lutzii* (Pb 01 and Pb 8334) and *P.brasiliensis* (Pb18, Epm83 and Pb265) species. Authors suggested that *P.lutzii* present less amount of gp43 compared to *P.brasiliensis*. In our study, we observed that depending on specie, the exoantigen also, promote increase in the production of TGF-β1. This could mean a capacity to promote the differentiation of fibroblasts in myofibroblasts and so corroborating to fibrosis establishment. Also, we observed that the gp43 did not show an important role in the induction of proliferation, but increased the production of TGF-β by human fibroblast.

Paracoccidioides ssp. exoantigens also promoted proliferation of human pulmonary fibroblasts; however, more discreetly. Furthermore, we verified a higher production of pro-collagen I in these cells, corroborating with the idea that exoantigens could participate on the establishment of pulmonary fibrosis. Similar to observed in the murine cells, we verified that gp43 did not participate in proliferation of pulmonary fibroblasts, however, we verified that gp43 promoted increase in production of TGF-β1 levels in human pulmonary fibroblasts. Also, we observed increased production of TGF-β1 in human pulmonary fibroblasts stimulated with Pb66, Pb8334, while in other isolates we verified decreased TGF-β1 levels compared to control culture. Interestingly, in human fibroblasts we verified proliferation but the levels of bFGF were lower than the control. Venturini et al. [26] verified *P. brasiliensis* antigens increased the production of IL1-β, TNF-α, TGF-β1 and bFGF by peripheral blood monocytes of CF-PCM patients, suggesting that fungal metabolites play an important role in the activation of these cells. Therefore, *Paracoccidioides* ssp antigens could participate in the establishment of fibrosis in PCM, activating monocytes that produce high levels of bFGF and TGF-β1, and also, activating fibroblasts that produce TGF-β1 as well, and pro-collagen I.

Other authors suggest the main role of fibroblasts during infections. Recently, Kühbacher et al [27] using a 3D cellular model culture composed by fibroblasts, keratinocytes $TCD4^+$ lymphocytes and mononuclear cells verified that dermal fibroblast transform to a antimicrobial phenotype with high expression of CXCL9, CXCL10 and CXCL11 in the presence of $TCD4^+$ lymphocytes during *Candida albicans* infection, and this process is dependent of TLR2 pathway and by pro-IL-1β cleavage. Our findings showed that both cell types did not produce IL-1 β indicating that exoantigens do not have the capacity alone activating inflamassome and producing IL-1β.

Herein, we also demonstrated that PCM patients serum influence the proliferation and/or functional activities of human pulmonary fibroblasts according to treatment moment. Our findings demonstrated that no alterations were observed in MCR-5 cells cultured in the presence of serum from no treated patients. Considering that before treatment, CF PCM patients present high levels of proinflammatory cytokines in the serum [28–30] and monocytes are able to produce *in vitro* growth factors, such as bFGF [26], we had expected intense proliferation and alterations in the activity of pulmonary fibroblast. Frankel et al [31] demonstrate that MRC-5 cells pre-incubated with TNF-α showed 41% of underwent Fas-induced apoptosis, suggesting that this pro-inflammatory cytokine is able to sensitize fibroblast and induce cell death. We hypothesized that the unresponsiveness of these fibroblasts could be a result of the balance of TNF-α-induced apoptosis and proliferation triggered by growth factors. Thus, we additionally determined the levels of TNF-α and bFGF in the serum of heath individual and PCM patients and, indeed, high levels of TNF-α and bFGF were observed in serum of PCM patients in M1 (Suppl. Figure 1).

Serum levels of patients in M2 showed higher TNF-α levels than normal individuals but it was lower than M1. In this scenario, M2 sera induced enhanced production of bFGF by MCR-5. Therefore, our findings confirm that the TNF-α plays a dual role in fibrogenesis: this

cytokine induces tissue damage and, thus, favor fibroblast and myofibroblast persistence and accumulation [32]. On the other hand, TNF-α also interferes in lung repair by sensitizing fibroblasts and myofibroblasts to cell death [31], and diminishes the pulmonary fibrosis induced by bleomycin-induced mice [33].

Interestingly, remarkable profibrogenic activity was observed in MCR-5 cells cultivated in presence of serum from patients that achieved serological cure (M3). It is well known that PCM patients show decreased serological titers of specific antibodies along the treatment what indicates improvement of immunity against the fungi [34]. In the serological cure, the serum showed the lowest levels of TNF-α and bFGF. Even we failed to determine the levels of TGF-β1 in the serum, we shall not rule out the role of others growth factors acting in the response of pulmonary fibroblast. Therefore our results suggest that during and/or after the recovery of immune response, the process of tissue repair seems to reach its maximal activity. It is important to highlight that our suggestion does not allow to correlate our *in vitro* findings with a possible increment of pulmonary fibrosis in patients after serological cure.

Serum from patients after treatment decreased the proliferation of MRC-5 cell, but induced high production of TGF-β1 by these cells. PCM patients exhibit, after treatment, completely restored cell-mediated immunity to *P. brasiliensis* [35] characterized by increased levels of IFN-γ and IL-2 by peripheral blood mononuclear cells. Frankel et al. [31] also observed that IFN-γ exhibited synergistic activity with TNF-α and promoted increased apoptosis of MRC-5 cells. As we detected high levels of TNF-α in these serums (Suppl Figure 1), it is possible that our findings are associated with the synergism of these cytokines. Intense production of TNF-α in PCM patients after treatment has been previously observed [26,28] and the authors suggest that hypoxia induced by pulmonary sequelae is responsible for continued systemic inflammation. Indeed, several reports have shown that hypoxia continues to interfere in inflammation and fibroblast activity [36–39]. Therefore, our findings suggest

that even after the treatment and the complete recovery of immunity, the activity of fibroblast is still affected by the host environment and could worsen the pulmonary fibrosis in PCM patients.

In summary, our results demonstrate for the first time that: 1) *Paracoccidioides* ssp exoantigens promote pulmonary fibroblast proliferation; 2) gp43 is not related to stimulation of fibroblast proliferation, but may have a role in maturation of fibroblasts to myofibroblasts; 3) Components of host serum interfere in proliferation and response of fibroblast. Our study has some limitations; for instance, the low number of patients enrolled in some moments of treatment; however, our results show, for the first time, the influence of the fungi and the host in the establishment of pulmonary fibrosis in PCM. Further studies to better understand the mechanistic explanations are needed.

ACKNOWLEDGMENTS

We thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for B.C.A. Master Scholarship.

FUNDING INFORMATION

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grants # 470221/2014-3).

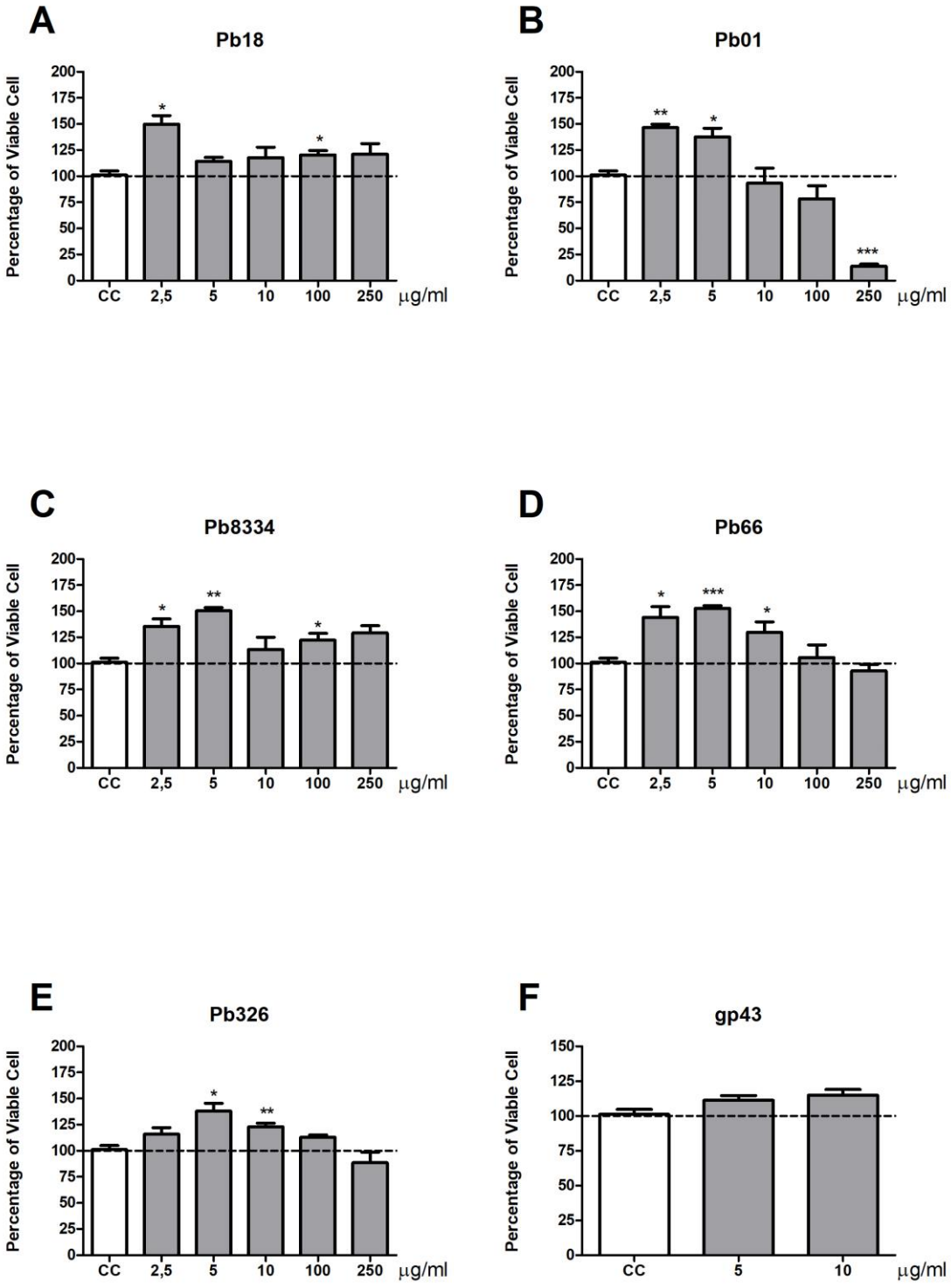


Figure 1. Percentage of viable murine pulmonary fibroblasts. Fibroblasts were cultured in the presence or absence of *Paracoccidioides* ssp exoantigens and, after 24 h were evaluated by MTT assay. A) Pb18 exoantigen, (B) Pb01 exoantigen, (C) Pb66 exoantigen, (D) Pb8334 exoantigen, (E) Pb326 exoantigen, (F) purified gp43. The proliferation was measured by the ratio of test culture (challenged with exoantigens): culture control (CC). Results are expressed as mean ± SEM, paired t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n = 4).

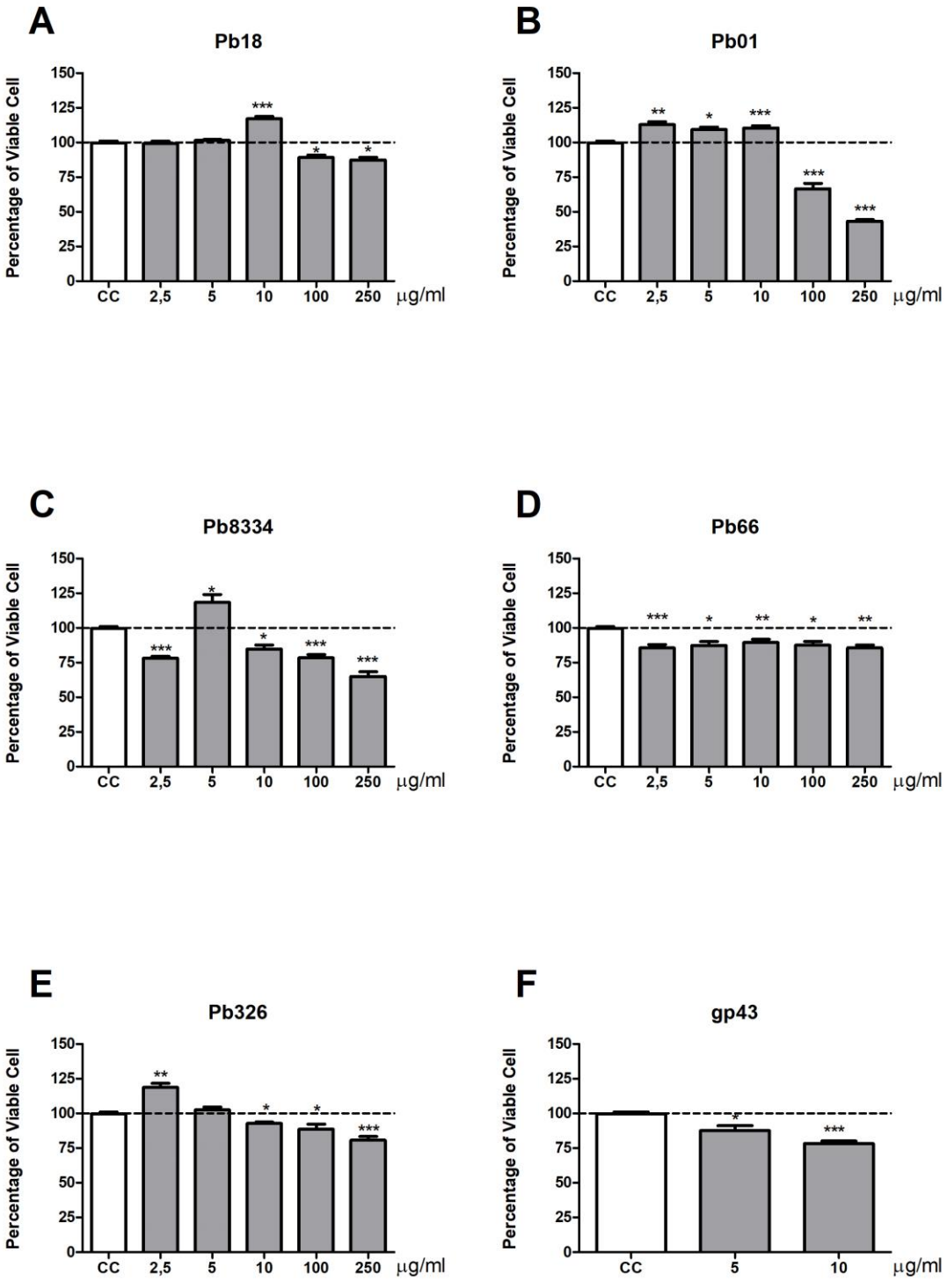


Figure 2. Percentage of viable human pulmonary fibroblasts. Fibroblasts were cultured in the presence or absence of *Paracoccidioides* ssp exoantigens and, after 24 h were evaluated by MTT assay. A) Pb18 exoantigen, (B) Pb01 exoantigen, (C) Pb66 exoantigen, (D) Pb8334 exoantigen, (E) Pb326 exoantigen, (F) purified gp43. The proliferation was measured by the ratio of test culture (challenged with exoantigens): culture control (CC). Results are expressed as mean ± SEM, paired t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n = 4).

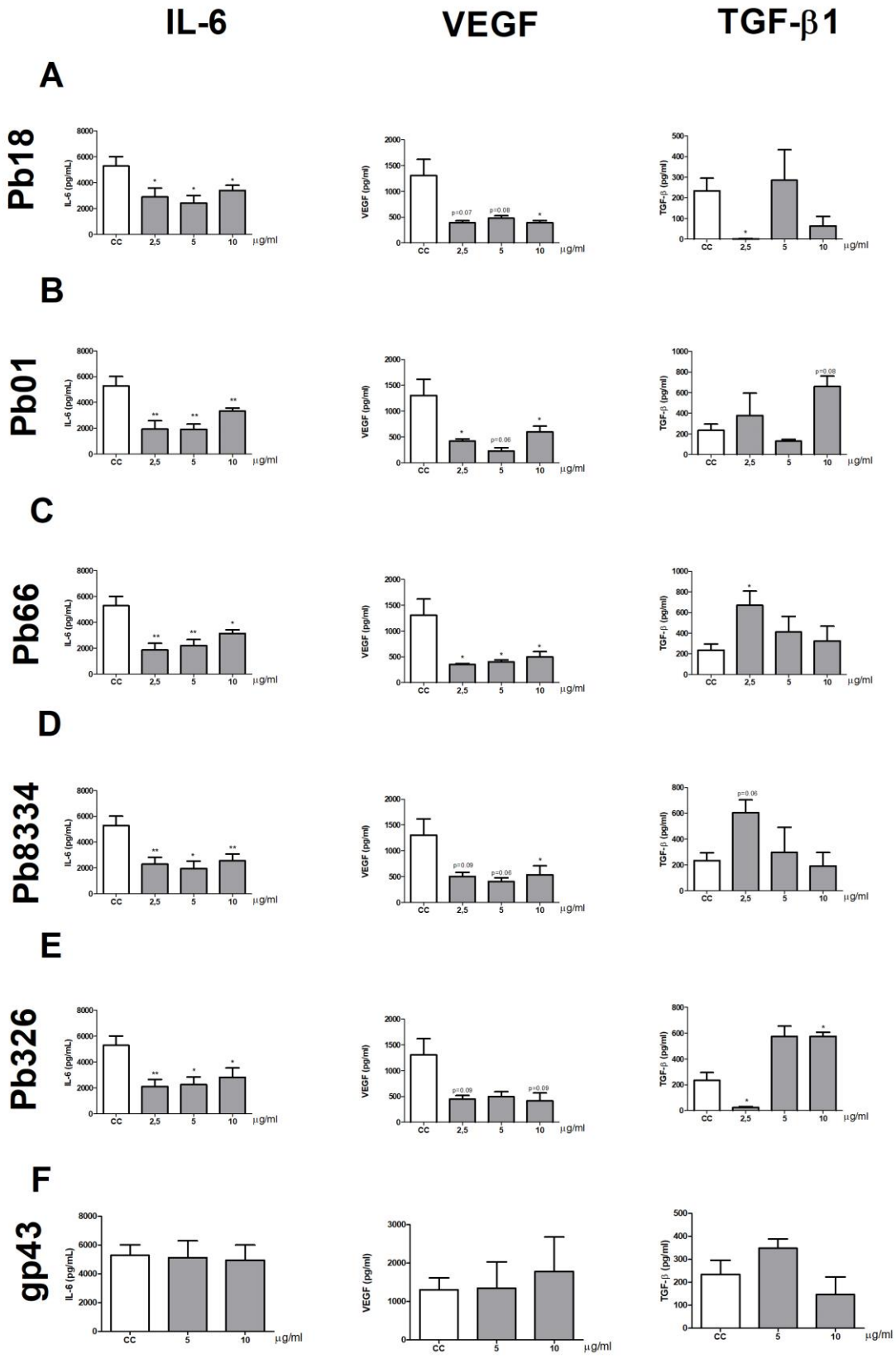


Figure 3 Murine pulmonary fibroblasts activity. Fibroblasts were cultured in the presence or absence of *Paracoccidioides* ssp exoantigens and, after 24 h the levels of IL-6, VEGF and TGF-β1 were determined in cell-free supernatants. (A) Pb18 exoantigen, (B) Pb01 exoantigen, (C) Pb66 exoantigen, (D) Pb8334 exoantigen, (E) Pb326 exoantigen, (F) purified gp43. Results are expressed as means ± SEM. (Paired t-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n = 4).

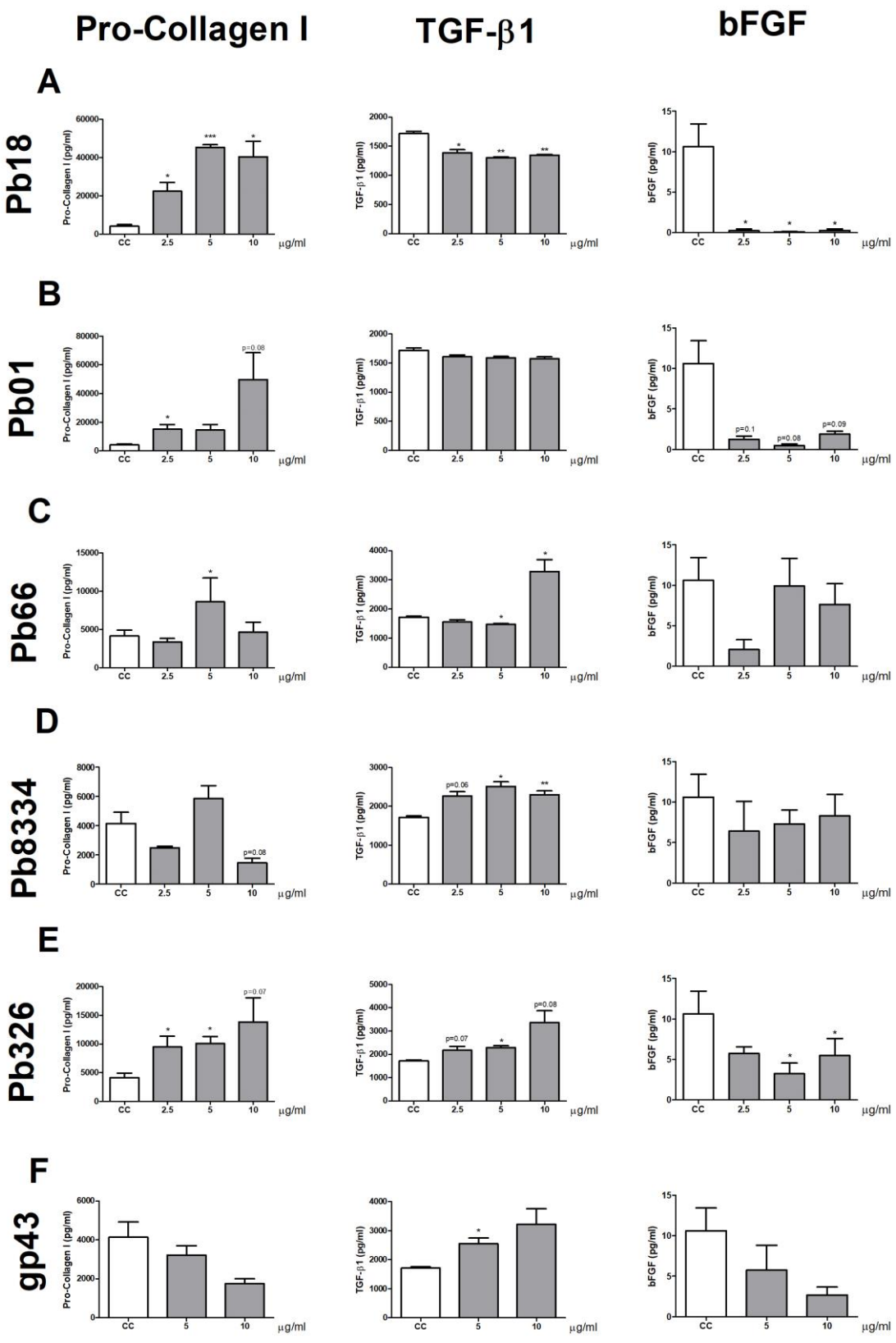


Figure 4. Human pulmonary fibroblasts activity. Fibroblasts were cultured in the presence or absence of *Paracoccidioides* ssp exoantigens and, after 24 h the levels of pro-collagen I, TGF-β1 and bFGF were determined in cell-free supernatants. (A) Pb18 exoantigen, (B) Pb01 exoantigen, (C) Pb66 exoantigen, (D) Pb8334 exoantigen, (E) Pb326 exoantigen, (F) purified gp43. Results are expressed as means ± SEM. (Paired t-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n = 4).

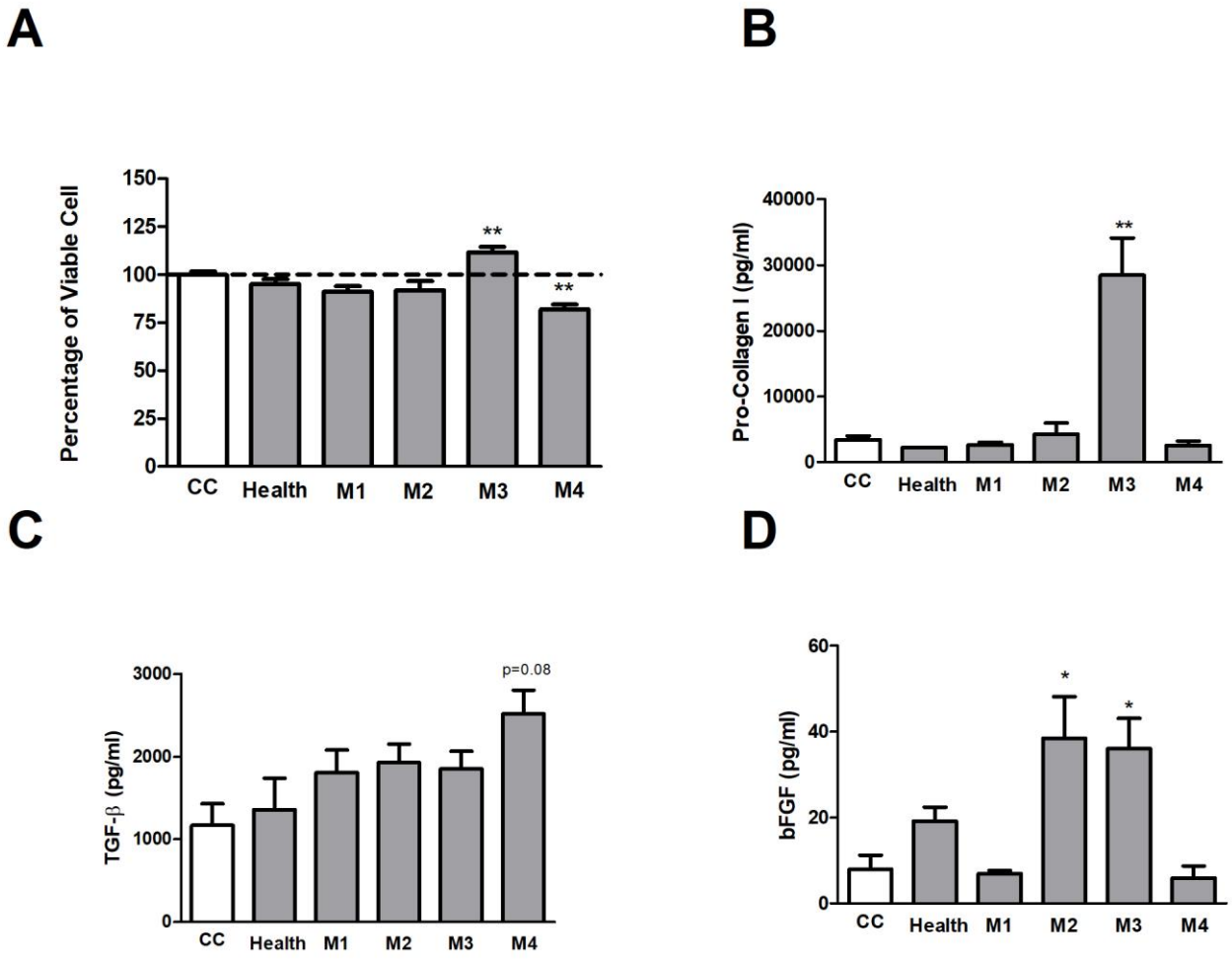
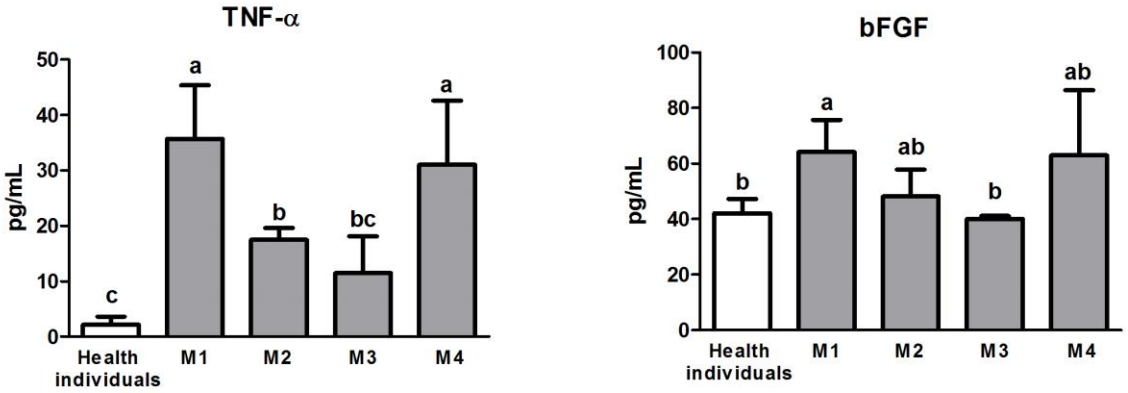


Figure 5. Influence of human serum of PCM patients in the activity of human pulmonary fibroblasts. MRC-5 cells were cultured in the absence (CC) or presence of serum from normal individual and PCM patients in different moments of treatment. M1: composed of non-treated patients newly diagnosed (n=12); M2: composed of patients with clinical cure (n=5); M3: composed of patients with serological cure (n=8); M4: composed of patients with apparent cure (n=4). After 24 hours of incubation, cells were submitted to proliferation assay (A) or determination of levels of pro-collagen I (B), TGF-β1 (C) and bFGF (C) in cell-free supernatants. Results are expressed as means ± SEM. (Paired t-test; * in comparison to CC; $P < 0.05$).



Supplementary Figure 1. Determination of serum levels of TNF-α and bFGF. Serum of PCM patients was distributed according to different moments of treatment. M1: composed of non-treated patients newly diagnosed (n=12); M2: composed of patients with clinical cure (n=5); M3: composed of patients with serological cure (n=8); M4: composed of patients with apparent cure (n=4). Results are expressed as means ± SEM. (Kruskal-Wallis test; * $P < 0.05$).

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Conclusão

6.CONCLUSÃO

Nossos resultados demonstram pela primeira vez uma nova metodologia de isolamento de fibroblastos pulmonares murinos, além de evidenciar a capacidade de exoantígenos de isolados de fungos do gênero *Paracoccidioides* ssp promoverem a proliferação e elevada produção de pró-colágeno I e TGF-β1 em fibroblastos pulmonares. Evidenciamos também que componentes do soro de pacientes com a forma crônica da paracoccidioidomicose interferem diferentemente na proliferação e ativação de fibroblastos pulmonares de acordo com cada momento do tratamento.