UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
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Bárbara Casella Amorim

Análise do inflamassoma na paracoccidioidomicose: correlação entre o tratamento antifúngico e a resposta imune mediada por monócitos e macrófagos alveolares.

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

Botucatu
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Palavras-chave: NLRP3; inflamassoma; monócitos; paracoccidioidomicose.

"A verdadeira viagem de descobrimento não consiste em procurar novas paisagens, mas em ter novos olhos".

(Marcel Proust)

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Resumo

A paracoccidioidomicose (PCM) é micose sistêmica causada por fungos do gênero *Paracoccidioides*. As principais formas clínicas da doença são aguda/subaguda e crônica (FC), sendo que nessa última, a maioria dos pacientes desenvolvem fibrose pulmonar e enfisema. Estudos prévios demonstraram que pacientes FC, na forma ativa da doença, apresentam elevada produção de mediadores inflamatórios, incluindo a IL-1β. Essa citocina, diferente das demais, é produzidas por uma plataforma protéica intracelular denominada inflamassoma que pode ser ativadas por patógenos e sinais de dano do hospedeiro. Considerando-se que os mecanismos de ativação do inflamassoma em pacientes com PCM não são conhecidos, o presente estudo teve por objetivo determinar a expressão de genes envolvidos na ativação do inflamassoma e a produção de citocinas por monócitos e macrófagos alveolares de pacientes com PCM em diferentes momentos do tratamento antifúngico.Nossos resultados demonstram a ativação do NLRP3-inflamassoma, caracterizada pela elevada expressão de *NLRP3*, *CASP1* e *IL1B* por monócitos. Esses achados corroboram a contribuição do NLRP3-inflamassoma na patogênese da PCM também em pacientes.

Palavras-chave: paracoccidioidomicose, inflamassoma, monócitos, tratamento antifúngico

Abstract

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by species of the genus *Paracoccidioides* . The main clinical forms of the disease are acute/subacute (AF) and chronic (CF), and in the latter, most patients develop pulmonary fibrosis and emphysema. Previous studies showed that CF patients in active disease, exhibit elevated production of inflammatory mediators, including IL-1β. This cytokine, different from the others, are produced by an intracellular multiprotein platform called inflammasome that can be activated by pathogens and host signs of damage. Considering that the activation mechanisms of the inflammasome in patients with PCM are not know, this study aimed to determine the expression of genes involved in inflammasome activation and cytokine production by monocytes and alveolar macrophages from PCM patients at different times of antifungal treatment. Our results demonstrate the activation of the NLRP3 inflammasome-characterized by high expression of *NLRP3*, *CASP1* and *IL1B* by monocytes. These findings corroborate the contribution of NRLP3-inflamassome in pathogenesis of PCM also in patients.

Keywords: paracoccidioidomycosis, inflammasome, monocytes, antifungal treatment

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1.INTRODUÇÃO:

1.1. Paracoccidioidomicose

A Paracoccidioidomicose (PCM), descrita por Adolfo Lutz em 1908, é micose sistêmica, granulomatosa, causada pelos fungos termo-dimórficos pertencentes ao gênero *Paracoccidioides* (1,2). Embora seu nicho e habitat natural não sejam totalmente conhecidos, sabe-se que esses fungos vivem em solos úmidos na fase micelial e o único hospedeiro natural bem caracterizado é o tatu *Dasypus novemcintus* (3-5). A instalação da doença ocorre pela inalação de esporos e fragmentos de hifas (forma infectante) que, ao atingirem os pulmões, se transformam em fungos da fase leveduriforme (forma patogênica)(6). Esses fungos se disseminam do parênquima pulmonar para linfonodos regionais, constituindo, em seu conjunto, o complexo primário. Alguns fungos se disseminam, ainda, pela corrente sanguínea e, ou, linfática, podendo atingir qualquer órgão, aparelho ou sistema. (7,8)

Os dados epidemiológicos revelam que a doença é endêmica na América Latina, com elevada prevalência no Brasil, Venezuela e Colômbia, afetando predominantemente indivíduos do sexo masculino (80% no Brasil), com idade entre 30 e 60 anos (9,10). O hormônio feminino estrógeno exerce papel protetor, em mulheres com idade reprodutiva, por inibir a transformação do fungo da fase micelial para a fase leveduriforme, o que favorece a ação de células fagocíticas, principalmente neutrófilos (11,12). Além disso, a maioria dos indivíduos acometidos pela doença são trabalhadores rurais, em fase produtiva, que ao desenvolverem a doença, deixam de exercer suas atividades por problemas respiratórios, levando a diversos problemas socioeconômicos e psicológicos ainda subestimados.(13)

As principais formas clínicas da PCM são aguda/subaguda e crônica. A forma aguda/sub-aguda, também chamada juvenil, em geral compromete crianças, adolescentes e adultos jovens, apresenta história clínica de curta duração (mediana de dois meses) e exibe manifestações clínicas compatíveis com o comprometimento do sistema fagocítico mononuclear. A forma crônica em geral compromete adultos com mais de 30 anos de idade, que apresentam doença de longa duração (em geral acima de seis meses), que acomete pulmões e mucosa das vias aerodigestivas superiores com grande freqüência. Muitos pacientes apresentam seqüelas após tratamento da PCM e são classificados como apresentando as formas residuais. (14,15)

O tratamento da PCM é realizado utilizando-se a associação sufametoxazol-trimetoprim, também chamada de cotrimoxazol (CMX), e os derivados azólicos cetoconazol e itraconazol(ITC)(16-19)

A sulfadiazina é pouco utilizada e a anfotericina B é reservada para casos mais graves, devido à sua toxicidade. Apesar da grande experiência de vários serviços no tratamento da PCM, são raros os estudos duplo-cegos e randomizados. Em 2014, Cavalcante e colaboradores estudaram a comparação entre a utilização do CMX e ITC no tratamento da PCM e observaram que o ITC propiciou cura clínica mais precoce e uma melhor tolerância, confirmando seu uso como a melhor escolha para o tratamento, exceto nos casos em que acomete o sistema nervoso central (20).

1.2. Imunidade na PCM

A resposta imune do indivíduo à infecção pelo *Paracoccidioides* spp determinará a evolução da infecção, uma vez que o desenvolvimento e a progressão da PCM apresentam forte dependência da sua relação com o hospedeiro, o parasita e o meio ambiente (21). Em sua configuração mais ampla, a imunidade do hospedeiro frente a *Paracoccidioides* spp está relacionada a três aspectos fundamentais: 1) a PCM é doença endêmica e acomete indivíduos saudáveis, isto é, que não apresentam condição imunossupressora prévia, como neoplasia ou uso de drogas que afetam a resposta imune, 2) o comprometimento da resposta imune está associado à deficiência específica a antígenos do fungo, ou seja, o paciente possui imunidade preservada para outros antígenos. (22), 3) a resposta do indivíduo ao desafio fúngico depende de fatores, como carga genética, gênero, estado nutricional e tamanho do inóculo inalado.

A morte do *Paracoccidioides* spp ocorre pela ação do peróxido de hidrogênio (H_2O_2) produzido pelos macrófagos (23), que são potencializados pela resposta imune adaptativa do tipo Th_1, caracterizada pela produção de IFN-y 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. Além disso, com a deficiência da resposta Th_1, que em geral inibe a produção de anticorpos, os pacientes passam a apresentar títulos elevados de anticorpos específicos circulantes.

O comportamento imunológico é distinto nas formas aguda/subaguda (FA) e crônica (FC) (24-27). Na FA, que se caracteriza por comprometer pacientes com baixa idade e por um pequeno tempo de instalação da doença – de algumas semanas a poucos meses, observa-se predomínio da 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 (28). Considerando

que este isotipo apresenta capacidade de fixação de complemento reduzida e pequena afinidade pelos receptores FcR, a fagocitose e, consequentemente, a eliminação do fungo, tornam-se comprometidas.

A FC, que ocorre devido à reativação de focos latentes (re-infecção endógena) 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, à semelhança de indivíduos saudáveis, 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 (28), 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) (29).

1.3. Fibrose pulmonar e PCM

Mesmo após tratamento antifúngico eficaz, a avaliação clínica e radiológica de pacientes com FC revela a presença de fibrose nos diferentes órgãos comprometidos, os pulmões, particularmente, também exibem o enfisema. Essas sequelas levam ao comprometimento funcional e incapacitação do paciente. Após tratamento, a função pulmonar poucas vezes é normal, revelando padrão obstrutivo em 85% dos casos, com freqüências iguais de obstrução leve, moderada e intensa (31-33).

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. A proliferação de fibras reticulares (colágeno III) também ocorre no septo alveolar, inclusive em áreas distantes do processo granulomatoso, sugerindo que o próprio fungo e, ou, seus antígenos podem atuar diretamente sobre a proliferação de fibras reticulares nos alvéolos

pulmonares (33).

Apesar da sua importância, poucos estudos têm focado especificamente a fibrogênese pulmonar que ocorre na PCM. Cock et al.(34) demonstraram em modelo experimental murino que esse processo é precoce. Araujo(35), em estudos necroscópicos demonstrou a presença de fibrose em pacientes que não receberam tratamento antifúngico.

Em geral, durante a evolução da PCM, a função pulmonar encontra-se alterada observando-se hipoxemia com predominância da perfusão sobre a ventilação pulmonar (36). 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 à grandes e pequenos esforços (Lemle et al., 1983). Nesta fase a hipoxemia é sequelar e observada em cerca de um terço dos casos (37).

1.4. Inflamassomas

Tradicionalmente, o sistema imune inato é considerado como a primeira linha de defesa do organismo, cuja função primária é eliminar patógenos por diversos mecanismos, dentre os quais estão as barreiras físicas e químicas e os processos biológicos, como a inflamação e a ativação do sistema complemento. Nos processos biológicos inatos que envolvem células, o reconhecimento de moléculas *nonself* ocorre por um sistema sofisticado de receptores de reconhecimento padrão (PRRs) que estão expressos em células como macrófagos, monócitos, células dendríticas, neutrófilos e células epiteliais. Famílias já descritas dos PRRs incluem receptores do tipo *toll-like* (TLRs), de lectinas do tipo-C e receptores *nucleotide-binding oligomerization domain* (NOD)-*like* (NLRs) (38,39). Estes últimos são, ainda, subdivididos em três famílias: NOD, NLRP e IPAF. Esses receptores são intracelulares com domínios bem característicos, podendo apresentar um domínio pirina (PYD) ou um domínio de recrutamento de caspase (CARD). Além disso, alguns deles estão envolvidos tanto no reconhecimento de PAMPs como na detecção de fatores endógenos derivados de estresse celular (DAMP)(40-45). Dentre as vias de sinalização associadas a estes receptores, destacamos a via que leva a montagem de uma estrutura intracelular denominada inflamassoma que tem por consequência a produção de IL-1β e IL-18 (46), citocinas que participam de maneira importante na indução das respostas Th17 e Th1, respectivamente (47-49).

Dentre os receptores NLRs que são associados ao inflamassoma, os melhores caracterizados são: NLRP1, NLRP3, IPAF ou também chamado NLRP4, AIM2 e IFI16,

pertencentes à família das proteínas HIN que, apesar de não ser estruturalmente associados à família NLRs, foram descritos como ativadores de inflamassoma (50). A formação do NLRP 1- inflamassoma é mediada por estímulos específicos como, por exemplo, a presença da toxina letal de *Bacillus anthrax* (51). O AIM2-inflamassoma e o NLRP4 têm possuem estímulos específicos, como DNA virale como flagelina, respectivamente (52,53). O NALP3-inflamassoma, por ser o mais estudado, se caracteriza por sua capacidade de responder a um grande número de agonistas que não possuem origem microbiana necessariamente, mas sinais endógenos. Estudos demonstram, ainda, que o NLRP3-inflamassoma responde a mudança nas concentrações iônicas celulares, especialmente potássio além da ativação por cristais de ácido úrico e ATP extracellular (54,55). A Figura 1 ilustra as estruturas das diferentes famílias dos receptores que ativam o inflamassoma.

Além dos receptores, a estrutura do inflamassoma é constituída por moléculas adaptadoras, coletivamente denominada proteína adaptadora associada a apoptose (ASC), molécula pró-caspase e os precursores de IL-1β e IL-18. A Figura 2 sumariza os eventos que caracterizam a ativação do NLRP3-inflamassoma. Para que ocorra a ativação do inflamassoma e como consequência a liberação da IL-1β e IL-18 são necessários dois sinais distintos em que pode-se observar a complementaridade entre os papéis das diferentes famílias de PRRs (54-56).

O primeiro sinal é o reconhecimento de PAMPs pelos PRRs (como por exemplo, receptores do tipo *toll*) que leva a ativação da via NF-kB com transcrição e tradução das moléculas pró- IL-1β e pró-IL-18. Um segundo sinal usualmente associados aos DAMPs são reconhecidos pelos receptores intracelulares NLRs que recrutam os demais componentes do inflamassoma e, assim, tem-se a clivagem, via Caspase-1, das citocinas resultando nas suas formas ativas (55-57).

As respostas dos inflamassomas podem ser induzidas por um grande número de estímulos citoplasmáticos que são associados a infecções e estresse celular. Entre os produtos microbianos que atuam como ativadores do inflamassoma estão moléculas bacterianas como flagelina, muramil dipeptideo, LPS além de RNA bacteriano. Outros indutores conhecidos são substâncias cristalinas derivadas do meio ambiente como asbestos e silica (58) além de outros estímulos endógenos como ATP extracellular (59) e cristais de ácido úrico (60, 61)

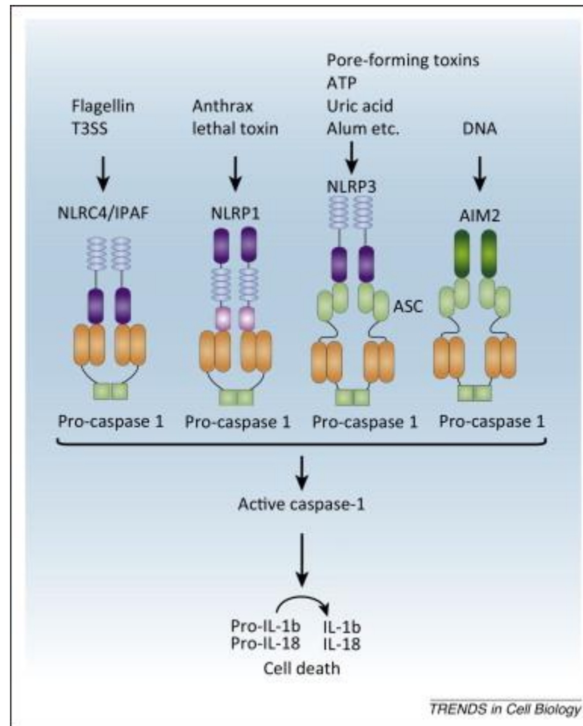

Figura 1. Estrutura dos inflamassomas canônicos e exemplos de agonistas (62).

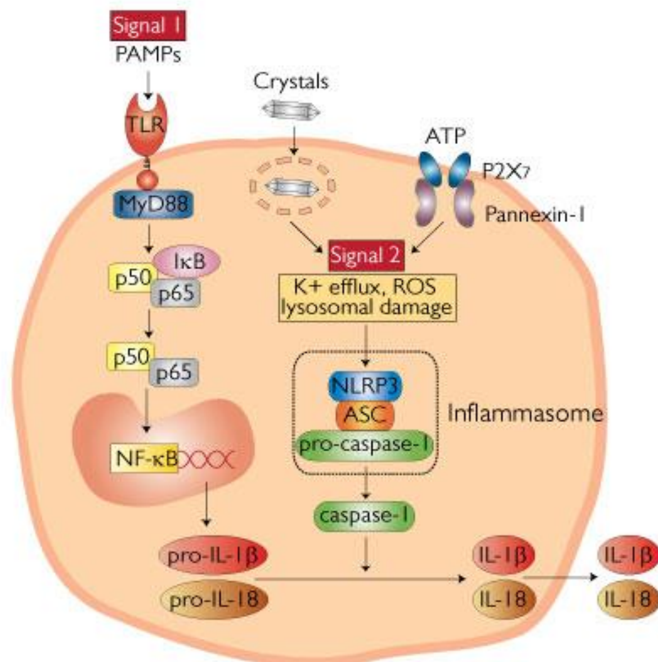

Figura 2. Esquema de ativação do inflamassoma NRLP3 (63). O primeiro sinal ocorre quando PAMPs são reconhecidos por um PRRs que ativa a via NF-κB induzido a transcrição e a tradução dos precursores da IL-1β e IL-18. O segundo sinal ocorre quando um receptor intracelular, como o NRLP3, reconhece PAMPs ou DAMPs induzindo a organização e ativação do inflamassoma, seguida da clivagem dos precursores de IL-1β e IL-18, pela caspase-1, nas suas formas ativas

1.5. Inflamassomas e infecções fúngicas

Pele e mucosa são exemplos típicos de locais frequentemente expostos à fungos e cabe ao sistema imune do hospedeiro diferenciar os patógenos que fazem parte da microbiota natural daqueles que podem causar as doenças mais graves. Desse modo, e apesar de pouco estudado, o inflamassoma parece ter um papel importante no equilíbrio entre a colonização simbiótica e infecção por fungos, como por exemplo, *Aspergillus fumigatus*, *Candida albicans, Cryptococcus neoformans* (64-68). Embora existam diferenças nos componentes das paredes celulares desses fungos patogênicos como a β- glucana presente na *C. albicans*, a galactomanana do *Aspergillus* spp e a glucoroxylomanana do *Cryptococcus* sp,(69-74) diversos estudos têm demonstrado a ativação do NLRP3-inflamassoma (75-79).

Os eventos iniciais da infecção fúngica inclui a participação de neutrófilos que controlam a infecção fúngica por atuar na fagocitose de conídios e leveduras e, ou, na liberação de diversas enzimas proteolícas e NETs (*neutrophil extracellular traps*) em respostas às hifas e micélios (80,81). Embora a IL-1β possua papel relevante na quimiotaxia dessas células e na amplificação dessa resposta, a IL-1β não é dependente do NLRP3-inflamassoma pois os neutrófilos são capazes de liberar uma enzima denominada proteinase 3 (PR3) que também possui capacidade de clivar a forma inativa da IL-1β na sua forma ativa (82). Desse modo, nos estágios mais tardios da infecção, quando a resposta inflamatória torna-se mais dependente das células mononucleares, o inflamassoma é o responsável por atuar na liberação da IL-1β e IL-18 que contribui na polarização das respostas Th17e Th1 respectivamente (83).

Estudos experimentais recentes têm relacionando a PCM e o inflamassoma. Em 2013, Tavares *et al.*(84) demonstraram que o NLRP3-inflamassoma é ativado pelo *P. brasiliensis* em células dendríticas murinas e que esse mecanismo seria de grande importância para a defesa do organismo contra o fungo. Recentemente, Feriotti *et al.* (2015) (85) observaram que a expressão do receptor Dectina-1 induz expressiva ativação do NLRP3-inflamassoma e que esse mecanismo estaria associado com a resistência do hospedeiro frente ao fungo e Ketelut-Carneiro *et al* (2015) (86) demonstraram que a IL-18 liberada pela ativação do NLRP3-inflamassoma induz a uma resistência antifúngica pelo hospedeiro.

2. OBJETIVOS

2.1. Objetivo Geral

O presente estudo teve por objetivo avaliar a expressão dos genes envolvidos na ativação do inflamassoma e a produção de citocinas inflamatórias por monócitos e macrófagos alveolares de pacientes com paracoccidioidomicose..

2.2 Objetivos Específicos

1. Determinar a expressão dos genes NALP1/NLRP1, NALP3/NLRP3, CASP1, CARD8 e IL1B por monócitos e macrófagos alveolares obtidos de pacientes com paracoccidiodomicose na forma crônica frente ao filtrado de cultura do *P. brasiliensis* (AgPb).

2. Determinar a produção de IL-1ß, IL-18, TNF-α, IL-6, Il-10, IL-12 e FGF por monócitos e macrófagos alveolares obtidos de pacientes com paracoccidiodomicose na forma crônica frente ao AgPb.

3. Correlacionar esses achados com o momento do tratamento antifúngico (pré-tratamento, durante tratamento e pós-tratamento).

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4. MANUSCRITO

Enhanced activity of NLRP3 inflammasome in patients with pulmonary paracoccidioidomycosis

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ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic and granulomatous mycosis caused by thermally dimorphic fungi of the gender *Paracoccidioides*. Endemic in Latin America, PCM affects predominantly 30-60 year-old rural worker males, mostly in working age, causing socio-economic problems. The main clinical forms of the disease are acute/subacute and chronic (CF); almost all CF patients develop pulmonary fibrosis possibly due to constant chronic inflammation and they also exhibit emphysema mainly due to the tobacco smoke. Previous studies showed that CF patients during active disease exhibit increased production of inflammatory mediators, including IL-1β. This cytokine, different from the others, is produced by an intracellular multimolecular protein complex called inflammasome that is activated by pathogens and/or host signs of damage. While inflammasome activation has been demonstrated in experimental models of *P. brasiliensis* infection, no information is available in humans, leading us to investigate the role of NRLP3-inflammasome in CF PCM patients from a Brazilian endemic area. Our results demonstrated for the first time the activation of NLRP3-inflammasome in monocytes from CF PCM individuals. Furthermore, we showed that CF treatment did not alter inflammasome activation. These findings corroborate the contribution of NRLP3-inflamassome in pathogenesis of PCM also in patients.

INTRODUCTION

Paracoccidioidomycosis (PCM) is a systemic and granulomatous mycosis caused by thermally dimorphic fungi of the *Paracoccidioides complex* (1). Described in 1908 by Adolf Lutz (2), PCM is endemic in Latin America and affects mainly farmer workers (3). There is considerable clinical variability among PCM patients, with clinical manifestations that ranges from acute/subacute (AF) to chronic (CF). AF affects mainly children and young adults in both gender, the duration of symptoms is around 2 months, there is involvement of lymph nodes, liver, spleen and bone-marrow; even displaying more degree of severity, there is rare sequels after treatment (4). Furthermore, the Th2/Th9 immune response is the hallmark in AF PCM patients (5). On the other hand, CF is more prevalent (around 75% of cases) and affects mainly male adults over 30 years with duration of symptoms more than 6 months and lungs and mucosa of the upper aerodigestive tract are the main affected organs/tissue (4). In general, CF occurs due to reactivation of latent foci (endogenous re-infection), i.e., the host had organized a Th1/Th17 adaptive immune response to *Paracoccidioides* spp (5) that was effective for undetermined time (6). These patients also exhibit intense production of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-17 that induces deleterious effects instead protection (5,7,8). In addition, CF PCM patients present sequels including pulmonary emphysema and fibrosis, oral mucousmembrane and adrenal fibrosis (4).

Among the pro-inflammatory cytokines involved in response to *Paracoccidioides brasiliensis* (PB) and in pulmonary damage, IL-1ß plays a central role in pathogenesis of PCM, as demonstrated in experimental model of infection (9,10). Biologic active form of IL-1ß is produced by the cleavage of pro-IL-1ß mediated by caspase-1 within the canonic inflammasome pathway. Both pathogen associated molecular pattern (PAMPs) and danger associated molecular pattern (DAMPs), such as monosodium urate crystals, extracellular

ATP, fibronectin, hyaluronic acid, are sensed by a cytoplasmic innate receptor (i.e.: NLRP1, NLRP3, NLRC4, AIM2), that directly or indirectly (through the adapter molecule ASC) recruits the effector molecule caspase-1 (11), leading to the production of IL-1β and IL-18 (12).

Inflammasome do not only plays a central role in innate immune response to pathogens or tissue damage, but also cooperate in adaptive response polarization (i.e: IL-1ß for Th17, IL-18 for Th1). Inflammasome activation is strictly regulated by transcriptional and post-transcriptional mechanisms, as well as by endogenous proteins such as CARD8. The transcription of components genes *NLRP3, AIM2* and *IL1B* is dependent of NF-kB activation, and mediated by TLRs, C-type lectin receptors and/or cytokine receptors signaling. Therefore, the priming of the complex happens during host/pathogen interaction and also in pro-inflammatory environmental. Inflammasome, especially NLRP3-inflammasome, has been recognized as a critical contributor in chronic inflammatory diseases as well as in fibrosis pathogenesis (i.e.: chronic obstructive pulmonary disease (COPD) caused by cigarette smoke exposure) (13,14).

Although there is emergent evidence for involvement of the NLRP3-inflammasome in *Paracoccioidoides*-host interplay (9,10,15) to our knowledge no studies have been conducted in PCM patients. All this considered, we hypothesized that inflammasome activation could be involved also in human infection and contribute to chronic inflammation. Moreover, we considered the possibility that a chronic inflammasome stimulation could be an adverse factor during PCM treatment. For this purpose, the activation of inflammasome was evaluated in Brazilian patients with pulmonary PCM and correlated it with the response to treatment.

MATERIALS AND METHODS

Patients. Nineteen CF PCM patients (PCM-p) were enrolled for this study at the Tropical Diseases Unit and Ambulatory of Systemic Mycosis at the University Hospital of the Faculty of Medicine/FMB, University of São Paulo State/UNESP (Botucatu , SP, Brazil). Patients were included in this study when presenting, at admission in the Service, clinical manifestations compatible with the CF of PCM and were considered either confirmed or probable (16). Cases were considered confirmed when the typical *Paracoccidioides* genus yeast forms were identified in the clinical specimens and probable when only serum-specific antibodies were detected using a double agar gel immunodiffusion test (DID). All patients were tobacco users and exhibited pulmonary involvement. Patients who exhibited co-morbidity such as neoplastic, inflammatory or other infectious diseases, or pregnancy were not enrolled in the study.(Table 1)

Ethics statement. This study was approved by the Research Ethics Committees of Botucatu Medical School – UNESP (CAEE #53235616.8.0000.5411) and School of Sciences – UNESP/Bauru (CAEE #53235616.8.3001.5398). After being informed of the study, written informed consent was obtained from all participating adults.

Experimental design. In this investigation, two types of experimental design were carried out. In the first, a comparison of variables evaluated in the same patients before treatment (moment M_o) and during antifungal therapy (M_1) was performed. In the second one, variables from independent individuals were compared at moment M_1 and at the last moment of evaluation, at apparent cure (M_2).

Groups of study. PCM-p were categorized into three groups. G_1: composed of non-treated

patients newly diagnosed (n=3), all of them presenting the first episode of PCM. G_2: composed of patients under treatment (n=9); consisting of seven patients under treatment of the first episode of PCM, including three patients from G_1 that were re-evaluated during treatment, and two were under treatment of the second episode of PCM (relapsed patients). All these patients presented clinical cure, but positive serology by DID; duration of the treatment ranged from 1 to 138.2 months, with a median of 31,5. G_3: composed of patients with apparent cure (n=10), consisting of patients who did not show any sign or symptom of active disease (clinically cured), negative serology, and at least two full years of non-treatment after complete antifungal therapy (17); two of them presented apparent cure in the second episode of PCM (relapsed patients). G_4: composed of six age- and gender-matched healthy individuals, selected among blood donors from the same geographical area (n=6).

Homogeneity of the groups G_2 and G_3 was evaluated based on gender, age, degree of severity at admission, serum levels of antibodies at admission, serum levels of IL-1β and TNF-α, respiratory parameters, and antifungal drugs used in the treatment (Table 1).

Pulmonary function tests (PFT). Forced expiratory volume in the first second (FEV1) and the forced vital capacity (FVC) based on the flow-volume curve were obtained using a spirometer Koko 606055 version 9.A (Ferraris Respiratory, Louisville, CO, USA) as the standard recommendations (18). The predicted values were derived based on the Brazilian population (19).

Biologic samples. Venous blood was collected from patients and healthy individuals for monocytes' isolation. Bronchoalveolar lavage (BAL) specimens were available for the three untreated patients (G_1; M_0) at the Service of Endoscopy of Hospital das Clínicas (UNESP, Botucatu, SP, Brazil). All donors were from the same geographical area.

***Peripheral blood monocyte culture*.** Peripheral blood mononuclear cells (PBMC), obtained by centrifugation over Ficoll-Hypaque (Sigma, St Louis, MO, USA) were distributed in 96-wells plates and monocytes isolated by adherence and cultured in RPMI-1640 medium containing 1mM L-glutamine, 25 mM HEPES buffer, penicillin/ streptomycin (Nutricell, Campinas, SP, Brazil) and 10% fetal calf serum (Nutricell) (complete medium). The cell concentration was adjusted to 0.2×10^6 monocytes/well, as judged by the uptake of 0.02% neutral red.

Alveolar macrophage (AM) culture. AM were isolated of the BAL. Briefly 60 ml of warmed sterile saline were instilled via a wedged fibreoptic bronchoscope. Aspirated BAL fluid was immediately cooled to 4°C and filtered through a cell strainer to remove particulate debris. BAL was centrifugated 250xg 10’ and pelleted cells were resuspended in complete medium and seeded at 0.2×10^6 AM/well in 96 well plates. Non-adherent cells were removed after one hour. The purity of adherent AMs was identified to be greater than 95% by morphology. The viability was greater than 80% as determined by Tripan blue 0.4%.

***Stimulation of monocyte cultures*.** LPS (10 μg/ml) (Sigma Aldrich) or PB exoantigen (20 μg/ml; Pb113 strain) was added to monocytes for three hours. After incubation, cell-free supernatants were used for cytokine and cells were lysed for mRNA isolation and gene expression analysis.

Cytokines quantification. The cytokines IL-1β, IL-6, IL-10, TNF-α, and IL-12p70 were quantified in the cell-free supernatants (3 hours of culture) by flow cytometry using the kit BD™ Human Inflammation Cytometric Bead Array (BD, Becton Dickinson, USA). Serum

levels of IL-1β and TNF-α were measured by ELISA using Duoset (R&D systems, Minneapolis, MI, USA).

Relative gene expression analysis. RNAm was isolated from monocytes using the RNAqueous micro kit (Ambion, Life Technologies). Five-hundred nanograms of RNA were retro-transcribed with SuperScritp-II kit (Invitrogen) according to manufacturer instructions. Inflammasome encoded genes *NLRP1, NLRP3, CARD8, CASP1* and *IL1B* were amplified using specific TaqMan® Gene Expression Assays (Applied Biosystems) in StepOne Plus equipment (Applied Biosystems). SDS 2.3 software was used to obtain cycle threshold (Ct) values for relative gene expression analysis. Relative gene expression was calculated by relative quantitation standard curve (20) ß-actina/ACTB was the housekeeping gene used for normalization (ΔCt).

***Statistical analysis*.** Statistical tests were performed using the GraphPad v.5.00 software (GraphPad Software Inc, San Diego, CA, USA), and significance was set up at $p \leq 0.05$ for all analyses. All data were submitted to normality test (Shapiro-Wilk). Comparisons of two independent samples were performed using t unpaired test or Mann-Whitney U test. Comparisons of two dependent samples were performed using t paired test. Correlations were tested using either Spearman tests.

RESULTS

Monocytes from PCM patients exhibited increased priming and activation of NLRP3-inflammasome

Inflammasome activation was first evaluated in peripheral blood monocytes from PCM patients isolated at admission (non-treated; n=3), during antifungal treatment (n= 10) and after treatment (apparent cure; n=9).

In untreated patients (G_1), basal expression of *NLRP1, NLRP3* and *IL1B* resulted higher than control individuals (Table 2). This increase in inflammasome encoded genes was accompanied by a higher basal production of IL1-β by non-treated PCM monocytes compared to controls (Table 2). These patients were newly evaluated (around the 10th month of antifungal treatment). Basal expression of *NLRP1, NLRP3* and *IL1B* as well as IL-1β production decreased when compared to untreated patients, but remained higher compared to controls (Table 2). In these monocytes, *P. brasiliensis* antigens did not change the expression of inflammasome genes as well as production of IL-1ß compared to unstimulated cells (data not shown).

Monocytes collected from PCM patients during (G_2) and after treatment (G_3) showed higher basal expression of *NLRP3* (Fig. 1A), *CASP1* (Fig. 1B) and *IL1B* (Fig. 1D) as well as constitutive production of IL1-β (Fig. 1E) than controls (G_4). *Pb*Ag-stimulated monocytes of G_2 patients showed similar expression of *NLRP3* (Fig 1A) and *CASP1* (Fig. 1C) compared to unstimulated monocytes. We did not observed modulation in the expression of *NLRP1* (Fig. 1B) and *CARD8* (data not shown). Relapsed patients exhibited same pattern of results among the patients.

In addition, the activation of inflammasome was evaluated in AM of untreated PCM patients (G_1; n=3). AM showed less *NLRP1*, *NLRP3*, and *IL1B* expression as well as

production of IL-1β compared to resting peripheral blood monocytes, independently of the stimulus (Supplementary Figure 1).

Monocytes from PCM patients exhibited increased secretion of TNF-α.

To evaluate the inflammatory state of the monocytes isolated from PCM patients, other pro-inflammatory cytokines were measured by unstimulated, PbAg, and LPS-stimulated monocytes. Higher levels of TNF-α produced by unstimulated and LPS-stimulated monocytes was detected in patients during treatment (G_2) and after treatment (G_2) than normal individuals (G_4) (Fig. 2). Low and/or no significant levels of IL-6, IL-10 and IL-12 produced by monocytes was observed, possibly duo to the short time of incubation (data nor shown).

Correlation between clinical parameters and inflammasome activation.

In order to better understanding the high and persistent inflammasome activation by PCM monocytes, we next evaluated the correlation between clinical variables, such as length of treatment, titers of specific *P. brasiliensis* antibody, PFT, and systemic inflammation activation (serum levels of IL-1β, TNF-α). We did not find correlations among basal expression of *NRLP3*, *NRLP1*, *CASP1*, *IL1B*, and length of treatment, titters of DID and serum levels of IL-1β and TNF-α (Table 3).

To evaluate the association between inflammasome activation and respiratory function, 14 patients were submitted to spirometry and the results showed one normal, one restrictive pattern and 12 obstructive defect (three severe, four moderate and five mild). No correlations were observed among the respiratory parameters and expression of genes *IL1B*, *NRLP3*, *NRLP1* and *CASP1* (Table 3) and production of cytokines (data not shown). Among the evaluated patients with obstructive pattern (n=12), seven showed FVC (% predicted) < 75, suggesting a mixed pattern (emphysema and fibrosis). Thus, we next compared the expression

of inflammasome genes and production of cytokines between patients that showed predominant emphysema and those with emphysema and fibrosis and no differences were observed (data not shown). Interestedly, the patient with restrictive pattern [FVC (% predicted) = 59; FEV1/FVC (% predicted) = 103] showed the highest levels of TNF-α (917.9 and 1046.5 pg/ml) and IL-6 (6074.6 and 8107.9 pg/ml) by monocytes (unstimulated and *Pb*Ag-stimulated, respectively) among all evaluated patients.

DISCUSSION

Deregulated inflammasome activation is emerging as a critical immunological mechanism involved in the pathogenesis of diverse pulmonary diseases, including infectious lung injury, pulmonary fibrosis, asthma, and COPD (21). In the present study, we demonstrated a significant increased priming and activation of NLRP3-inflammasome in peripheral blood monocytes of patients with chronic PCM unstimulated or stimulated. Apparently, this pro-inflammatory state is independent of the antifungal treatment. The constitutive activation of inflammasome in peripheral blood monocytes is a common hallmark of several chronic inflammatory diseases (22–24) and it is suggested to be a epigenetic phenomenon (25). Systemic inflammation in PCM has been reported since the first studies focused on the immunopathology of PCM and it has been associated to pathogenesis by elicits depression of T cell-mediated immunity and induces hypergammaglobulinaemia (26). In addition, imbalanced immunoregulatory responses have been not only observed in PCM patients during active disease, but also after complete and successful treatment (27,28), as observed in the present study.

Herein, we observed that *Pb*Ag did not enhance NLRP-3 inflammasome activation in monocytes of controls as well as PCM individuals. This finding may be occurred due to impaired ability of monocytes from patients to respond to stimulation, since persistent inflammation ultimately leads to monocyte exhaustion. In addition, the exoantigen *Pb*Ag used in our experiments is a yeast cell-free filtrate culture of *P. brasiliensis* (Pb113 strain) that is rich in glycoproteins specially gp43, the immunodominat antigen (29), however it lack cell wall components, such as β-glucan which is a well-known activator of NLRP-3 (9,10,30). Indeed, Kurocawa et al. (31) showed that yeasts of *P. brasiliensis*, Pb18 and Pb265 strains, induce high production of IL-1β by monocytes from heathy individuals and, in a previous

study, we showed that *Pb*Ag does not enhance the production of IL-1β by monocytes of PCM patients and heathy individuals (27). Another explication for the missing activation of inflammasome in *Pb*Ag-treated monocytes is that we did not add a second signal, essential for inflammasome activation. In order to understand about the activation of the inflammasome by *P. brasiliensis,* we additionality determined the production of active IL-1 β by resting monocytes from patients and healthy individuals challenged with LPS (20μg/ml),viable yeast of of *P. brasiliensis* (virulent strain Pb 18), beta-glucan (10 μg/ml) (Sigma-Aldrish), *Pb*Ag and ATP (1mM) (Sigma-Aldrish), as second signal (Supplementary Table 1). Interestedly, our results showed that yeast forms of *P. brasiliensis* was able to stimulate high production of IL-1 β in the absence of ATP. This finding suggest that the viable yeasts are able to activate both signals; first signal is induced by PAMPs and the second signal is a result of ROS and K+ efflux induced by cell damage (9). Monocytes stimulated with PbAg and beta-glucan only showed increased production of active IL-1β in the presence of ATP. It is important to highlight that our purpose was to evaluate fresh monocytes isolated in order to preserve the inflammatory background of the patients.

Alveolar macrophages did not showed increased basal inflammasome activation compared to peripheral blood monocytes from PCM as well as from control individuals. Lasithiotaki et al (32) showed similar results in idiopathic pulmonary fibrosis, but not in rheumatoid arthritis–usual interstitial pneumonia, suggesting the complex immunobiology of AM and the involvement of others lung parenchymal cell types in the activation of inflammasome during fibrosis development. Moreover, previously data reported that different cell types present different mechanism and timing of priming and activation of inflammasome, suggesting differences in peripheral blood monocytes and AM.

Considering the complexity of chronic form, we tried to identify some clinical variables that could affect the activation of NLRP3 inflammasome, and that could explain the

constitutive activation after the introduction of antifungal therapy and/or successful treatment. Chronic inflammation triggered by microbial pathogens or danger signals derived from the host culminates in distressing signals that can lead to organ dysfunction due to non-functional fibrous tissue. Collagen deposition is a result of proliferation and maturation of fibroblast resulted from the several cytokines and growth factors, including TGF-β that is also induced by IL-1β. Therefore, IL-1β acts by perpetuates and exacerbates the inflammatory process and contributes for fibrogenesis (21). It has been proposed that activation of NLRP3 by sterile signals is more relevant in fibrogenesis than PAMPs (33,34). *In vitro* studies have shown activation of NLRP3 and subsequent production of active IL-1β by asbestos and silica crystals (35,36). NLRP3$^{-/-}$ mice challenged with silica showed less collagen deposition in the lungs than wild-type mice (37).

Functional analyses of respiratory parameters achieved in the present study failed to demonstrate involvement of NRLP3-inflamassome activation in the fibrogenesis in PCM. We have considered the lower number of enrolled patients as the main limitation for these analyses. Furthermore, simultaneous occurrence of emphysema and fibrosis in the lungs of PCM patients is a complex challenge to understand the fibrogenesis as well as its repercussion. Important discrepancies between intense radiological abnormalities and mild altered respiratory functions of PCM patients has been noticed (38). According to the authors, this observation is most likely secondary to overinflating and increased pulmonary compliance due to the loss of elasticity in areas with emphysema that are counterbalanced by the loss of volume and decreased compliance elicited by fibrosis (38,39). Therefore, we suggest that spirometry as well as arterial gasometry are limited parameters to evaluate fibrogenesis and the results of PCM patients should be carefully interpreted. In the present study, we evaluated a rare patient that exhibited only restrictive pattern (i.e. presence of fibrosis and absence of emphysema) and the results showed exacerbated production of TNF-α

and IL-6 and absence of inflammasome activation. These results are very interesting and confirm that under hypoxia induced by fibrosis, cells attempt to restore homeostasis through the activation of hypoxia-inducible factors (HIFs), that in turn, trigger several immune-related processes, including release of proinflammatory cytokines (40). In addition, we suggest that inflammasome activation seems to be more relevant in the fibrosis development, possibly in the initial process, than the late and consolidated fibrotic stage. Nevertheless, further studies are necessary to confirm the involvement of NLRP3- inflammasome activation in the PCM fibrogenesis.

In summary, our results showed that peripheral blood monocytes of PCM patients, independently from the anti-fungal treatment, exhibited a constitutively augmented activation of NLRP3-inflammasome and IL-1β production, concordant with a chronic inflammatory state.

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Disclosure

The authors declare no conflict of interest.

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Table 1. Clinical, immunological, biochemical and respiratory characterization of PCM patients during (G_2) and after treatment (apparent cure, G_3).

Parameters	During treatment (G_2)	Apparent cure (G_3)	*P* value[1]
Severity	Mild = 1 Moderate = 7 Severe = 2	Mild = 1 Moderate = 5 Severe = 3	0.62
Antifungal treatment	CMX = 5 ITC = 4 CMX/ITC = 1	CMX = 6 ITC = 2 CMX/ITC = 1	0.62
Length of treatment	15 (1 – 46)	32 (24 – 138)	0.03
DID – admission (1:)	16 (NR – 64)	16 (NR – 512)	0.77
DID - collection of samples (1:)	NR (NR – 128)	0	-
Serum			
IL-1β (pg/mL)	0 (0 – 26)	0 (0 – 19)	0.63
TNF-α (pg/mL)	0 (0 – 15)	0 (0 – 12)	0.67
Spirometry			
FVC	2.90 (2.81-4.45)	3.37 (1.13-6.22)	0.70
% predicted	62 (58-99)	81 (27-121)	0.24
FEV1/FVC	0.56 (0.5-0.7)	0.70 (0.38-0.93)	0.12
% predicted	72 (64-88)	86 (48-121)	0.15

[1]Homogeneity of the groups was determined by Mann-Whitney U test and Fisher's exact test.
DID = double agar gel immunodiffusion test; NR = non-reagent; CMX = cotrimoxazole; ITC = itraconazole; n = number of patients; FEV1 = forced expiratory volume in 1 s; FVC = forced vital capacity; Apparent group is composed with PCM-p submitted to antifungal treatment who present clinical and radiological cure, negative serological tests (DID) during at least 24 months after antifungal discontinuation.Data were showed in Median(min-max).

Table 2. Gene expression and inflammasome activation in monocytes of non-treated patients and healthy donors. Relative expression of inflammasome encoded genes *NLRP1, NLRP3, IL1B* and secretion of IL-1ß were measured in unstimulated monocytes from three patients recently diagnosed (G_1) before treatment (M_0) and during treatment after clinical cure[1] (M_1) and six healthy individuals (G4). The results are expressed as mean ± SEM. G_1 x G_4 were compared by unpaired *t*-test ($p<0.05$; different capital letters mean statistical differences; A > B). M_0 x M_1 were compared by paired *t*-test ($p<0.05$; different lowercase letters mean statistical differences; a > b).

	NLRP1[2]	***NLRP3***[2]	***IL1B***[2]	**IL-1β**[3]
Healthy individuals (G_4)	2.97 ± 2.36 **B**	0.22 ± 0.08 **B**	0.11 ± 0.02 **B**	56.7±21.5 **B**
PCM patients (G_1) – M_0	65.0 ± 32.5 **Aa**	66.0 ± 31.6 **Aa**	97.5 ± 2.5 **Aa**	1079±265 **Aa**
PCM patients (G_1) – M_1	7.55 ± 5.24 **Bb**	38.6 ± 25.4 **Ab**	9.92 ± 4.8 **Ab**	476.7±245.1 **Ab**

[1]Clinical cure is defined as the moment when the patients are under treatment, with disappearance of the initial symptomatology, but maintain positive immunodiffusion test (Mendes, 1994)
[2]Values expressed as relative expression
[3]Valures expressed as pg/ml.

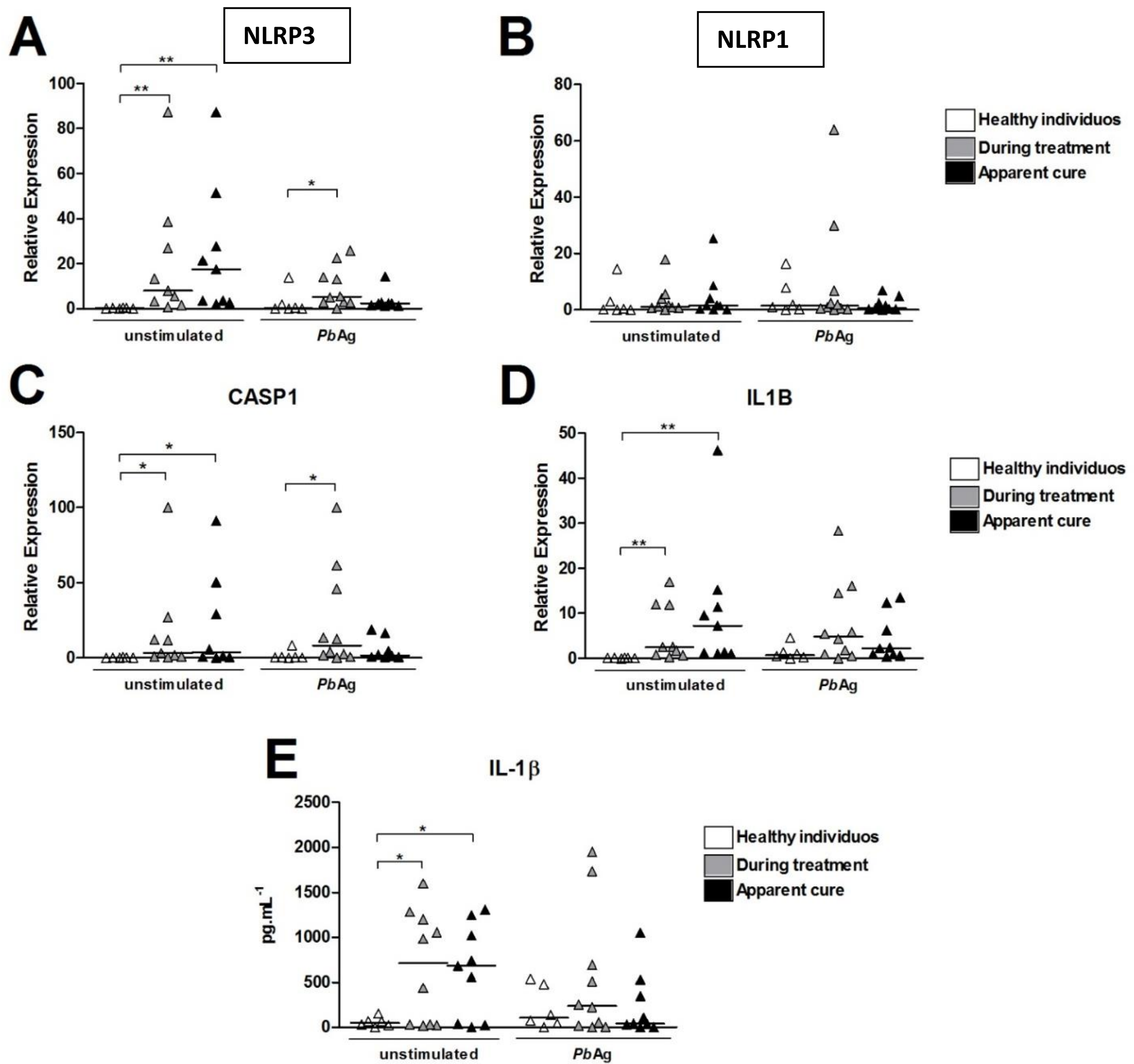


Figure 1. Relative expression of *NLRP3*, *NLRP1*, *CASP1* and *IL1B* and production of IL-1β by monocytes. Unstimulated and *P. brasiliensis* antigens-stimulated monocytes of patients during treatment (G_2; n=9) and after treatment (apparent cure) (G_3, n=10) and healthy individuals (G_4; n=6) were incubated for 3 hours. Data are expressed as median (horizontal bars) with the individual values. * $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney test).

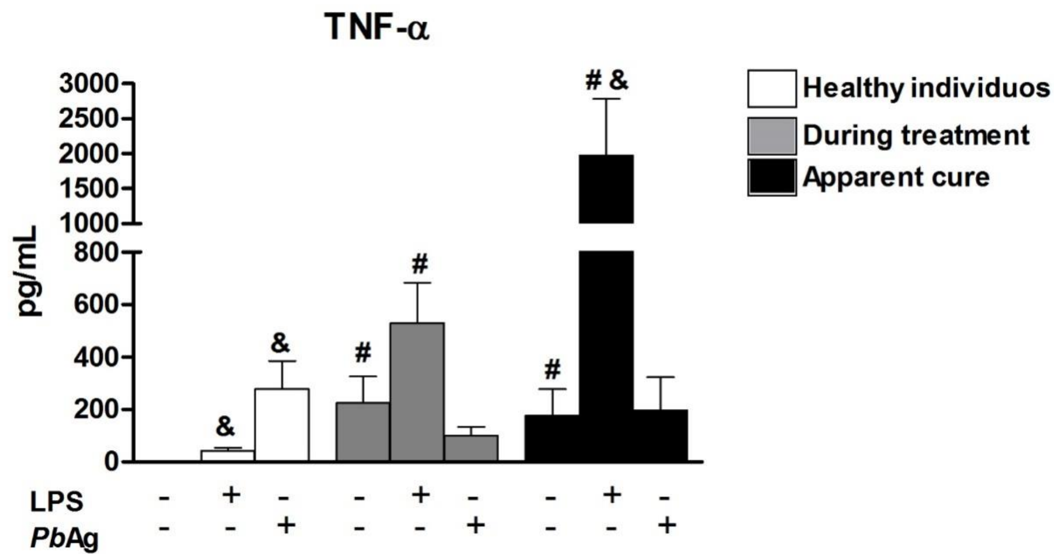
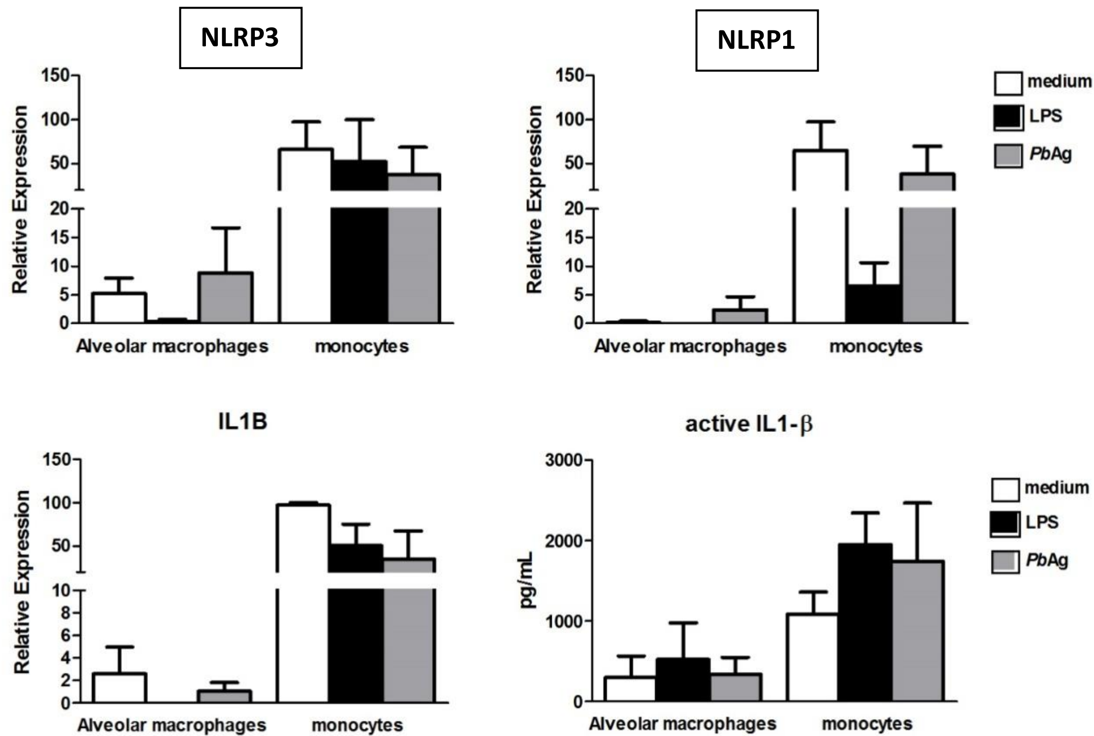


Figure 2. Production of TNF-α by monocytes. Unstimulated, lipopolysaccharide and *P. brasiliensis* antigens-stimulated monocytes of patients during treatment (G_2; n=9) and after treatment (apparent cure) (G_3, n=10) and healthy individuals (G_4; n=6) were cultured for 3 hours. Data are expressed as mean ± SEM. # means significant differences between patients and health individuals ($p < 0.05$, unpaired *t*-test). & means significant differences between *Pb*Ag or LPS-stimulated and unstimulated monocytes ($p < 0.05$, paired *t*-test).

Table 3. Correlations of the expression of genes *IL1B*, *NLRP3*, *NLRP1*, *CASP1* and clinical parameters of PCM patients. Unstimulated monocytes of PCM patients were cultivated for 3 hours. Expression of encoded genes *IL1B*, *NLRP3*, *NLRP1*, *CASP1* and clinical, immunological, respiratory functions of PCM patients were submitted to Spearman's test. Data are expressed as Spearman's correlation coefficient (p>0.05).

Parameters	IL1B	NRLP3	NRLP1	CASP1
Length of treatment	-0.47	-0.49	-0.57	-0.31
DID – admission (1:)	0.17	0.32	0.01	0.05
DID - collection of samples (1:)	0.24	0.21	0.11	0.09
Serum				
IL-1β	0.13	0.13	0.02	-0.16
TNF-α	-0.28	-0.21	0.01	-0.28
Spirometry				
FVC	0.17	0.11	0.11	0.12
% predicted	0.29	0.17	0.21	0.39
FEV1/FVC	-0.01	0.01	-0.09	0.01
% predicted	-0.07	-0.05	-0.10	-0.05

DID = double agar gel immunodiffusion test, FEV1 = forced expiratory volume in 1 s, FVC = forced vital capacity.



Supplementary Figure 1. Relative expression of *NLRP3*, *NLRP1* and *IL1B* inflammasome genes and active IL-1β by alveolar macrophages and monocytes. Unstimulated, lipopolysaccharide and *P. brasiliensis* antigens-stimulated alveolar macrophages and monocytes of non-treated patients newly diagnosed (G1; n=3) were cultured for 3 hours. Data are expressed as media ± SEM ($p < 0.05$; paired *t*-test).

Supplementary Table 1. Production of IL-1β by monocytes from patients with chronic form of paracoccidioidomycosis and health individuals. Venous blood collected from three patients with chronic form of paracoccidioidomycosis and three healthy individuals for monocytes isolation. Peripheral blood mononuclear cells (PBMC), obtained by centrifugation over Ficoll-Hypaque (Sigma) and monocytes (concentration was adjusted to 0,2x10^6 /well) isolated by adherence in 96-wells plate and cultured in RPMI-1640 medium containing 1mM L-glutamine, 25 mM HEPES buffer, penicillin/ streptomycin (Nutricell, Campinas, SP, Brazil) and 10% fetal calf serum (Nutricell) (complete medium) and rest for 24 hours. After this, cells were challenged with LPS (20µg/ml),viable yeast of of *P. brasiliensis* (virulent strain Pb 18), beta-glucan (10 µg/ml) (Sigma-Aldrish), *Pb*Ag and ATP (1mM) for three hours.As positive control we used ATP and LPS and as a negative control unstimulated monocytes. After incubation, cell-free supernatants were used for IL-1β quantification.

Condition	Pac I	Pac II	Pac III	Control I	Control II	Control III
Baseline	60,05	217,10	53,89	69,80	0,21	45,44
LPS + ATP	2097,02	14082,38	6987,38	1565,25	1839,65	3633,60
Pb18 1:5	4292,71	1119,74	847,84	4608,82	1154,84	4482,05
Pb18 1:5 + ATP	1347,72	1600,79	1226,58	4292,71	627,24	2128,11
b-glucan	63,24	92,80	51,32	18,06	0,00	50,97
b-glucan + ATP	1839,65	1446,77	542,37	662,62	1170,85	3473,86
PbAg	0,00	0,00	42,99	0,00	38,28	78,92
PbAg + ATP	1560,58	1612,86	5384,38	961,59	3164,34	8213,22

Values are expressed in pg/ml

5. CONCLUSÃO

Nossos resultados demonstram pela primeira vez a ativação do NLRP3-inflamassoma, caracterizada pela elevada expressão de *NLRP3*, *CASP1* e *IL1B* por monócitos de pacientes com a forma crônica da paracoccidioidomice em relação aos indivíduos saudáveis.