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Dissertação

Introdução

Paracoccidioides brasiliensis é um fungo dimórfico, agente etiológico da paracoccidioidomicose (PCM), que se apresenta endêmica na América Latina [1]. As manifestações clínicas da micose são de doença granulomatosa crônica, comprometendo especialmente tecidos pulmonares, mucosas e o sistema fagocítico mononuclear, com disseminação para fígado, baço, adrenais e outros órgãos [2,3]. Na PCM como em outras infecções granulomatosas crônicas, a resposta imune celular é o principal mecanismo de defesa. O granuloma na PCM representa uma resposta tecidual específica do hospedeiro contra o fungo, na tentativa de destruir e circunscrever o parasita, evitando sua multiplicação e disseminação [4,5].

Estudos experimentais de PCM em camundongos, demonstraram associação entre resposta dominante Th2 e gravidade da infecção [6]. Na doença humana, alguns autores observaram o predomínio de resposta imune Th2 sobre a resposta Th1, com produção de Interleucina (IL)-4, IL-5 e IL-10 e níveis elevados de IgG4, IgA e IgE [7-9]. Esses parâmetros associados a baixos níveis de IFN-γ foram correlacionados com as formas graves da micose [9]. Células mononucleares de sangue periférico de pacientes com as formas aguda e crônica da micose, apresentam baixa resposta proliferativa e produção de citocinas de padrão Th1, interferon-gamma (IFN-γ) e IL-2 em resposta a antígenos de *P. brasiliensis*, enquanto os níveis IL-10 se encontram elevados [7,10]. Assim, a imunossupressão na PCM poderia ser entendida como decorrente da perturbação do equilíbrio das células do sistema imune, causada pelo *P. brasiliensis*, levando ao aparecimento dos diferentes fenômenos envolvidos na modulação da resposta imune. Os trabalhos citados acima, mostram claramente que uma resposta protetora contra o *P. brasiliensis* depende de um padrão de resposta do tipo Th1, envolvendo como citocinas principais, IFN-γ, fator de necrose tumoral-alfa (TNF-α) e IL-12 [11,12]. O desenvolvimento dessa resposta seria representado, principalmente pelas células fagocitárias, os monócitos e macrófagos, que necessitam de ativação fornecida por essas citocinas para atuarem na resposta inflamatória e na atividade fungicida.

Na PCM humana e experimental, os macrófagos estão presentes em grande número nas lesões granulomatosas [4,13,14] e representam a principal célula de defesa contra *P. brasiliensis* [15,16]. Considerando que *P.brasiliensis* é um microrganismo intracelular facultativo [17], os macrófagos podem ter papel essencial na patogênese da doença. Portanto, a investigação de mecanismos relacionados com a fagocitose do

fungo por macrófagos e sua atividade funcional, torna-se importante para a compreensão da relação hospedeiro-parasita na PCM.

O contato inicial de *P. brasiliensis* com o hospedeiro se faz através das células fagociticas, que constituem um importante compartimento da defesa inata do organismo. O fungo é fagocitado por macrófagos in vivo [15] e in vitro é internalizado e se multiplica em monócitos e macrófagos não ativados [18]. Apenas monócitos e macrófagos ativados *in vitro* com IFN-γ e TNF-α apresentam atividade fungicida contra *P. brasiliensis* [19,20].

P. brasiliensis apresenta em sua constituição uma multiplicidade de componentes antigênicos, que podem ser extraídos da parede do fungo, obtido do conteúdo citoplasmático (intracelulares) ou do filtrado de cultura (metabólicos ou exocelulares) [21]. Componentes do próprio fungo, como a glicoproteina de 43 kDa (gp43), considerada o principal antígeno secretado por *P. brasiliensis,* pode promover a adesão inicial e a internalização da levedura por células fagocíticas [22]. Assim, a gp43 parece estar envolvida na adesão de *P.brasiliensis* aos macrófagos peritoneais murinos, fenômeno que leva à fagocitose e pode favorecer a morte do fungo. Esse efeito é inibido por anticorpos anti-gp43. Ensaios de inibição indicam o envolvimento de resíduos de fucose e manose, na fagocitose de *P. brasiliensis* [22].

POPI et al. [23] avaliaram o efeito da gp43 da interação entre macrófagos peritoneais murinos e *P. brasiliensis.* A fagocitose de células leveduriformes do fungo pelas células peritoneais de camundongos das linhagens B10.A e A/Sn, considerados, respectivamente, suscetíveis e resistentes à infecção, foi inibida pela adição de diferentes concentrações de gp43 ao meio de cultura. Os autores sugerem que a produção de gp43 pelo fungo, pode ser considerada um mecanismo de evasão do *P. brasiliensis* na instalação da infecção em hospedeiros suscetíveis.

FERREIRA & ALMEIDA [24] estudaram o efeito da imunização prévia com células dendríticas estimuladas com gp43 sobre a infecção experimental de camundongos com cepa virulenta de *P. brasiliensis*. Verificaram que a gp43 apresenta efeito regulador negativo sobre a expressão de moléculas MHC-classe II e de adesão CD80, CD54 e CD40 e sobre a produção de IL-12 por essas células. Segundo os autores, a baixa expressão de moléculas co-estimulatórias e de IL-12 por células dendríticas, ambas induzidas por gp43, poderia estar envolvida na depressão de resposta Th1 in vivo, levando ao desenvolvimento de infecção mais grave nos animais.

Por outro lado, outros autores relataram que gp43 é capaz de induzir a produção de níveis elevados de IL-2, IFN-γ e IL-10 por células mononucleares de sangue periférico de indivíduos saudáveis, previamente infectados com *P.brasiliensis* e IL-10 por monócitos de pacientes com a forma ativa da doença [7].

A imunidade inata se constitui na primeira linha de defesa contra infecções, reconhecendo um largo espectro de patógenos, sem necessidade de sensibilização prévia. Foi inicialmente considerada como mecanismo inespecífico da resposta imune, caracterizado por fagocitose e digestão de microrganismos e substâncias estranhas por macrófagos e leucócitos. Entretanto, atualmente sabe-se que os mecanismos da imunidade inata reconhecem e reagem contra os microrganismos, discriminando entre os patógenos e o *self* [25,26]. Além disso, a ativação da resposta imune inata desencadeia uma rede de citocinas, resultando em resposta inflamatória e podendo ser um pré-requisito para o desencadeamento da imunidade adquirida [27,28].

O reconhecimento inicial de microrganismos é mediado por receptores celulares expressos em células da imunidade inata. Segundo TRICKER & CHENG [29] a fagocitose é iniciada por fagócitos incluindo macrófagos e células polimorfonucleares através do reconhecimento de partículas tais como patógenos ou células apoptóticas. Esses componentes da imunidade natural reconhecem estruturas que são características dos patógenos microbianos e não estão presentes nas células dos mamíferos. O reconhecimento ocorre através dos receptores da superfície celular, cujos ligantes estão presentes na superfície do patógeno. As substâncias dos microrganismos que estimulam a imunidade natural são chamadas de padrões moleculares associados aos patógenos (PAMPs) e os receptores que se ligam a essas estruturas preservadas são chamados de receptores de reconhecimento de padrões (PRRs). Diferentes classes de microrganismos como vírus, bactérias e fungos expressam diferentes PAMPs, estruturas que incluem ácidos nucléicos específicos de microrganismos, proteínas típicas de bactérias e complexos de lipídeos e carboidratos sintetizados pelos microrganismos, tais como lipopolissacarídeos (LPS), ácidos teicóicos e oligossacarídeos ricos em manose encontrados em glicoproteínas microbianas, mas não nos tecidos de mamíferos [30]. Assim, a interação entre essas moléculas de superfície do microrganismo e receptores homólogos, presentes na membrana celular de macrófagos, modulam a fagocitose e a ativação da célula [31,32]. Portanto, monócitos e macrófagos são células da imunidade inata que expressam receptores de superfície para manose, CD14, componentes do

sistema complemento, porção Fc de moléculas de imunoglobulinas e receptores semelhantes a Toll (TLR, *Toll-like receptor*) capazes de reconhecer produtos microbianos, levando à estimulação da fagocitose, atividade microbicida e produção de citocinas [30,33].

Receptores semelhantes ao *Toll* são uma família de proteínas de transmembrana, evolutivamente conservadas entre insetos e humanos [34] que foram primeiramente identificados como moléculas determinantes para o padrão embriogênico em *Drosophila* e, posteriomente como receptores essenciais na imunidade antifúngica [35]. Essas proteínas servem como receptores de reconhecimento padrão para uma variedade de moléculas derivadas de microrganismos e estimulam a resposta imune inata. Uma família de receptores Toll, designada TLRs foi descrita em mamíferos [25]. Até agora, pelo menos 11 TLRs foram identificados em humanos e 13 em camundongos, sendo fundamentais no reconhecimento de PAMPs [36]. Esses receptores podem ser divididos de acordo com a sua localização na célula: TLR-1/2/4/6/10 são expressos na superfície celular, enquanto que TLR-3/7/8/9 são expressos em compartimentos endossomais intracelulares. Assim, a expressão e ativação de TLRs contribuem para a defesa do hospedeiro contra infecções em *Drosophila*, camundongos e humanos [35,37,38]. A ativação de TLRs pode regular, não apenas a fagocitose e atividade microbicida, mas também a liberação de citocinas e diferenciação de células dendríticas imaturas em maduras, capacitando o sistema imune inato a induzir a resposta imune adaptativa [25,39].

Todos os receptores TLR contêm repetições ricas em leucina, flanqueadas por motivos ricos em cisteína em suas regiões extracelulares e um domínio de homologia ao receptor Toll/IL-1R (TIR) em suas regiões citoplasmáticas, o que é essencial para a sinalização [30]. Todo TLR sinaliza através de uma proteína adaptadora MyD88, que também contém um domínio Toll/IL-1R, resultando na translocação do fator de transcrição NF-κB e subseqüente transcrição de genes para citocinas pro-inflamatórias [40]. Além dessa via, uma via dependente de TRIF pode também ser ativada, que interage com TRAF 6 e RIP 1, mediando assim a ativação de NFkB [25,41].

A expressão de TLRs na superfície celular pode ser detectada por anticorpos monoclonais principalmente em monócitos e células dendríticas imaturas [42]. Entretanto, a expressão de TLR é observada em outras células, incluindo neutrófilos, células endoteliais vasculares, adipócitos, miócitos cardíacos e células epiteliais intestinais. A expressão de vários TLR é também modulada em resposta a diferentes estímulos [27].

Alguns TLRs podem reconhecer uma variedade de ligantes. Em muitos casos, dois diferentes TLRs colaboram entre si ou com outro co-receptor para envio de sinais, após interação com o ligante microbiano [43]. TLR4 e seu co-receptor MD-2, reconhecem LPS de bactérias Gram-negativas bem como polissacáride de *Cryptococcus neoformans* [44]. Por outro lado, TLR2 medeia resposta celular a peptidoglicanos de bactérias, lipoproteinas e zimosan, em cooperação com TLR1 ou TLR6 [43]. A resposta imune inata a uma espécie de microrganismo pode refletir a integração das respostas de vários TLRs para diferentes moléculas produzidas pelo microrganismo [30].

Muitos componentes da parede celular de fungos podem atuar como PAMPs, que são reconhecidos por TLRs expressos por fagócitos e células dendríticas. Os principais TLRs envolvidos no reconhecimento de diferentes formas dos fungos como conídios, hifas e leveduras são TLR1, TLR2, TLR4 e TLR9 [45]. Estudos in vitro envolvendo células fúngicas têm mostrado que *C. neoformans, Candida albicans* e *Aspergillus fumigatus*, podem interagir com TLRs, particularmente TLR2, TLR4 e TLR9 presentes em células da imunidade inata, [44,46-49]. Tem sido descrito que a resposta de macrófagos a *A. fumigatus* é dependente da interação com TLR-2, TLR4 e MyD88 [50], enquanto a resposta a *C. neoformans* é principalmente mediada por TLR4 [44].

Estudos realizados com macrófagos peritoneais murinos demonstraram que esférulas de *Coccidioides posadasii* estimulam a produção de TNF-α, via interação com TLR2, MyD88 e Dectin-1 [51]. VAN DER GRAAF et al. [52] investigaram o reconhecimento diferencial de blastoconidios e de hifas de *C. albicans* por TLR, presentes em células mononucleares de sangue periférico humano e em macrófagos de camundongos. Os autores verificaram que TLR4 é capaz de mediar a indução de citocinas pró-inflamatórias após estimulação com o fungo, enquanto o reconhecimento desse microrganismo por TLR2 conduz principalmente à liberação de citocinas anti-inflamatórias. Assim, é provável que diferenças nos componentes de superfície desses fungos, sejam responsáveis pela ligação a diferentes TLRs e por diferentes padrões de citocinas, produzidas no confronto fungo-célula hospedeira.

A interação de uma única espécie fúngica com diferentes TLRs pode resultar em diferentes atividades biológicas. Estudos com componentes purificados da parede celular revelam o principal PRR e via de sinalização utilizada pelas células do hospedeiro para reconhecer PAMPs, ao contrário de quando se usa o patógeno inteiro para infectar hospedeiros normais ou deficientes de PRR. A ativação final, embora

influenciada pelo receptor ausente, é mediada por PRRs remanescentes que podem compensar ou não ao receptor deficiente [53]. Por outro lado, constituintes purificados da parede celular fúngica podem ativar mais de uma via de sinalização dependente de TLR; por exemplo, a produção de TNF-α por macrófagos in vitro, em resposta a fosfolipomanana de *C. albicans* é dependente de TLR2, TLR4 e TLR6 [46].

O principal componente da cápsula de *C. neoformans,* a glicuronoxilomanana liga-se a vários receptores da superfície de macrófagos, como CD14, CD18 e TLR-4, afetando algumas funções biológicas dessas células [54]. Além disso, manoproteinas secretadas pelo fungo são reconhecidas por receptores para manose [44,55]. CROSS & BANCROFT [56] demonstraram que a ingestão de formas não-capsuladas de *C. neoformans* é mediada por receptores para manose e β-glucana na superfície do macrófago e, que esse processo induz a produção das citocinas, TNF-α e fator estimulador de colônias de granulócitos e macrófagos (GM-CSF), resultando na ativação das células e fagocitose das formas encapsuladas do fungo. Portanto, durante a infecção fúngica, a geração de uma resposta inflamatória, a morte do fungo e a sobrevida do hospedeiro envolvem múltiplas vias dependentes ou não de TLR [45,57,58].

Além dos receptores TLR, outro receptor de superfície de macrófagos bem caracterizado é o receptor para manose, capaz de mediar a fagocitose e a morte intracelular de microrganismos patogênicos [59]. Esse receptor está envolvido na imunidade antifúngica e seu papel não tem sido totalmente esclarecido [60].

O receptor de manose (MR, CD206) é uma proteína transmembrânica que possui oito domínios de lectina do tipo C, um domínio com repetições de fibronectina do tipo II, um domínio rico em cisteína e uma pequena porção citoplasmática [60]. O receptor para manose é uma lectina do macrófago que interage com resíduos terminais de manose e fucose de glicoproteinas e glicolipídeos. Esses açúcares são moléculas tipicamente observadas na parede celular de microrganismos, que são assim, reconhecidas pelas células do hospedeiro [30]. A expressão do receptor de manose tem sido demonstrada em macrófagos peritoneais [61] e alveolares [62], bem como fagócitos mononucleares humanos [63]. Têm sido sugerido que o principal papel do MR é o clearance endocítico de glicoproteínas derivadas do hospedeiro [64] podendo mediar a fagocitose de microrganismo não-opsonizado, interagindo com polissacarídeos da parede celular, bem como com manana fúngica, cápsula bacteriana, lipopolissacáride e lipoarabonomanana [65]. O receptor de manose é essencial na produção de citocinas

tanto pró-inflamatórias quanto anti-inflamatórias, sendo capaz de interagir com outros PRRs, para mediar a sinalização intracelular [66]. Esse receptor é capaz de interagir com uma ampla variedade de microrganismos, como bactérias Gram-positivas, Gram-negativas, fungos, protozoários e micobactérias [59,62,67-69]. Macrófagos derivados de monócitos humanos fagocitam leveduras de *C.albicans* não opsonizadas, via receptor para manose [63,70-73]. Além da interação com *C. albicans* esse receptor também está envolvido na ligação de outros microrganismos como *Pseudomonas* [74], *Pneumocystis carinii* [75], *Leishmania donovani* [76], *Mycobacterium avium* [77,78] e *Paracoccidioides brasiliensis* [22].

Portanto, a produção de citocinas pelas células da imunidade inata parece ser um evento importante que ocorre após a interação com os patógenos, levando à ativação celular e resultando na destruição do microrganismo ou na instalação da doença. A produção de citocinas como TNF-α, IL-1, IL-6, IL-8, IL-10 e IL-12 é observada em estudos in vitro, após estímulo de macrófagos e monócitos humanos com diferentes fungos, como *C. immitis*, [79], *C. neoformans* [56,80], *C. albicans* [81,82], *Malassezia furfur* [83,84] e *P. brasiliensis* [85,86], demonstrando que essas células podem ser fontes importantes de citocinas, após interação com esses microrganismos.

Embora a importância da imunidade inata na resistência a infecções fúngicas já seja bem reconhecida [45], os PRRs que reconhecem *P. brasiliensis e os mecanismos moleculares envolvidos* não estão ainda bem caracterizados [53,87]. É possível que TLR2 e TLR4 estejam envolvidos, uma vez que vários fungos como *C. albicans*, *A. niger*, *A. fumigatus,* e *Sacharomyces cerevisiae* são reconhecidos por esses receptores [88]. A interação de *P.brasiliensis* com macrófagos peritoneais é aumentada por opsonização das células leveduriformes com iC3b [89] e, a fagocitose de conidios do fungo por linhagens de macrófagos murinos ocorre via CR3 e receptor de manose (MR) [90]. Estudos recentes sobre o papel dos receptores TLR na PCM experimental murina sugerem que células leveduriformes de *P.brasiliensis* podem interagir tanto com TLR2 como TLR4 para entrar e infectar os macrófagos, resultando em aumento da atividade fagocitica, secreção de NO e infecção dos macrófagos. Na infecção in vivo, a deficiência de TLR resultou em diminuição da carga fúngica nos animais e sobrevida semelhante à de animais normais, novamente sugerindo que TLRs são usados pelo fungo para infectar o hospedeiro. Assim, a interação com TLR pode ser considerada um mecanismo de patogenicidade do *P.brasiliensis,* que usa os receptores da imunidade inata (TLR2 e TLR4) para infectar as células e garantir sua própria multiplicação [53].

BONFIM et al. [91] avaliaram a expressão de TLR1, TLR2, TLR4 e dectina-1 em monócitos e neutrófilos de indivíduos saudáveis após estimulação de células leveduriformes de *P. brasiliensis*, com alta ou baixa viruência e sugeriram a participação de TLR2, TLR4 edectina-1 no reconhecimento, internalização e conseqüente ativação da resposta imune contra o fungo.

Em trabalho recente, avaliamos a modulação da expressão de TLR2 e TLR4 na superfície de monócitos humanos estimulados in vitro com células leveduriformes de *P.brasiliensis* ou com gp43, seu principal antígeno e, a produção de TNF-α e IL-10 por citometria de fluxo e Elisa, respectivamente. Os resultados mostram que tanto o fungo como a gp43 são capazes de modular a expressão de TLR2 e TLR4 em relação às células controle não estimuladas. Entretanto, os resultados obtidos com estímulo de gp43 foram mais evidentes. Baixa expressão de TLR2 e alta de TLR4 por monócitos foram induzidas por gp43 em 4h de cultura, associadas com elevada produção de TNF-α. Entretanto, esse perfil inverteu-se após 18 h de cultura, observando-se maior expressão de TLR2 e menor de TLR4, associadas com maior produção de IL-10. Assim, a persistência de gp43 na cultura de monócitos por 18h parece aumentar a expressão de TLR2 e a produção de IL-10 por essas células, sugerindo um mecanismo de escape do fungo na célula hospedeira. A produção da citocina antiinflamatória poderia levar a um estado de supressão da resposta do hospedeiro, permitindo a instalação do fungo nos tecidos. Por outro lado, considerando o envolvimento de níveis elevados de TNF-α na patogênese da PCM [92], é possível que a produção sustentada de IL-10, induzida por gp43, poderia controlar a resposta inflamatória excessiva induzida por citocinas pró-inflamatórias, que resultaria na lesão tecidual observada na PCM [93].

Esses resultados de modulação TLR2 associada a aumento da síntese de IL-10 por monócitos induzidos por gp43, associados aos resultados de alta produção de IL-10 por monócitos de pacientes com PCM [92] e aumento do número de células T reguladoras (CD4+CD25+ FoxP3) nesses pacientes [94] poderiam explicar a imunossupressão observada na PCM e mostram que a imunidade inata pode direcionar ou interferir nos resultados da imunidade adaptativa.

Assim, tanto células leveduriformes de *P. brasiliensis* como a gp43 purificada são capazes de induzir a produção de citocinas por monócitos humanos. O ambiente de citocinas presente na interação inicial fagócito-*P. brasiliensis*, poderia ser eficiente para eliminação do fungo ou permitir sua implantação e multiplicação nos tecidos do

hospedeiro, causando doença progressiva. Dessa forma, o perfil e a quantidade de diferentes citocinas produzidas, com atividade supressora ou estimulatória sobre a resposta imune, pode determinar diferenças na evolução da PCM. A compreensão dos mecanismos envolvidos durante o contato inicial entre o fungo e monócitos, pode auxiliar na compreensão da patogênese desta micose. Portanto, o estudo da interação da gp43 de *P. brasiliensis* com receptores TLRs e MR da superfície de monócitos humanos, permitirá a melhor compreensão das vias de ativação dessas células, que levam à produção de citocinas pró e anti-inflamatórias durante o confronto fungo-monócito.

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The glicoprotein (gp43) of *Paracoccidioides brasiliensis* modulates TLR2 and TLR4 expression and cytokine production by human monocytes

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Abstract

Toll-like receptors recognize distinct components of fungi to initiate the innate immune response. We examined whether *Paracoccidioides brasiliensis* or its immunodominant antigen gp43 can modulate *in vitro* TLR2 and TLR4 expression and trigger cytokine production by human monocytes. Monocytes from healthy individuals were incubated with gp43, lypopolysaccharide (LPS) or heat-killed yeast forms of *P. brasiliensis* in a ratio of 50 monocytes per fungal cell (Pb18) at 37^{o}C for 4h and 18h. The expression of TLR2 and TLR4 on monocyte surface, and TNF-alpha, IL-10 and IL-12p40 production were determined by flow cytometry and ELISA respectively. The results showed that monocyte stimulation with LPS or Pb18 promoted up-regulation of the TLR2 and TLR4 expression on monocyte surface in relation to the non-stimulated cells at both 4h and 18h of culture, and induced higher levels of TNF-alpha, IL-10 and IL-12p40 mainly at 18h of culture. On the other hand, high TLR4 and low TLR2 expression were elicited by gp43 at 4h of culture, associated with higher levels of TNF-alpha. However, after 18h a change to high TLR2 and low TLR4 expressions was followed by elevated levels of IL-10 and IL-12p40. These results suggest that gp43 might induce an imbalance between pro- and anti-inflammatory responses in fungal-monocyte interactions by a modulatory effect on TLR pathway. As TNF-alpha may be involved in the pathogenesis of paracoccidioidomycosis, the regulatory effect induced by gp43, via upregulation of TLR2 expression and IL-10 production, can be important to protect against tissue injury which is described in this mycosis.

Keywords: *Paracoccidioides brasiliensis;* gp43; human monocytes; TLR2; TLR4

Introduction

Paracoccidioidomycosis is the most prevalent systemic human mycosis in Latin America, and is caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis* [1]. Infection can be acquired by inhalation of fungal mycelial propagules which are transformed into infective yeast cells in the lung [2,3]. The disease presents as an entity with a systemic course or as a chronic localized mycosis, depending on several factors, including host immunocompetence, parasite strain and the environment [4].

Monocytes from patients with active forms of paracoccidioidomycosis are an important source of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 that may be associated with pathogenesis of the mycosis [5]. In previous work we demonstrated that *in vitro* infection of monocytes from healthy individuals with a virulent strain of *P. brasiliensis*, induced higher levels of both pro- and anti-inflammatory cytokines in comparison with the low virulence strain of the fungus [6]. Thus, the cytokine environment during the early fungus-monocyte interactions may be important to fungal clearance or may promote their growth and implantation in host tissues, leading to progressive disease.

Innate immunity, mainly involving monocytes and macrophages, is considered an important defense mechanism against Gram-positive and Gram-negative bacteria, viruses and pathogenic fungi. Macrophages and dendritic cells from this immune system directly kill the microorganism through phagocytosis and inactivation of invading organisms, cytokine production, and interactions with other adaptive immunity cells such as T and B lymphocytes [7-9].

Macrophage activation is one of the first events in innate resistance to intracellular fungal infection by recognition of microorganism surface components. The specific detection of microorganisms by innate cells is mediated by a limited repertoire

of germ-line-encoded proteins, the pattern recognition receptors (PRRs), that recognize microbial structures referred as pathogen-associated molecular patterns (PAMPs), considered to be essencial for the pathogen survival [8-10]. PAMPs recognition by PRRs allows self-nonself discrimination, because PAMPs are not produced by host cells [11]. A variety of receptors that mediate PAMP recognition from phagocytic cells signal to the host the presence of infection, include mannose receptor (MR), β-glucan receptor (βGR), dectin-1, and Toll-like receptors (TLRs) [12-14]. TLRs not only mediate recognition of microbial structures, such as those of fungi, but also trigger the release of cytokines and the differentiation of immature to mature dendritic cells, enabling the innate immune system to instruct the adaptative immune response [10,15,16].

The involvement of TLRs on fungi recognition and resistance of mammalian hosts has been described for *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* [17-20], and more recently for *P. brasiliensis.* Bonfim et al. [21] evaluating the expression of TLR1, TLR2, TLR4 and dectin-1 in monocytes and neutrophils from healthy individuals after stimulation with high and low virulence yeast of *P. brasiliensis*, suggested the participation of TLR2, TLR4 and dectin-1 in fungus recognition, internalization and consequent activation of immune response against the fungus. A study on the role of TLRs in a murine model of paracoccidioidomycosis employing normal and $TLR2^{-}$ or $TLR4^{-}$-gene knockout mice (KO) in a C57BL/6 background suggested that *P. brasiliensis* yeasts use TLR2 and TLR4 to gain entry into macrophages and infect mammalian hosts, and allowing the fungus own multiplication [14]. Experimental model of chronic pulmonary infection with the fungus in TLR2-deficient mice shows that TLR2 deficiency results in increased Th17 immunity associated with diminished expansion of regulatory T cells and increased lung pathology due to unrestrained inflammatory reactions [22].

It has been reported that a single fungal species can use different TLRs resulting in a diverse biological activities. Recognition of *C. albicans* at the level of cell membrane by TLR induces different types of cytokines. TLR4 induces mainly pro-inflammatory signals in monocytes, macrophages and dendritic cells, while TLR2 stimulates the production of moderate amounts of pro-inflammatory cytokines, and strong IL-10 and TGF-β responses [23]. Different research groups have demonstrated divergent roles for TLR2 and TLR4, and their importance in the control of *C. albicans* and *C. neoformans* infections is still unclear [12,20]. Thus, studies with purified components of fungal cell walls may reveal the major PRR and signaling pathways used by host cells to recognize fungal PAMPs [14].

The main antigenic component of *P. brasiliensis* is a 43-kDa secreted as high-mannose glycoprotein (gp43) [24,25] employed for diagnosis and prognosis of paracoccidiodomycosis [26,27]. Gp43 may participate in the installation mechanisms of primary infection by inhibiting phagocytosis, nitric oxide (NO) and hydrogen peroxide (H2O2) production, and fungal intracellular killing [28,29], thus playing different roles in the pathogenesis of the mycosis [30].

The molecular mechanisms of innate recognition and the receptors involved in paracoccidioidomycosis are poorly understood. In this study we analyzed whether *P. brasiliensis* or its immunodominant antigen gp43 can modulate in vitro TLR2 and TLR4 expression and trigger cytokine production by human monocytes.

Materials and Methods

Reagents and media

All reagents were obtained from Sigma-Aldrich, Inc., (St Louis, MO. USA), unless stated otherwise. Phycoerythrin-Cy7 (PE/Cy7)-labeled mouse monoclonal antibodies (MAbs) to human CD14 (M5E2), Phycoerythrin (PE)-labeled MAbs to human TLR4 (HTA125), Fluorescein (FITC)-labeled MAbs to human TLR2 (TLR2.1), and PE/Cy7-, PE- and FITC-labeled control isotype MAbs were purchased from Biolegend (San Diego, CA, USA). Quantikine ELISA kit and respective standards of TNF-alpha, IL-10 and IL-12p40 were acquired from R&D Systems (Minneapolis, MN, USA) and used according to the manufacturer's instructions.

Healthy individuals

Twenty healthy blood donors were recruited from the University Hospital, Botucatu Medical School, São Paulo State University, Brazil, age range 20–50 years (mean age 32.5 ± 10.2 years). The study was approved by Botucatu Medical School Ethics Committee, and informed consent was obtained from all the blood donors.

Fungus

Paracoccidioides brasiliensis strain 18 (Pb18) was maintained in yeast-like form cells at 35 ^{o}C on 2% glucose, 1% peptone, 0.5% yeast extract, and 2% agar medium (GPY medium) all from Gibco Laboratories, Grand Island, NY, USA) and used on the sixth day of growth culture. Yeast cells were washed and suspended in 0.15 M phosphate-buffered saline (PBS-pH 7.2). In order to obtain individual cells, the fungal suspension was homogenized with glass beads in a Vortex homogenizer (three cycles of 10 seconds) [31]. Fungal suspensions were washed twice with PBS at 400 g for 10 min,

and submitted to autoclavation at 121 ^{o}C for 15 min to obtain heat killed yeast forms of *P. brasiliensis.* The dead yeast cell concentration was adjusted to 1 x 10^6 cells/mL in RPMI 1640 medium.

Gp43 from *Paracoccidioides brasiliensis*

Gp43 was kindly provided by Professor Rosana Puccia of Federal University of Sao Paulo (UNIFESP) Brazil. A soluble recombinant gp43 (gp43r) was expressed in the yeast *Pichia pastoris*, and purified in affinity columns of Affi-Gel 10 bound to Mab17c, a monoclonal antibody anti-gp43, according to Carvalho et al. [27].

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized (50U/mL heparin) venous blood by Histopaque [density (d) = 1.077] (Sigma-Aldrich) density-gradient centrifugation. Briefly, 5 mL of heparinized blood was mixed with an equal volume of RPMI-1640 tissue culture medium (Sigma-Aldrich) containing 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 20 mM HEPES, and 40 ug/mL gentamicin (complete medium). Samples were layered over 5-mL Histopaque in a 15-mL conical plastic centrifuge tube. After centrifuging at 400 g for 30 min at room temperature, the interface layer of PBMC was carefully aspirated and washed twice with PBS containing 0.05 mM ethylenediaminetetraacetic acid (PBS-EDTA) and once with complete medium at 300 g for 10 min. Cell viability, as determined by 0.2% Trypan Blue dye exclusion, was > 95% in all experiments. Monocytes were counted using neutral red (0.02%), and were suspended at a concentration of 1 x 10^6 monocytes/mL in complete medium.

Production of monocyte culture supernatants

The monocyte suspension (1 x 10^6/mL) was distributed (1 mL/well) in 24-well flat-bottomed plates (Nunc, Life Tech. Inc., Maryland, USA). After incubation for 2 hr at 37 ^oC in a humidified 5% CO_2 atmosphere, non-adherent cells were removed by aspiration and each well was rinsed twice with complete medium. Monocyte preparations routinely contained > 90% monocytes as determined by morphologic examination and staining for nonspecific esterase [32]. In the experiments for evaluating cytokine production, monocytes were incubated with or without monoclonal antibodies (MAbs) anti-TLR2 or anti-TLR4 (all from Biolegend) at 0.5 μg/mL for 60 min at 37º C in 5% CO_2. After incubation, the monocytes were washed and treated with complete medium, in the presence or absence of gp43 (10ng/mL) or lipopolysaccharide (LPS) of *Escherichia coli* $O_{55}B_5$ (Sigma-Aldrich) (10 ug/mL) or heat-killed yeast forms of Pb18 (1:50 fungus/monocytes ratio) for 4h and 18h at 37º C in 5% CO_2. Culture supernatants were harvested and stored at -80 ^oC until assayed.

Determination of cytokines

Cytokine concentrations were determined in cell-free supernatants obtained after 4h or 18h monocyte cultures with gp43, LPS or heat- killed yeast forms of Pb18 (1:50 fungus/monocytes ratio) by enzyme-linked immunosorbent assay (ELISA), as described above, using Quantikine ELISA kits (R&D Systems) for TNF-α, IL-10, IL-12p40 and IL-12p70 according to the manufacturer's instructions. Assay sensitivity limit was 10 pg/mL for TNF-α, 7.5 pg/mL for IL-10 and 15 pg/mL for IL-12p40 and IL-12p70.

Flow-cytometry analysis of TLR2 and TLR4 expression on monocytes

Cell surface expression of TLR2 and TLR4 on monocyte was assessed by flow cytometry, by use of a FACScalibur flow cytometer with Cell Quest software (both from Becton Dickinson). PBMC containing 5 x 10^5 monocytes/mL from healthy subjects were distributed into polystyrene tubes for cytometric analysis (BD Biosciences, San Diego, CA, USA), and were incubated for 4h or 18h at 37 ^oC in a humidified 5% CO2 atmosphere with complete medium with or without gp43 (10 ng/mL) or LPS (10 ug/mL) or heat-killed yeast forms of Pb18 (1:50 fungus/monocytes ratio). Cells were washed and incubated with monoclonal antibodies, according to the manufacturer's instructions: 0.5 ug of PE/Cy7-labeled anti-CD14, 0.5 ug of PE-labeled anti-TLR2, and 0.5ug of FITC-labeled anti-TLR4 (all from Biolegend). The cells were incubated for 30 min in the dark at room temperature, washed and fixed with 2% paraformaldehyde in PBS. The back-ground staining was determined by cell incubation with 0.5 ug of FITC- or PE- or PE/Cy7-labeled control isotype antibodies, for 30 min at room temperature in the dark. The samples were then washed twice with PBS and were analyzed by flow cytometry. Ten thousand monocyte events, defined as cells with respective side scatter (SSC) and CD14 staining characteristics were acquired in the list mode file from each sample, and corresponding levels of TLR2 and TLR4 were obtained from the CD14 cell gate. Results were expressed as mean percentage of positive CD14+ cells or fluorescence intensity (MFI) of positive events gated.

Statistical analysis

The results are presented as mean ± standard error (SEM). The data were compared by analysis of variance (ANOVA) followed by the Tukey test using INSTAT 3.05 software (GraphPad San Diego, CA, USA.). A p value < 0.05 was considered significant [33].

Results

TLR2 and TLR4 expression on monocytes surface

Monocytes were cultured in the absence (control culture) or in the presence of heat-killed yeast forms of *P. brasiliensis* (Pb18), in a ratio of 50 monocytes per fungal cell, or gp43 (1ng, 5ng, 10ng and 20 ng/mL) at 37º C for 4h and 18h. LPS (10 ug/mL) was employed as a positive control for monocyte stimulation. Figures 1A and 1B show that more than 90% of monocytes were positive for TLR2 and TLR4 respectively with the different stimuli employed. No significant increase in percentage of cells expressing TLR2 or TLR4 after LPS, Pb18 or gp43 in both periods of incubation were observed in relation to control, non-stimulated cultures. High percentage of cells co-expressing both TLR2 and TLR4 is also represented (Fig. 1C). The high co-expression (more than 80% cells) of TLR2 and TLR4 in control, non-stimulated cultures suggests that monocytes constitutively express these receptors.

The mean fluorescence intensity (MFI) analysis showed significant increase in TLR2 expression on monocyte surface after 4h and 18h of PB18 and LPS stimulation in comparison with control non-stimulated cultures. Monocyte stimulation with different concentrations of gp43 (1 ng, 5 ng, 10 or 20 ng/mL) demonstrated higher MFI expression with 10 ng/mL (data not shown). Although the culture with 10 ng/mL gp43 enhanced TLR2 expression at 4h of monocyte culture, significant MFI values were observed only after 18h of culture. This increase on TLR2 expression was significantly higher than those observed in control or gp43-stimulated cells at 4h of culture (Fig. 1E). These results showed that TLR2 expression was modulated by Pb18 and LPS after 4h and 18h of culture, while gp43 induces significant increase of TLR2 expression after 18h of culture.

The MFI analysis of TLR4 expression (Fig 1F) after 4h of culture showed that this receptor expression on monocyte surface is higher after the three stimuli employed than in control cultures. No significant difference between 4h and 18h of TLR4 expression were detected in monocyte non-stimulated cultures or cultures stimulated with Pb18 or LPS. On the other hand, TLR4 expression induced by gp43 at 4h of culture was significant higher than in control, LPS and Pb18-stimulated cultures. However, TLR4 expression after 18h of monocyte cultures stimulated with gp43 was significantly lower than at 4h cultures and did not show statistical difference in comparison with control cultures. These results showed that monocyte cultured with gp43 exhibit higher TLR4 expression at 4h and higher TLR2 expression after 18h of stimulation.

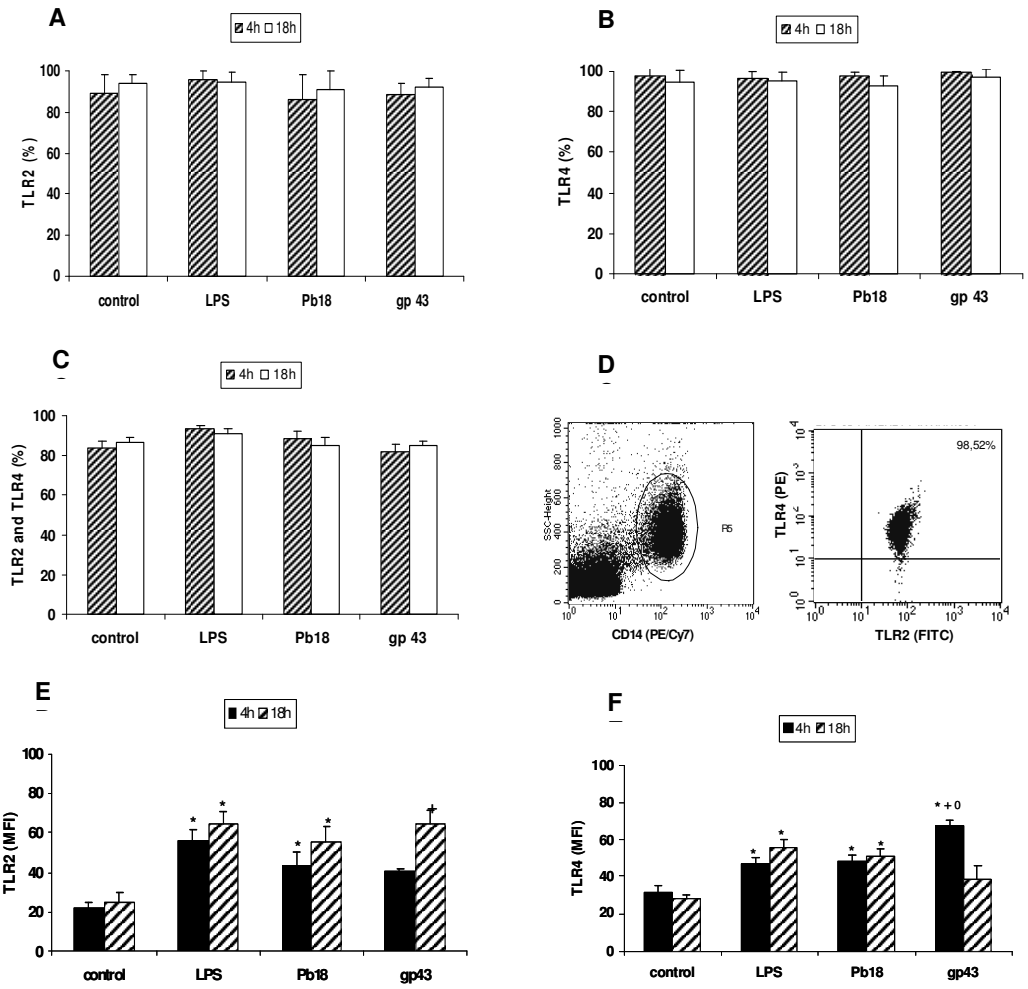

Fig 1. TLR2 and TLR4 expression on monocyte surface. Monocytes were incubated in the absence (control culture), or presence of lipopolysaccharide (LPS) (10 ug/mL), heat-killed yeast forms of *P. brasiliensis* (Pb18), in a ratio of 50 monocytes per fungal cell, or gp43 (10 ng/mL) at 37º C for 4h and 18h, and TLR2 and TLR4 expression were analyzed by flow cytometry. Percentage of monocytes expressing TLR2 (A), TLR4 (B) and co-expressing TLR2 and TLR4 (C). Representative dot-plots of gated CD14+ cells co-expressing TLR2 and TLR4 (D), and the mean fluorescence intensity (MFI) for TLR2 (E) or TLR4 (F) on monocyte surface. Results are expressed as the mean $\pm$ SEM for 20 healthy individuals.

* ($p < 0.05$) *versus* control (4h and 18h); + ($p < 0.05$) *versus* Pb18, LPS (4h); 0 ($p < 0.05$) *versus* gp43 (18h).

Comparison between TLR2 and TLR4 after 4h and 18h of Pb18, LPS and gp43 stimuli may be seen at Figure 2A and 2B respectively. The results showed that both Pb18 and LPS positively modulate TLR2 and TLR4 expression, and no significant differences in MFI expression were observed in both periods of culture. On the other hand, gp43 stimulation induced higher expression of TLR4 and TLR2 at 4h and 18h respectively.

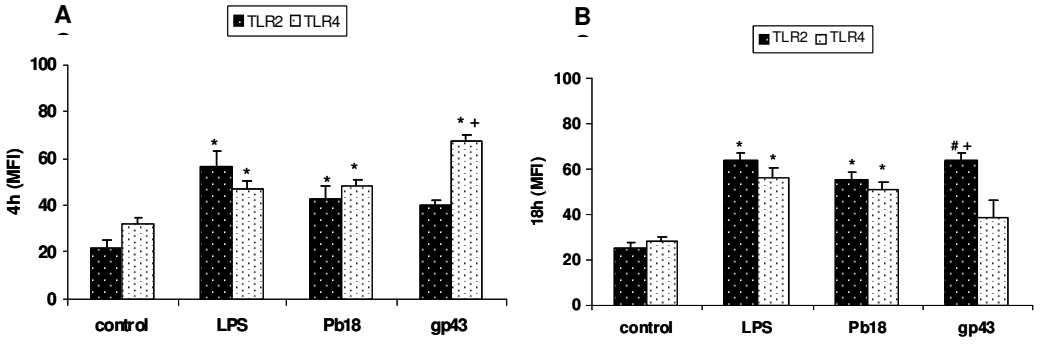

Fig 2. Comparison between TLR2 and TLR4 expression on monocytes surface according to the time of culture. Monocytes were incubated in the absence (control culture), or presence of lipopolysaccharide (LPS) (10 ug/mL), heat-killed yeast forms of *P. brasiliensis* (Pb18), in a ratio of 50 monocytes per fungal cell, or gp43 (10 ng/mL) at 37º C for 4h and 18h, and TLR2 and TLR4 expression were analyzed by flow cytometry. Results represent the mean $\pm$ SEM of the mean fluorescence intensity (MFI) for TLR2 or TLR4 on monocyte surface obtained from 20 healthy individuals at 4h (A) and 18h (B) of culture.

* ($p < 0.05$) *versus* control; + ($p < 0.05$) *versus* TLR2; # ($p < 0.05$) *versus* TLR4

Cytokine production by monocytes stimulated with *P. brasiliensis* and gp43

Production of IL-10, IL-12p40 and TNF-α in monocyte cultures after 4h and 18h after LPS, Pb18 and gp43 stimulation can be observed in Figure 3. The levels of IL-10 and IL-12p40 detected after 18h of stimulation with gp43 were significant higher than the same stimulus at 4h. However, lower levels of these cytokines were observed after Pb18

stimulation than after LPS and gp43 (Fig. 3A and 3B). There were no significant differences in IL-12p40 concentration detected in supernatant cultures of monocytes stimulated with Pb18 during 4h or 18h. Interestingly, the levels of IL-10 and IL-12p40 produced at 18h of monocyte culture with gp43 were 5-fold higher than those detected at 4h of cultures, suggesting later induction of these cytokines by gp43. Concentration of IL-12 p70 was not detected in the supernatant cultures of monocytes stimulated with Pb18 or gp43 (data not shown).

On the other hand, TNF-α production after monocyte stimulation with gp43 was higher at 4h than at 18h of culture (Figure 3C). This result was not expected, since higher levels of this cytokine were produced after 18h of monocyte stimulation with Pb18 or LPS. These results suggest a control of TNF-α production by gp43.

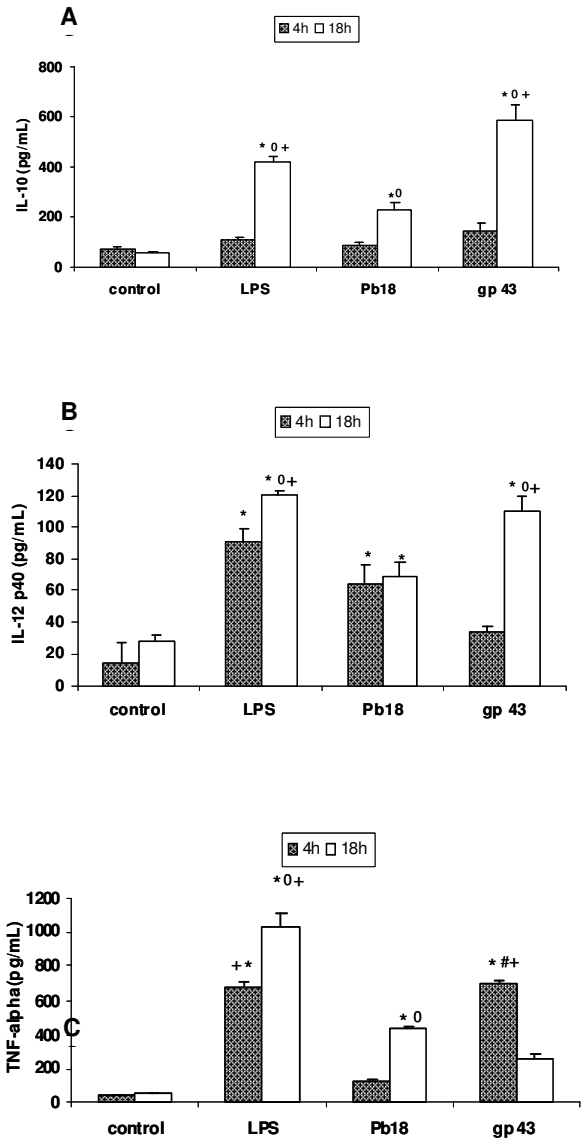

Fig 3. Cytokine production by human monocytes stimulated without (control culture), or with lipopolysaccharide (LPS) (10 ug/mL), with heat-killed yeast forms of *P. brasiliensis* (Pb18), in a ratio of 50 monocytes per fungal cell, or gp43 (10 ng/mL) at 37º C for 4h and 18h. Results represent the mean ± SEM of the cytokine levels produced by monocytes obtained from 20 healthy individuals.

* ($p < 0.05$) *versus* control; + ($p < 0.05$) *versus* 4h; 0 ($p < 0.05$) *versus* Pb18; # ($p < 0.05$) *versus* 18h

Involvement of TLR2 and TLR4 on cytokine production

The role of TLR2 and TLR4 on IL-10, IL-12p40 and TNF-α production by human monocytes stimulated with gp43 (10 ug/mL) was evaluated by blockade of these receptors with specific MAbs, before cell culture with the antigen for 4h and 18h. The blockade of TLR2 or TLR4 individually, as well as both receptors before gp43 stimulation led to lower levels of IL-10 and IL-12p40 in comparison with monocytes cultures not submitted to TLR2 and TLR4 blockade. These effects were more evident after 18h of monocyte stimulation with gp43 (Fig. 4A and 4B). Although there is a tendency to lower levels of IL-10 and IL-12p40 after TLR2 blockage in relation to TLR4 blockage, no significant differences were observed when the cytokine levels were compared. These results suggest that IL-10 and IL-12p40 production may be dependent on gp43 interaction with TLR2 and TLR4.

The concentration of TNF-α in supernatant culture of monocytes submitted or not to TLR2, TLR4 or TLR2 plus TLR4 blockade with MAbs, and stimulated with gp43 (10 ng/mL) for 4h was significantly higher than the observed at 18h of culture. The blockade of TLR4 before gp43 stimulation led to lower levels of TNF-α produced by monocytes both at 4h and 18h of culture than in cultures with TLR2 blockade or cultures not submitted to TLR blockade. On the other hand, TLR2 blockade did not show inhibitory effect on TNF-a production. The concentration of this cytokine was similar to the values obtained in non-blockade monocyte cultures stimulated with gp43. The lower levels of TNF-α.detected in cultures with TLR4 blockade before gp43 stimulation suggest that TLR4 play a role in the interaction with gp43 for TNF-α production (Fig. 4C).

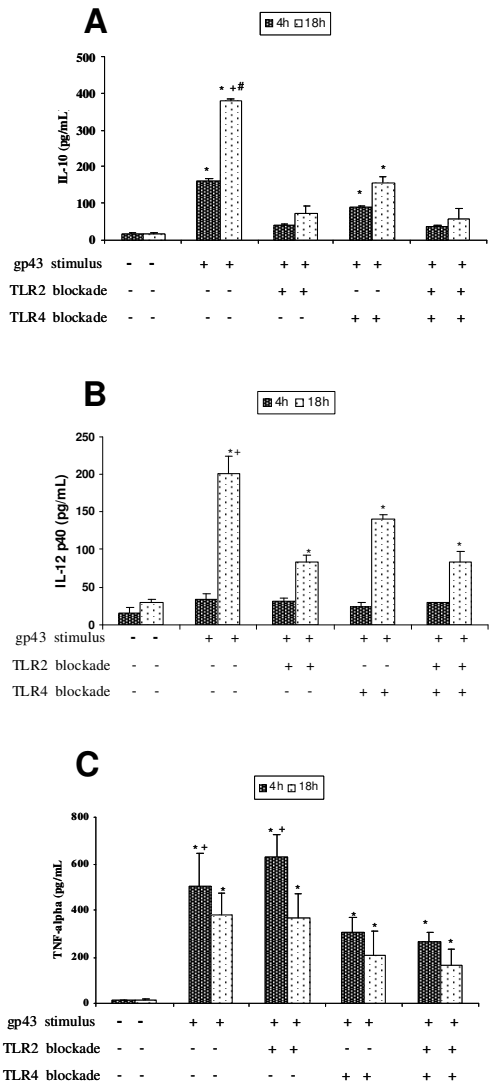

Fig 4. Involvement of TLR2 and TLR4 on IL-10, IL-12 and TNF-α production by human monocytes stimulated with gp43. Monocytes were incubated in the absence (control culture), or presence of anti-TLR2, anti-TLR4 or anti-TLR2 plus anti-TLR4 monoclonal antibodies for 60 min, and then stimulated with gp43 (10 ng/mL) at 37º C. After 4h and 18h of culture supernatants were collected for cytokine detection by ELISA. Results represent the mean ± SEM of IL-10 (A), IL-12p40 (B) and TNF-α (C) concentrations obtained from 20 healthy individuals.

IL-10: * ($p < 0.01$) *versus* control; + ($p < 0.05$) *versus* TLR2, TLR4, TLR2 plus TLR4 (4h and 18h); # ($p < 0.01$) *versus* gp43 (4h);

IL-12p40: * ($p < 0.05$) *versus* control and gp43-4h; + ($p < 0.05$) *versus* TLR2, TLR4 and TLR2 plus TLR4 (18h)

TNF-α: * ($p < 0.05$) *versus* control and gp43-18h; + ($p < 0.05$) *versus* 18h;
+ ($p < 0.05$) *versus* TLR2, TLR4, TLR2 plus TLR4 (4h and 18h)

Discussion

The central role of TLRs in innate immune recognition of fungal pathogens has been proposed [12,19], but so far, only one study evaluated expression of these receptors in monocytes and neutrophils stimulated by yeast cells of *P. brasiliensis* [21] The results of the present study demonstrated that *P. brasiliensis* and its main antigen gp43 promote monocyte activation, by cytokine production and exerting a modulatory effect on TLR expression by these cells. Gp43 is the best-studied *P. brasiliensis* component employed for diagnosis and prognosis of paracoccidioidomycosis described in the literature [24,26,27]. However so far, studies on modulatory effect of gp43 over TLR expression by monocytes have not yet been performed.

First, we observed that monocytes from healthy individuals constitutively express TLR2 and TLR4, since no significant increase in percentage of cells expressing TLR2 or TLR4 were detected 4h and 18h after LPS, Pb18 or gp43 stimulation in relation to control, non-stimulated cultures. High percentage of monocytes (80 – 90%) co-expressed both TLR2 and TLR4. According to Netea et al. [23] monocytes and neutrophils are the major cells of the innate immune response that recognize invader pathogens in blood, and express high levels of TLR on their cell membranes. During differentiation into macrophages, monocytes retain expression of TLR and increase their expression of lectin receptors. This information might explain the high percentage of monocytes expressing TLR2 and TLR4 detected in control, non-stimulated cells observed in the present study.

However, the mean fluorescence intensity (MFI) analysis showed significant increase in TLR2 expression on monocyte surface after 4h and 18h of Pb18 and LPS stimulation, while TLR4 expression was higher after 4h of culture with the three stimuli employed than in control, non-stimulated cultures. The comparison between the MFI

values and percentage of monocytes expressing TLR2 and TLR4 after stimulation with Pb18, LPS and gp43 showed that the MFI parameter allowed the better discrimination of the modulatory effect induced by the three stimuli employed. Thus, we utilized the MFI parameter to analyze the modulatory effect of *P.brasiliensis* and its antigen gp43 on monocyte TLR expression.

Whole yeast cells of Pb18 induced high expression of TLR2 and TLR4 on monocyte surface both at 4h and 18h of culture, whereas the stimulus with gp43 significantly enhanced TLR2 expression after 18h of culture. On the other hand, TLR4 expression induced by gp43 in 4h was significant higher than LPS and Pb18 stimuli in this period, suggesting a greater ability to modulate earlier TLR4 expression. After 18h of monocyte stimulation with gp43, TLR4 expression was significantly lower than at 4h cultures. These results showed that gp43 upregulates TLR4 expression at 4h, and TLR2 after 18h of stimulation, whereas these differences are not so evident with monocyte stimulation with Pb18. Our results showing that Pb18 and gp43 positively modulate TLR2 and TLR4 expression differ from those recently reported by Bonfim et al. [21] demonstrating a decrease of TLR1, TLR2, TLR4 and dectin-1 expression on monocytes, as soon as 30 to 60 min after *P. brasiliensis* yeast cells stimulation, and suggesting the participation of these receptors in *P. brasiliensis* recognition, internalization and consequent activation of the immune response against the fungus. Since the authors evaluated the expression of these receptors after few minutes of fungus stimulation, our results might be explained by the longer period (4h and 18h) of monocyte stimulation with Pb18 and gp43. It is possible that after internalization TLRs receptors can be re-synthesized and appear at monocyte membrane, explaining the higher MFI of TLR2 and TLR4 observed after 4h and 18h of stimulation with the fungus or gp43.

Results concerning TLR expression and cytokine production by monocytes stimulated by Pb18 were not always in accordance with those obtained after cell stimulation with gp43, in relation to the period of 4h or 18h of culture. Thus, Pb18 stimulated the expression of TLR2 and TLR4 in both periods of culture, and induced higher levels of TNF-α and IL-10 only after 18h of culture. On the other hand, gp43 preferentially upregulates TLR4 and TNF-α production at 4h, with moderate levels of IL-10, whereas high TLR2 expression and higher levels of IL-10 and IL-12p40 were detected at 18h of culture. Therefore, these results were confirmed by experiments of TLR4 or TLR2 blockade with specific MAbs, and suggest that gp43 stimulates earlier higher levels of TNF-α, and elevated production of IL-10 latter. The persistence of gp43 in monocyte cultures, for 18h, may induce downregulation of the inflammatory response, by high production of IL-10 in association with high TLR2 and low TLR4 expression, and followed by low TNF-α production in this period. This antigen maintenance for a long period of monocyte culture might cause chronic stimulation of TLR2 with higher IL-10 production which could autocrinally inhibit TNF-α production by monocytes.

The discrepancy among the results employing Pb18 and gp43 might be explained by the presence of different antigenic components in the cell wall of the yeast form of Pb18, capable to interact with different TLR or non-TLR receptors, present in the cell surface of monocytes, in comparison with gp43. It is possible that the purified antigen of *P. brasiliensis* might be better available to interact and stimulate monocyte cultures than whole fungal cells. The gp43 employed in the present study was obtained as a soluble recombinant gp43 (gp43r) isoform, a N-mannosylated protein expressed in the yeast *Pichia pastoris* and purified in affinity columns containing anti-gp43 antibodies by Carvalho et al. [27]. According to the authors gp43r may replace the

native gp43 (gp43n) in assays for diagnosis of paracoccidioidomycosis, because gp43n expression in supernatant fluid of *P. brasiliensis* cultures can be unstable, and varies with the isolate. The advantage of gp43r from *P. pastoris* relies mainly in reproducibility for the production of large amounts of a known sequence of gp43, which is expressed in culture supernatants under inducible conditions in non-pathogenic, fast-growing yeast. Some authors suggest that the employement of purified cell wall components of pathogenic fungi could point out the main PRR receptor and the signaling pathway of the host cell to recognize PAMPs associated with fungi [14,23].

In the present study, monocyte stimulation with gp43 after TLR4 blockade employing MAbs induced lower levels of TNF-α, while no inhibitory effect on this cytokine production was observed after TLR2 blockade. Thus, TLR4 may play a role in gp43 recognition for TNF-α production, as observed in other studies showing association between TNF-α production and TLR4 recognition of fungal components by human monocytes [34,35]. On the other hand, TLR2 and TLR4 blockade before stimulation with gp43 led to low production of IL-10 and IL-12p40, suggesting that these cytokines release may be dependent on gp43 interaction with TLR2 and TLR4. Association between TLR2 expression and IL-10 production against pathogenic fungi, such as *A. fumigatus* and *C. albicans* has been described in the literature [36,37]. The deleterious effect of TLR2 signaling during *C. albicans* infection is associated with increased production of IL-10 and development of T regulatory (CD4+CD25+) cells, resulting in deficient cellular immune response and lower ability to the fungus elimination [37]. The same association was observed in other infection models with *Schistosoma mansoni* [38] and *Borrelia burgdorferi* [39], suggesting an important role of TLR2 in inducing the expansion and control of regulatory T cells [40]. In mice infected with *P. brasiliensis*, pulmonary dendritic cells showed increased TLR2 gene

expression, associated with higher production of IL-10, and contributing to higher susceptibility to infection [41]. High concentrations of IL-10 were detected in serum, and as IL-10 mRNA expression and protein release in peripheral blood cell culture supernatants of patients with paracoccidioidomycosis [42-45]. Monocytes from patients spontaneously release high levels of IL-10 [5] which decrease after antifungal treatment and clinical cure [46]. These results corroborates the participation of T regulatory cells in paracoccidioidomycosis, since it was reported elevated number of these cells in patients with active disease both in peripheral blood and lesions [47]. However, experimental model of paracoccidioidomycosis demonstrated that TLR2 deficiency results in increased Th17 immunity associated with diminished expansion of CD4+CD25+Foxp3+ T cells and increased lung pathology due to unrestrained inflammatory response [22]. Therefore, activation of TLR2 and IL-10 production may have a protective role against the pathogenesis of the mycosis.

The similar profile of IL-10 and IL-12p40 production by monocytes submitted to TLR2 blockade and gp43 stimulation suggest that *P. brasiliensis* antigen may interact with this receptor, leading to both cytokine production. The high production of IL-10 after 18h of monocytes stimulation with gp43 suggest its role in downregulation of inflammatory response against *P. brasiliensis*. It has been suggested that IL-12p40 subunit can play anti-inflammatory role by controlling IL-12p70 production, which is involved in excessive inflammation observed in severe sepses [48]. Release of high concentration of IL-12p40 has been also reported in mononuclear phagocytes stimulation with intracellular microorganisms such as *Leishmania*, whereas low levels of IL-12 p70 were produced [49]. Thus, in the present study, high levels of IL-10 and IL-12p40 produced after gp43 monocyte stimulation might be involved in regulation of inflammatory cytokines produced during excessive monocyte activation. Corroborating

this hypothesis, our results showed that monocytes stimulated with Pb18 and gp43 produced higher levels of IL-12p40, whereas production of IL-12 p70 was not detected in these cultures (data not shown).

Although our data suggest the involvement of TLR2 and TLR4 in gp43 recognition, leading to preferentially IL-10 and TNF-α production respectively, we cannot discard the possibility that other TLR and non-TLRs may participate in these cytokine release. Gp43 has been reported to interact with mannose receptor and play a role in the installation mechanisms of primary infection by inhibiting both phagocytosis and fungal intracellular killing [28,29]. Recent study demonstrated that peptides P4 and P23 from gp43 inhibit macrophage function and show non-specific and specific anti-inflammatory properties [30].

In summary, our results showed that gp43 considered the immunodominant antigen of *P. brasiliensis* modulates TLR2 and TLR4 expression during human monocytes interaction. Association among high TLR4 expression and elevated production of TNF-α and moderate levels of IL-10 at 4h, and between high TLR2 expression and high production of IL-10 and IL-12p40 at 18h of culture suggest that this component of the fungus might induce an imbalance between pro-inflammatory and anti-inflammatory response in host/fungal interactions. Considering that high production of TNF-α has been described as involved in pathogenesis of paracoccidioidomycosis [5] it is possible that gp43, by trigger sustained production of IL-10, might control excessive inflammatory response induced by pro-inflammatory cytokines which result in tissue injury observed in this mycosis. The TLR blockade experiments suggest the interaction of gp43 with TLR2 and TLR4 signaling the cytokine profile obtained. However, experiments showing TLRs blockade and gp43

uptake are in progress in our laboratory and will be important to confirm this hypothesis.

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Conflict of interest

There is no conflict of interest.

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Interaction of TLR2, TLR4 and mannose receptor with gp43 of *Paracoccidioides brasiliensis* induces cytokine production by human monocytes

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Abstract

The glycoprotein gp43 is the immunodominant antigen secreted by *Paracoccidioides brasiliensis* that naturally infects human hosts. We investigated whether gp43 can interact with MR, TLR2 and TLR4 on human monocyte surface, modulates the expression of these receptors and triggers pro- and anti-inflammatory cytokines production. Monocytes from 20 healthy individuals were incubated in the absence or presence of monoclonal antibodies (MAbs) anti-TLR2, anti-TLR4 or anti-MR, or association of these MAbs and the gp43 binding to monocyte surface was analyzed by flow cytometry. The expression of TLR2, TLR4 and MR, and TNF-alpha and IL-10 production by monocytes submitted or not to these receptors blockade and gp43 stimulation were determined by flow cytometry and ELISA respectively. The results, confirmed by receptors blockade, showed that gp43 binds to TLR2, TLR4 and MR, being MR and TLR2 the most involved receptors in gp43 uptake. All receptors seem to play a role in IL-10 release, while TNF-alpha production was associated with TLR4 and MR. Modulatory effect of gp43 was evidenced by high TLR4 and low TLR2 expression after 4h of culture, associated with higher levels of TNF-alpha, whereas high TLR2 and low TLR4 expressions followed by elevated levels of IL-10 was detected at 18h. Gp43 uptake by the monocyte receptors studied might affect cell activation and this to determine fungal establishment or its elimination during host-fungus interaction.

Keywords: *Paracoccidioides brasiliensis;* gp43; human monocytes; Toll-like receptor; mannose receptor; cytokine production

1. Introduction

Paracoccidioides brasiliensis is a thermal dimorphic fungus that causes paracoccidioidomycosis, the most prevalent systemic mycosis in Latin America [1,2]. The fungus enter the host probably through inhalation of the conidia produced by its mycelial form, which reach lung alveoli, and establish infection after transformation into yeast, the infective form [2,3]. The clinical manifestations of the disease are those of a chronic granulomatous disease with involvement of the lung, reticuloendothelial system, mucocutaneous areas and other organs [4]. In paracoccidioidomycosis as in other systemic mycosis, immunity efficiency depends on the interplay between innate and adaptive host defenses and fungal pathogenic mechanisms. Although the importance of innate immunity in resistance to fungal infection is well recognized [5], the molecular mechanisms underlying recognition of *P.brasiliensis* by innate immune cells remains poorly defined [6].

Monocyte and macrophage activation is one of the first events in innate resistance to fungal infections resultant from recognition of microorganism surface components. Pathogen recognition is mediated by a series of germline encoded pattern recognition receptors (PRRs) that are either soluble or membrane-bound. These PRRs recognize conserved microbial structures, such as bacterial lipopolysaccharide, or fungal β-glucan which are known as pathogen associated molecular patterns (PAMPs) [7,8,9]. Fungi contain many components that can be considered non-self for the host, the majority of which are cell-wall-based carbohydrates [10]. These PAMPs can decorate the surface of the fungus, and include β-glucans, chitin, and mannoproteins [8,11]. Over recent years, a growing number of opsonic and non-opsonic PRRs that recognize fungal PAMPs have been identified; however, of particular interest are those

of the Toll-like receptor (TLR) and C-type lectin families, which appear to have a central roles in antifungal immunity [10].

The involvement of TLRs on fungi recognition and resistance of mammalian hosts has been described for *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* [5,12-14], and more recently for *P. brasiliensis.* Bonfim et al. [15] evaluating the expression of TLR1, TLR2, TLR4 and dectin-1 in monocytes and neutrophils from healthy individuals after stimulation with high and low virulence yeast of *P. brasiliensis*, suggested the participation of TLR2, TLR4 and dectin-1 in fungus recognition, internalization and consequent activation of the immune response against the fungus.

The MR (CD206) is a group VI C-type lectin receptor (CLR), possessing eight extracellular C-type lectin-like domains (CTLDs), a fibronectin type II repeat domain, a cysteine-rich domain and a short cytoplasmic tail. A soluble form of the MR is shed into the serum, which is generated through proteolytic cleavage of the membrane-bound receptor [10]. The MR is present widely on peritoneal macrophages [11] and alveolar macrophages [16], as well as human monocytes, and recognizes oligosaccharides that terminate in mannose, fucose and N-acetylglucosamine [17].

MR receptor has been implicated in endocytosis and phagocytosis, and the engagement with fungi leads to pro-inflammatory and anti-inflammatory cytokine production [18,19]. MR is able to interact with other canonical pattern recognition receptors in order to mediate intracellular signaling [20]. Stimulation via the TLR family leads to initiation of signaling cascades that culminate in activation of nuclear factor kB (NFkB) and mitogen-activated protein kinases. This process facilities the transcription of genes that regulate the adaptative immune response, including those for many cytokines and chemokines [21].

The gp43 glycoprotein is the main antigenic component secreted by *P. brasiliensis* which is recognized by most sera of patients with active paracoccidioidomycosis [22,23] and may be used for monitoring patients under treatment with antifungals [24]. Additionally, gp43 may be a virulence factor due to its adhesive properties [25,26], modulating *P.brasiliensis* infection in a hamster intratesticular model [27]. It has been reported that gp43 plays a role in the installation mechanisms of primary infection by inhibiting phagocytosis, nitric oxide (NO) and hydrogen peroxide (H2O2) production, and fungal intracellular killing by macrophages [28,29]. According to Konno et al. [30] inhibition of macrophage function may facilitate the homing of the fungus in host tissue, at least in the initial phase of infection, since phagocytosis of *P. brasiliensis* is mediated by mannose receptors on macrophage surface [28,29]. On the other hand, one peptide named P10, derived from gp43 was shown to mediate specific T-cell activation, leading to protection against paracoccidioidomycosis in BALB/c mice [31]. Thus, so far, gp43 of *P. brasiliensis* has been well recognized as an important fungal component which plays mainly pathogenic [30], than protective [31] roles for the host. Since studies on its binding to human monocytes has not yet performed, the present study was undertaken in order to determine whether the immunodominant antigen gp43 of *P. brasiliensis* can interact with MR, TLR2 and TLR4 on human monocyte surface, modulates the expression of these receptors and trigger pro- and anti-inflammatory cytokine production by these cells.

2. Materials and methods

2.1. Healthy individuals

Twenty healthy blood donors were recruited from the University Hospital, Botucatu Medical School, São Paulo State University, Brazil, age range 20–50 years (mean age

30.8 ± 8.9 years). The study was approved by Botucatu Medical School Ethics Committee, and informed consent was obtained from all the blood donors.

2.2. Gp43 from Paracoccidioides brasiliensis

Gp43 was kindly provided by Professor Rosana Puccia of Federal University of Sao Paulo (UNIFESP) Brazil. A soluble recombinant gp43 (gp43r) was expressed in the yeast *Pichia pastoris*, and purified in affinity columns of Affi-Gel 10 bound to Mab17c, a monoclonal antibody anti-gp43, according to Carvalho et al. [32].

2.3. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized (50U/mL heparin) venous blood by Histopaque [density (d) = 1.077] (Sigma-Aldrich, Inc., (St Louis, MO. USA) density-gradient centrifugation. Briefly, 5 ml of heparinized blood was mixed with an equal volume of RPMI-1640 tissue culture medium (Sigma-Aldrich) containing 2 mM L-glutamine, 10% heat-inactivated fetal calf serum (FCS), 20 mM HEPES, and 40 ug/ml gentamicin (complete medium). Samples were layered over 5-ml Histopaque in a 15-ml conical plastic centrifuge tube. After centrifuging at 400 g for 30 min at room temperature, the interface layer of PBMC was carefully aspirated and washed twice with phosphate buffer saline 0.1M pH 7.2 (PBS) containing 0.05 mM ethylenediaminetetraacetic acid (PBS-EDTA) and once with complete medium at 300 g for 10 min. Cell viability, as determined by 0.2% Trypan Blue dye exclusion, was > 95% in all experiments. Monocytes were counted using neutral red (0.02%), and were suspended at a concentration of 1 x 10^6 monocytes/ml in complete medium.

2.4. Production of monocyte culture supernatants

The monocyte suspension (1 x 10^6/mL) was distributed (1 mL/well) in 24-well flat-bottomed plates (Nunc, Life Tech. Inc., Maryland, USA) and incubated for 2 hr at 37 ^oC in a humidified 5% CO_2 atmosphere. Non-adherent cells were removed by aspiration and each well was rinsed twice with complete medium. Monocyte preparations routinely contained > 90% monocytes as determined by morphologic examination and staining for nonspecific esterase (Li et al., 1973). In the experiments for evaluating cytokine production, monocytes were incubated with or without monoclonal antibodies (MAbs) at 0.5 μg/mL anti-TLR2 (TLR2.1) or anti-TLR4 (HTA125), or anti-MR (15-2), all from Biolegend (San Diego, CA, USA) for 60 min at 37º C in 5% CO_2. After incubation, the monocytes were washed and treated with complete medium, in the presence or absence of gp43 (10ng/mL) or lipopolysaccharide (LPS) of *Escherichia coli* O55B5 (Sigma-Aldrich) (10 ug/mL) for 4h and 18h at 37oC with 5% CO2. Culture supernatants were harvested and stored at -80^{o}C until assayed.

2.5. Determination of cytokines

Cytokine concentrations were determined in cell-free supernatants obtained after 4h or 18 h monocyte cultures with gp43 or LPS by enzyme- linked immunosorbent assay (ELISA), using Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) for TNF-α and IL-10 according to the manufacturer's instructions. Assay sensitivity limit was 10 pg/ml for TNF-α and 7.5 pg/ml for IL-10.

2.6. Preparation of biotin-labeled gp43r

Recombinant gp43 (100 ng/ml) in 1 ml PBS were incubated for 2h on ice with 20ul EZ-link sulpho-NHS-biotin reagent solution, prepared according to the manufacturer's

instructions (Pierce, Rockford, USA). Afterwards, non-labelled biotin was removed with a dialysis chamber (Pierce) in PBS overnight.

2.7. Flow-cytometric analysis of uptake of gp43r by monocytes

In the experiments for evaluating gp43r binding to TLR2, TLR4 and MR, 5 x 10^5 monocytes/ml in complete medium were distributed into poliesterene tubes for cytometric analysis (BD Biosciences, San Diego, CA, USA) and were incubated in the absence or presence of 0.5 ug/ml of the following MAbs: anti-TLR2 (TLR2.1) or anti-TLR4 (HTA125), or anti-MR (15-2) all from Biolegend (San Diego, CA, USA), or association of these MAbs for 60 min at room temperature for receptors blockade. Cells were washed in PBS plus 1% FCS and incubated with 10 ng biotin-labeled gp43r for 30 min on ice. After another washing in PBS plus 1% FCS monocytes were subsequently incubated with streptavidin-FITC (SoutherBiotech, Birmingham, AL, USA) and with 0.5 ug of Pe/Cy7-labeled anti-CD14 (M5E2) for another 30 min on ice, washed once again in PBS with 1% FCS, fixed with 4% paraformaldehyde and analyzed by use a FACScalibur flow cytometer with Cell Quest software (both from Becton Dickinson). The back-ground staining was determined by incubation of the cells, in 0.5 ug of FITC-labeled control isotype antibodies, for 30 min at room temperature in the dark. Ten thousand monocyte events, defined as cells with respective side scatter (SSC) and CD14 staining characteristics were acquired in the list mode file from each sample, and corresponding levels of monocyte positive for gp43r were obtained from the CD14+ cell gate. Results were expressed as fluorescence intensity (MFI) of positive events gated.

2.8. Flow-cytometry analysis of MR, TLR2, TLR4 expression on monocytes

Cell surface expression of MR, TLR2, TLR4 on monocyte were assessed by flow cytometry, by use of a FACScalibur flow cytometer with Cell Quest software (both from Becton Dickinson). Peripheral blood mononuclear cells containing 5 x 10^5 monocytes/ml from healthy subjects were distributed into poliesterene tubes for cytometric analysis (BD Biosciences), and were incubated in complete medium with or without gp43 (10 ng/ml) or LPS (10 ug/mL) for 4h or 18h at 37°C and 5% CO2 atmosphere. Cells were washed and incubated with MAbs, according to the manufacturer's instructions: 0.5 ug of Pe/Cy7-labeled anti-CD14 (M5E2), 0.5 ug of FITC-labeled anti-MR (15-2), 0.5 ug of PE-labeled anti-TLR2 (TL2.1), 0.5ug of FITC-labeled anti-TLR4 (HTA125) (all from Biolegend). The cells were incubated for 30 min in the dark at room temperature, washed and fixed with 2% paraformaldehyde in PBS. The back-ground staining was determined by incubation of the cells, in 0.5 ug of FITC- or PE- or Pe/Cy7-labeled control isotype antibodies, for 30 min at room temperature in the dark. The samples were then washed twice with PBS and were analyzed by flow cytometry. Ten thousand monocyte events, defined as cells with respective side scatter (SSC) and CD14 staining characteristics were acquired in the list mode file from each sample, and corresponding levels of MR, TLR2 and TLR4 were obtained from the CD14+ cell gate. Results were expressed as mean percentage of positive cells or fluorescence intensity (MFI) of positive events gated.

2.9. Statistical analysis

The results are presented as mean ± standard deviation. The data were analyzed by analysis of variance (ANOVA) followed by the Tukey test using INSTAT 3.05 software (GraphPad San Diego, Calif., U.S.A.). A p value < 0.05 was considered significant [33].

3. Results

3.1. Binding of gp43r to TLR2, TLR4 and MR on monocytes

The extent of monocyte binding with gp43r was evaluated by mean intensity fluorescence (MFI) of the total CD14+ cell population. Monocytes treatment with different concentrations of gp43 biotin-labeled (1, 5, 10 or 20 ng) and other times of incubation (10 min, 30 min and 60 min) showed that the highest MFI expression was obtained with gp43r (10ng/ml) and 30 min of incubation (data not shown). The viability of monocytes cultured in the presence or absence of gp43r was > 95% in all determinations performed, indicating that the lack of uptake was not due to cell death. Results obtained from cells cultured with gp43r without TLR2, TLR4 and MR receptors blockade showed an elevated loading of gp43r on monocyte surface after 30 min of incubation. Blockade of TLR2, TLR4 and MR with specific MAbs, individually or in association, led to a significant decrease in MFI compared with cultures not submitted to receptors blockade (Fig. 1), suggesting that all the evaluated receptors may be involved in gp43r uptake. The highest inhibition of MFI was detected in monocytes after MR blockade (71.1%) or its association with TLR2 and TLR4 blockade (76.7%), showing values significant higher than after only TLR4 blockade (29.6%). The results suggest that MR and TLR2 are the most important receptors involved in gp43 uptake.

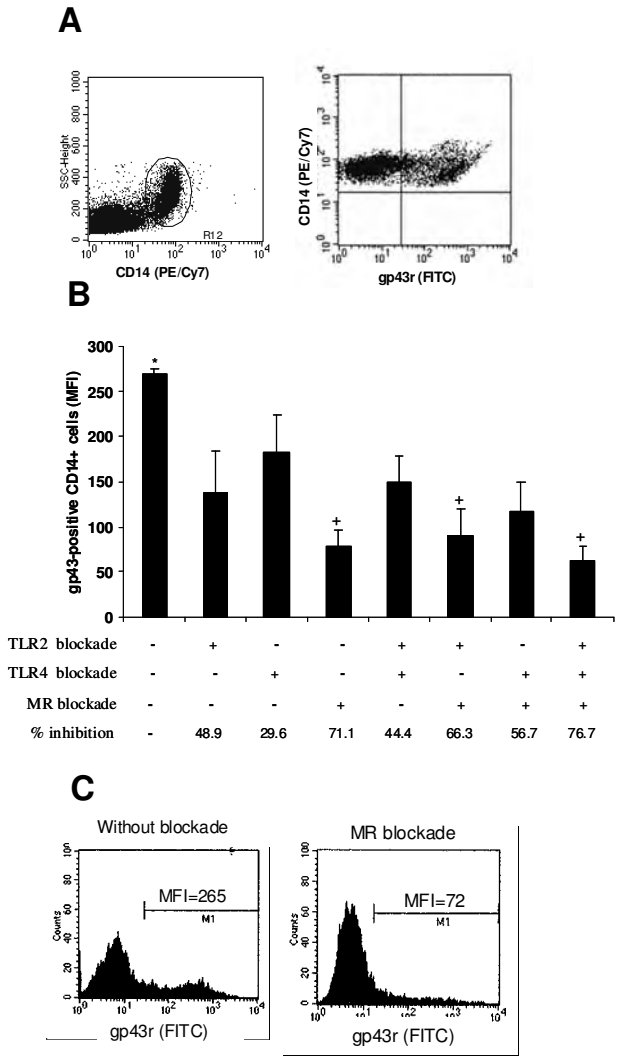

Fig 1. Binding of gp43 on human monocyte surface. Monocytes (5 x 10^5/ml) were incubated in the absence (without blockade), or presence of anti-TLR2, anti-TLR4, anti-MR, anti-TLR2 plus anti-TLR4, anti-TLR2 plus anti-MR, anti-TLR4 plus anti-MR or anti-TL2 plus anti-TLR4 plus anti-MR for 60 min at at room temperature . After incubation, the cells were treated with biotin-labeled gp43r (10 ng/ml) for 30 min on ice, and analyzed by flow cytometry. A) Representative dot-plots of one experiment showing gated $CD14^+$ cells expressing uptake of gp43; B) MFI of gp43-positive $CD14^+$ cells expressed as mean $\pm$ SEM of experiments from 20 healthy individuals. Percentage inhibition of gp43 binding after receptors blockade is also represented. C)

Representative histograms of one experiment showing gp43-positive $CD14^+$ cells before and after MR blockade.

(* $p < 0.05$) when compared with TLR2, TLR4, MR, TLR2 plus TLR4, TLR2 plus MR, TLR4 plus MR and TLR2 plus TLR4 plus MR blockade; (+ p , 0.05) when compared with TLR4 blockade.

3.2. Involvement of MR, TLR2 and TLR4 on IL-10 and TNF-alpha production

The role of MR, TLR2 and TLR4 played on IL-10 and TNF-α production by human monocytes stimulated with gp43 (10 ng/ml) was evaluated by blockade of these receptors with specific MAbs, for 60 min before cell culture with the antigen for 4h and 18h. IL-10 production after 18h of gp43 stimulation was significantly higher than the observed at 4h in cultures only stimulated with the antigen. Blockade of MR, TLR2 and TLR4, individually or in association, by specific MAbs followed by gp43 stimulation, inhibited IL-10 production, leading to cytokine levels significantly lower than in monocyte cultures non-submitted to these receptors blockade (Fig. 2A). These results suggest that IL-10 production may be dependent on gp43r binding to TLR2, TLR4 and MR

The concentration of TNF-α in supernatant of monocytes cultures was significantly higher after 4h of stimulation with gp43 (10 ng/ml) than after 18h and than in non-stimulated cultures. The blockade of TLR4 before gp43 stimulation led to lower levels of TNF-α produced by monocytes both at 4h and 18h of culture than in cultures with TLR2 blockade or cultures not submitted to TLR blockade. Similar results were observed after MR blockade. On the other hand, TLR2 blockade did not show inhibitory effect on TNF-α production. The concentration of this cytokine was similar to the values obtained in non-blockade monocyte cultures stimulated with gp43.

Downregulation of TNF-α production in cultures submitted to TLR2 blockade was observed only when there were association between TLR2, with TLR4 and MR blockade. The lowest levels of TNF-α.detected in cultures which TLR4 and MR were blockade before gp43 stimulation suggest that these receptors play a role in the interaction with gp43 for TNF-α production (Fig. 2B).

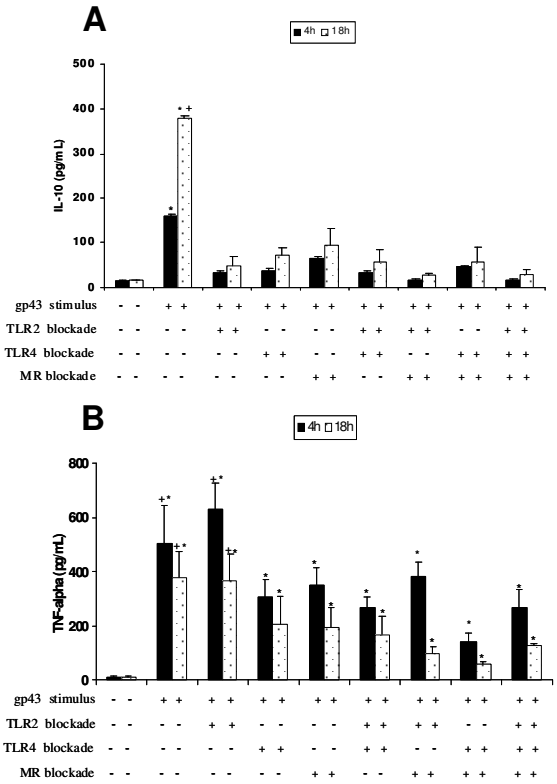

Fig 2. Involvement of MR, TLR2 and TLR4 on IL-10 and TNF-α production by human monocytes stimulated with gp43. Monocytes (5 x 10^5/ml) were incubated in the absence (without blockage), or presence of anti-TLR2, anti-TLR4, anti-MR, anti-TLR2 plus anti-TLR4, anti-TLR2 plus anti-MR, anti-TLR4 plus anti-MR or anti-TL2 plus anti-TLR4 plus anti-MR for 60 min at 37°C. After incubation, the cells were stimulated with

gp43 (10 ng/ml) for 4h and 18h at 37°C. Supernatants of cultures were collected for cytokine detection by ELISA. Results represent the mean ± SEM of IL-10 (A) and TNF-α (B) concentrations obtained from 20 healthy individuals.

IL-10 production: * ($p < 0,01$) when compared with control, TLR2, TLR4, MR, TLR2 plus TLR4, TLR2 plus MR, TLR4 plus MR, TLR2 plus TLR4 plus MR (4h and 18h); + ($p < 0,01$) vs gp43 (4h)

TNF-α production: * ($p < 0,05$) when compared with control and 4h; + ($p < 0.05$) when compared with TLR4, MR, TLR2 plus TLR4, TLR2 plus MR, TLR4 plus MR, TLR2 plus TLR4 plus MR (4h and 18h)

3.3. Modulatory effect of gp43r on TLR2, TLR4 and MR expression on monocytes

Monocytes were cultured in the absence (control culture) or in the presence of gp43r (10ng/ml) for 4h and 18h at 37°C. LPS (10 ug/mL) was employed as a positive control for monocyte stimulation. Figures 3A, 3B, 3C and 3D show the percentage of monocytes positive for MR, TLR2 and TLR4. High percentage of MR-positive monocytes were detected in cultures stimulated with gp43 or LPS, while no significant increase in percentage of cells expressing TLR2 or TLR4 after LPS or gp43 in both periods of incubation were observed in relation to control, non-stimulated cultures. Comparison among the percentage of TLR2-, TLR4- and MR-positive cells showed that few monocytes (less than 2%) express constitutively MR receptor before stimulation with LPS and gp43, while TLR2 and TLR4 were expressed in more than 90% CD14+ cells (Fig. 3B). The percentage of MR-positive monocytes increased three- and five-fold after LPS and gp43 stimulation respectively.

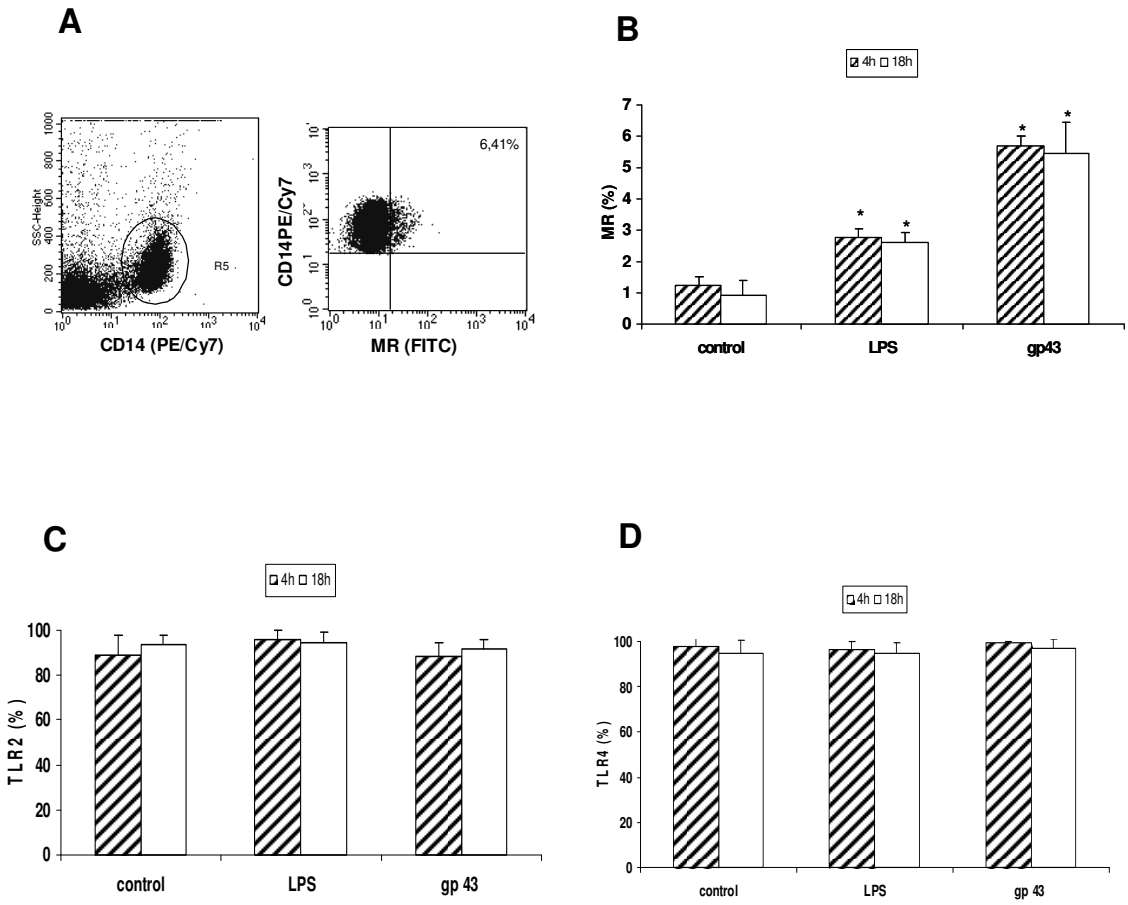

Fig 3. MR, TLR2 and TLR4 expression on human monocytes surface. Monocytes ($5x10^5$/ml) were incubated in the absence (control culture), or presence of lipopolysaccharide (LPS) (10 ug/ml), or gp43 (10 ng/ml) at 37° C for 4h and 18h, and MR, TLR2 and TLR4 expression were analyzed by flow cytometry. A) Representative dot-plots of one experiment showing gated $CD14^+$ cells expressing MR. Results represent the mean ± SEM percentage of monocytes expressing MR (B), TLR2 (C) or TLR4 (D) obtained from 20 healthy individuals.

* ($p< 0,05$) when compared with control.

The mean fluorescence intensity (MFI) analysis showed that the treatment of monocytes with LPS or gp43r did not increase MR expression both at 4h and 18h of periods of stimulation (Fig. 4A). On the other hand, significant increase on TLR2 expression was detected on monocyte surface after 4h and 18h of LPS stimulation in comparison with control non-stimulated cultures. Although the culture with gp43r enhanced TLR2 expression at 4h of monocyte culture, significant MFI values were only observed after 18h of culture. This increase in TLR2 expression was significantly higher than those observed in control or gp43r-stimulated cells at 4h of culture (Fig. 4B). TLR4 expression induced by gp43r at 4h of culture was significant higher than in control and LPS-stimulated cultures. However, TLR4 expression after 18 h of monocyte cultures stimulated with gp43 was significantly lower than at 4h cultures and did not show statistical difference in comparison with control cultures. These results showed that gp43 upregulates TLR4 expression at 4 h and TLR2 expression after 18 h of stimulation (Fig. 4C), but did not interfere with MR expression (Fig. 4A).

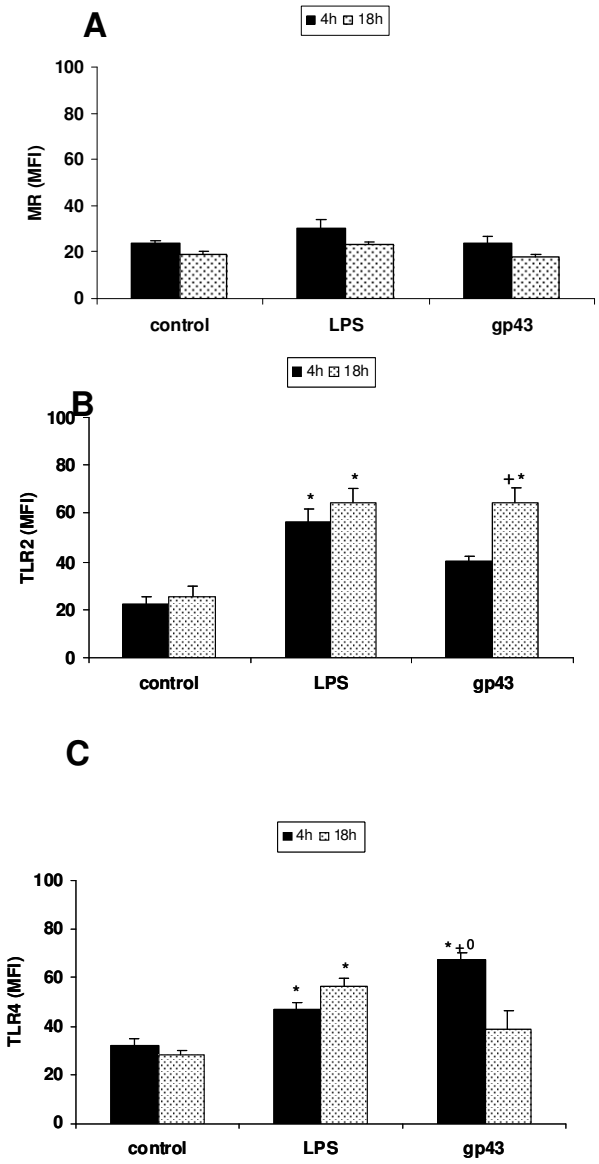

Fig 4. MR, TLR2 and TLR4 expression on human monocytes surface. Monocytes ($5x10^5$/ml) were incubated in the absence (control culture), or presence of lipopolysaccharide (LPS) (10 ug/ml), or gp43 (10 ng/ml) at 37° C for 4h and 18h, and MR, TLR2 and TLR4 expression were analyzed by flow cytometry. Results represent the mean $\pm$ SEM of the mean fluorescence intensity (MFI) for MR (A), TLR2 (B) or TLR4 (C) on monocyte surface obtained from 20 healthy individuals.

TLR2 expression: * ($p < 0,05$) vs control (4h e 18h); + ($p < 0,05$) vs gp43 (4h)
TLR4 expression: * ($p < 0,05$) vs control (4h e 18h); + ($p < 0,05$) vs LPS (4h); 0 ($p < 0,05$) vs gp43 (18h)

4. Discussion

In human paracoccidioidomycosis the exact route of infection with *P. brasiliensis* has not yet very well characterized, except for showing that the fungus can infect tissue macrophages and monocytes [34]. Recent studies reported that *P. brasiliensis* yeast cells may interact with TLR2, TLR4 and dectin-1 receptors expressed by monocytes and neutrophils, leading to cell activation and provoking an intense inflammatory response [15]. Besides, a virulent strain of the fungus can induce high levels of pro- and anti-inflammatory cytokines during human monocytes infection in vitro [35]. The present study has examined whether the immunodominant antigen gp43 of *P. brasiliensis* can interact with MR, TLR2 and TLR4 on human monocytes surface and modulates the expression of these receptors and pro- and anti-inflammatory cytokines production. Up- and down-regulatory effects were observed. First we analyzed the interaction of gp43 with PRRs and showed that all the receptors studied are involved in gp43 uptake. Monocytes cultured with gp43 showed an elevated loading of antigen on monocyte surface after 30 min of incubation. Gp43 binding to monocytes was significantly inhibited when TLR2, TLR4 and MR were blockade with specific MAbs. The highest inhibition of MFI was detected in monocytes after MR blockade (71.1%) or its association with TLR2 and TLR4 blockade (76.7%), showing significant inhibitory values than after only TLR4 blockade (29.6%). The results suggest, for the first time, that MR and TLR2 are the most important receptors involved in gp43 uptake by human monocytes. The inability of a cocktail of MAbs to produce complete inhibition of gp43r binding could be caused by the presence of other receptor not evaluated.

Binding of *Penicilliun marneffei* conidia to human monocytes was significantly inhibited by monoclonal antibodies against MR, TLR1, TLR2, TLR6, CD14, CD11b and CD18, showing that various PRRs on human monocytes surface participate in the initial fungus recognition [19]. The MR has previously been reported to serve as recognition site for pathogenic fungi, including *C. albicans, Pneumocystis jiroveci* and *P. marneffei* yeasts [36], and for phagocytosis of *P. marneffei* [19] and *P. brasiliensis* [28,37,38], supporting the hypothesis that MR is a common phagocytic receptor for a wide variety of fungal pathogens. However, recent evidence challenges this view, suggesting that MR mediates binding of these organisms primarily and not their ingestion [39,40]. Besides, MR engagement leads to pro-inflammatory cytokine production [18]. Dectin-1 and MR bind to a variety of fungal pathogens including *C. albicans* and *A. fumigatus* [17]. Generally, Dectin-1 was also demonstrated to respond to β-glucan of *A. fumigatus* [41,42] and *C. albicans* [43]. Few studies reported the interaction of fungal antigens with cells of host innate immune system. Components of *C. albicans,* such as phospholipomannan is sensed by TLR2 and TLR6 [44], and glucoronoxilomanan of *C. neoformans* is recognized by TLR4 [45], whereas cryptococcal mannoproteins requires recognition of terminal mannose groups by MR [46].

More is known about the receptors involved in the induction of cytokine production by fungal pathogens. At least four TLRs (TLR2, TLR2, TLR6 and TLR9) and MR take part in triggering these responses. TLR4 can strongly stimulate pro-inflammatory cytokines [47,48] whereas TLR2 is associated with IL-10 release [49]. Srinoulprasert et al. [19] reported that treatment of human monocytes with MAbs specific to CD14 or TLR4 diminished TNF-α production by these cells stimulated with *P. marneffei*. These results were consistent with those obtained by others showing the

involvement of TLR4 and CD14 on TNF–α production by monocytes and macrophages activated by *A. fumigatus* [50-52]. Association between TLR2 expression and IL-10 production against pathogenic fungi, such as *A. fumigatus* and *C. albicans* has been described in the literature [49,53]. The deleterious effect of TLR2 signaling during *C. albicans* infection is associated with increased production of IL-10 and development of T regulatory (CD4+CD25+) cells, resulting in deficient cellular immune response and lower ability to the fungus elimination [49]. According to Bonfim et al. [15] while the high virulence strain of *P. brasiliensis* (Pb18) predominantly induced TNF-α, the low virulence strain (Pb265) was preferentially recognized by TLR2 and dectin-1, resulting in the production of adequate concentration of IL-10, which might induce a controlled immune response beneficial to the host. Differently, our results concerning to MR, TLR2 and TLR4 involvement in cytokine production after gp43 recognition by human monocytes, and confirmed by TLRs and MR blockade, showed that all receptors studied may be involved in IL-10 release, whereas TLR4 and MR may be key receptors for TNF-α production after gp43 binding. Thus, it is possible that a cross-talk between TLRs and MR after gp43 binding is necessary to mediate intracellular signaling for cytokine production. After bind with *Pneumocystis carini,* MR forms a functional complex with TLR2 on cell surface and facilitate signal transduction to cytokine production [54]. The discrepancy between our results and those reported by Bonfim et al [15] may be explained by the employment of gp43, a purified antigen of *P. brasiliensis* for binding studies to PRRs on monocyte surface while the authors used whole yeast cells to stimulate monocytes. According to Calich et al. [6] studies with purified components of fungal cells may reveal the major PRR and signaling pathways used by the host cells to recognize fungal PAMPs.

We also investigated the modulatory effect of gp43 on TLR2, TLR4 and MR expression after binding to these receptors on monocyte surface. Gp43 increased the percentage of MR-positive monocytes both at 4h and 18h of stimulation, but did not interfere with the percentage of TLR2 and TLR4-positive cells. In contrast to TLR2- and TLR4-positive monocytes that expressed, constitutively these receptors in more than 90% of cells, few monocytes (less than 2%) express MR before stimulation with gp43. Monocytes express less MR than TLRs on their membranes [17]. At the steady state, 10 – 30% of MR is found at the cell surface, and the remaining 70% is localized intracellularly [20]. The higher percentage of monocytes expressing TLR2 and TLR4 before gp43 stimulation may be explained by their role in recognition of invading pathogen in the circulation. On the other hand, results of MFI analysis showed that gp43 preferentially upregulates TLR4 expression and TNF-α production at 4h with moderate levels of IL-10, whereas high TLR2 expression and higher levels of IL-10 were detected after 18h of stimulation. The results showed that after binding to TLRs, gp43 modulates their expression on cell surface, a phenomenon that may be attributed to cell activation, represented by the higher levels of cytokines released in monocyte cultures.

Although gp43 significantly increase the number of MR- positive cells, the antigen did not interfere with MFI expression. These results may be attributed to the low percentage of MR-positive cells. However, it is possible that after gp43 uptake by MR, this receptor may be internalized and its *de novo* synthesis and appearance on monocyte surface may be downregulated due to cell activation induced by pro-inflammatory cytokines produced during monocyte activation with gp43. It has been reported that macrophages MR recycles between plasma membrane and the early endossomal compartment, even in the absence of any ligand [20] or after binding to terminal mannose residues on the surface of *Trypanosoma cruzi* amastigotes, resulting

in their phagocytosis and intracellular multiplication [55]. Activation of macrophages by interferon-gamma (INF-γ) treatment downregulates MR expression on cell surface [56]. Then, adherence of *T.cruzi* mediated by MR may select macrophage that have not been activated with IFN-γ and are permissive to parasite intracellular reproduction [55].Thus, gp43 binding to MR present on surface of non-activated monocytes or macrophages could represent an evasion mechanism of *P. brasiliensis* from host immune response, as previously suggested [29,34].

In conclusion, the results showed that gp43 of *P. brasiliensis* binds to TLR2, TLR4 and MR and affects many functions of innate immune response by exerting modulatory effects on PRRs expression and inducing pro- and anti-inflammatory cytokines production. The balance between these effects, during interaction with host cells may be influenced by the amount of gp43 produced by the fungus and the susceptibility of the host, and could be determinant in fungal establishment or its elimination during primary host-fungal cell interaction.

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