UNIVERSIDADE ESTADUAL PAULISTA "JULIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA
CAMPUS DE BOTUCATU

O PAPEL DE RECEPTORES DE IMUNIDADE INATA βGR, MR, TLR2 E TLR4 NO RECONHECIMENTO DA *Candida albicans* POR MONÓCITOS HUMANOS ESTIMULADOS COM POLISSACARÍDEOS EXTRAÍDOS DO COGUMELO *Agaricus brasiliensis*

PRISCILA RAQUEL MARTINS

Tese apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista – UNESP para obtenção do título de Doutor em Patologia.

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Orientadora: Profa. Dra. Ângela M. V. de Campos Soares
Co-orientador: Prof. Dr. Ramon Kaneno

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" Grandes foram as lutas, maiores as vitórias.

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Queria recuar ou parar.

No entanto, Tu sempre estavas presente,

Na alegria ou na tristeza,

Fazendo da derrota uma vitória,

Da fraqueza uma força.

Com a tua ajuda venci.

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" Bom mesmo é a luta com determinação,

abraçar a vida com paixão,

perder com classe e vencer com ousadia,

pois o triunfo pertence a quem se atreve...

A vida é muita para ser

insignificante."

Charles Chaplin

Sumário

Revisão de Literatura

O fungo dimórfico *Candida albicans (C. albicans)* pertencente ao grupo dos Deuteromicetos se apresenta como levedura, de forma oval, com brotamento e produz pseudo-hifas tanto em cultura quanto em tecidos e exsudatos (Edman, 1998). Está presente na microflora do trato digestivo e nas membranas mucocutâneas de indivíduos saudáveis, mantendo-se inofensivo aos hospedeiros em condições normais. Já em hospedeiros com imunodeficiência ou quando há uma desregulação na microflora normal, pode ocorrer o desenvolvimento da infecção. Estas infecções podem se manifestar tanto em candidíase aguda ou crônica de pele e mucosa, quanto candidíase invasiva, sistêmica ou disseminada (Netea et al., 2006).

O mecanismo de defesa contra candidíase sistêmica envolve principalmente a ingestão e eliminação do fungo pelas células do sistema inume inato, especialmente neutrófilos, monócitos e macrófagos (Van't Wout et al., 1988; Marodi et al., 1993). Após ativação dessas células pela *C. albicans*, ocorre a liberação de citocinas pró-inflamatórias como TNF-α, IL-1β, IL-6 e IFN-γ. Essas citocinas ativam neutrófilos e macrófagos a fagocitarem o fungo e liberarem reativos do oxigênio e nitrogênio que são tóxicos ao patógeno invasor, promovendo assim a sua eliminação (Djeu, 1990; Kullberg et al., 1993).

Calderone et al. (1994) sugeriram que a ligação dos fungos às células polimorfonucleares (PMN) levaria à produção de oxidantes citotóxicos como peróxido de hidrogênio (H_2O_2) e ânion superóxido (O_2^-), os quais exerceriam atividade fungicida. O peróxido de hidrogênio é gerado a partir da redução do NADPH, que passa os elétrons para o citocromo da membrana celular e faz com que ocorra a redução do ânion superóxido pela NADPH oxidase. Pela influência da superóxido dismutase, transforma-se em peróxido de hidrogênio e depois em radicais hidroxilas (OH) microbicidas, agindo contra os microrganismos no ambiente extracelular (Root & Metcalf, 1977 apud Pick & Keisare, 1980). Estes metabólitos, além das atividades microbicidas e citotóxicas, possuem importantes propriedades imunoreguladoras e inflamatórias (Los et al., 1995). Stevenhagen & Furth (1993) demonstraram que o aumento da atividade candidacida por granulócitos ativados com IFN-γ foi devido a um aumento na produção de radicais reativos do oxigênio.

Em relação ao óxido nítrico (NO), sua síntese ocorre a partir da oxidação do aminoácido L-arginina, por ação de enzimas chamadas de NO-sintase (NOS) que removem o nitrogênio guanidina terminal da L-arginina formando L-citrulina e NO.

Há três isoformas de NOS, com diferentes distribuições nos tecidos. O tipo I (nNOS) é uma NOS neuronal expressa constitutivamente e que não tem papel significativo na inflamação. O tipo II (iNOS) é uma enzima induzível, presente nos macrófagos e células endoteliais, induzida por várias citocinas e mediadores inflamatórios como IL-1, TNF, IFN-γ e por endotoxinas bacterianas. Já o tipo III (eNOS) é uma NOS sintetizada constituvamente, encontrada princpalmente no endotélio. Níveis altos de produção de NO por uma variedade de células parecem limitar a replicação de bactérias, helmintos, protozoários, vírus, bem como células tumorais, sob o risco de lesão das células e tecidos do hospedeiro (Moilanen et al., 1999).

A estimulação da produção de citocinas pró-inflamatórias e a ativação da imunidade inata dependem do reconhecimento do patógeno invasor. A estratégia para reconhecimento do patógeno pelas células do sistema imune inato consiste no reconhecimento não-clonal de estruturas conservadas dos microrganismos, chamadas de padrões moleculares associados à patógenos (*pathogen-associated molecular patterns*, PAMPs), os quais não estão presentes nas células do hospedeiro (Netea et al., 2006).

Várias classes de receptores de reconhecimento de padrões (*pattern-recognition receptors*, PRRs) reconhecem os vários PAMPs, contudo há um maior interesse nos receptores semelhantes a Toll (*Toll-like receptors*, TLRs) e uma família de lectinas do tipo-C (*C-type lectin families*, CLRs), os quais parecem ter papel central na imunidade antifúngica (Willment & Brown, 2008).

O receptor Toll foi originalmente identificado em *Drosophila melanogaster*, devido ao seu papel na determinação do padrão dorso-ventral durante a embriogênese da mosca. Mais tarde, observou-se que ele também participava na sinalização em resposta à infecção em moscas adultas. Um homólogo de Toll foi identificado em mamíferos e proteínas semelhantes são também usadas por plantas em sua defesa contra vírus, indicando que a via Toll é uma antiga via de sinalização utilizada em defesas inatas na maioria dos organismos multicelulares (Netea et al., 2004a). São expressos em várias células do sistema imune como macrófagos, células dendríticas, células B, tipos específicos de células T além de células não imunes como fibroblastos e células epiteliais (Akira et al., 2006). Há 12 genes TLR em mamíferos, sendo 11 expressos em humanos; todos os quais são glicoproteínas de membrana tipo I que contêm repetições ricas em leucinas 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 (Akira et al., 2006). Todo TLR sinaliza através de uma proteína adaptadora MyD88 (*myeloid differentiation factor 88*) que também contém o domínio TIR, resultando na translocação do fator de transcrição NF-κB e subseqüente transcrição de genes para citocinas proinflamatórias e quimiocinas (Takeda & Akira, 2004; Akira et al., 2006). A estimulação da maioria dos TLRs leva preferencialmente a uma resposta do tipo Th1 do que Th2 (Akira et al., 2006).

Tem sido descrito que os TLRs reconhecem uma ampla variedade de estruturas patogênicas como lipopeptídeos triacilados bacterianos (TLR1 em associação com o TLR2), lipoproteínas bacterianas, ácido lipoteicóico e zymosan (TLR2), RNA de fita dupla (TLR3), lipopolissacarídeos de bactérias Gram negativas (TLR4), flagelina bacteriana, lipopeptídeos diacilados (TLR6 em associação com TLR2), RNA de fita simples (TLR7) e CpG DNA não metilado bacteriano (Takeda & Akira 2004).

Efeitos sinérgicos também têm sido reportados através da co-ativação de dois TLRs. A co-ativação do TLR2 e TLR4 levam a uma grande produção de TNF-α, IL-6 e proteína inflamatória de macrófagos-1 α (MIP-1 α) tanto em macrófagos murinos quanto em monócitos humanos (Bagchi et al, 2007). Esses receptores também exercem efeito sinérgico quanto à produção de NO pelos macrófagos (Paul-Clark et al., 2006).

Vários trabalhos relatam o envolvimento do TLR2 e TLR4 no reconhecimento da *C. albicans* (Netea et al., 2002; Netea et al., 2004b; Villamon et al., 2004a; Villamon et al., 2004b).

Netea et al. (2002) foram os primeiros a estudar a participação dos TLRs no reconhecimento de patógenos fúngicos, avaliando o envolvimento do TLR4 na candidiase experimental. Os autores observaram que camundongos C3H/HeJ, os quais possuem uma mutação de ponto que os confere um TLR4 defeituoso, apresentaram um aumento do crescimento de *C. albicans* nos rins, órgãos chave da candidíase disseminada. Este defeito no TLR4 não alterou a produção de citocinas proinflamatórias, como o TNF, IL-1α e IL-1β e nem os mecanismos de morte fúngica, como produção de óxido nítrico e ânion superóxido. Esses mesmos autores observaram uma inibição no recrutamento de neutrófilos para o sítio da infecção , processo relacionado com a inibição da produção de quimiocinas como KC e MIP-2, sugerindo que o TLR4 estimula a produção dessas quimiocinas, mas não das

citocinas proinflamatórias. Nesse mesmo trabalho, utilizando células mononucleares do sangue periférico humano (PBMC), os autores novamente confirmaram que o TLR4 não está envolvido com a produção de TNF e IL-1. Surpreendentemente, eles também não encontraram diferenças quanto a produção de IL-8, uma quimiocina humana CXC homóloga as murinas KC e MIP-2 sugerindo então, que o efeito do TLR é específico para cada espécie. Usando anticorpos bloqueadores eles observaram ainda, que o TLR2 parece ser o receptor envolvido na produção de citocinas proinflamatórias (Netea et al., 2002).

Esses achados de que camundongos C3H/HeJ (TLR4 defeituosos) tem um aumento da suscetibilidade à candidíase disseminada estão de acordo com estudos que demonstraram que o TLR4 está envolvido no reconhecimento e na defesa do hospedeiro contra *A. fumigatus* e *C. neoformans* que são outros dois importantes patógenos fúngicos (Shoham et al., 2001; Mambula et al., 2002).

Villamón et al. (2004a) também estudaram o papel dos TLRs em modelo animal na defesa contra *C. albicans* e observaram que camundongos deficientes de TLR2, experimentalmente infectados por *C. albicans,* apresentaram menor sobrevida quando comparada com animais controle, concluindo que a expressão desse receptor é crucial para a proteção dos camundongos contra *C. albicans* disseminada. A produção *in vitro* de TNF-α e MIP-2 por macrófagos de camundongos TLR2$^{-/-}$, em resposta a *C. albicans,* foi significativamente mais prejudicada nesses animais o que poderia contribuir para a diminuição do recrutamento de neutrófilos para o sítio de infecção. Esses autores observaram ainda que a fagocitose das leveduras e produção de reativos intermediários do oxigênio (ROIS) não foi afetada nos camundongos TLR2$^{-/-}$. Os autores sugerem que o TLR2 exerce um papel importante na resposta de macrófagos contra *C. albicans,* levando à produção de citocinas e quimiocinas, essenciais para a proteção do indivíduo contra a infecção. Em outro estudo, esses mesmos autores observaram que no início da infecção por *C. albicans*, a produção de citocinas como TNF-α, IL-12 e IFN-γ, foram afetadas nos camundongos deficientes de TLR2 (Villamon et al., 2004b).

Por outro lado, resultados contraditórios foram encontrados por Netea et al (2004b) que demonstraram que animais TLR2$^{-/-}$ são mais resistentes a candidíase disseminada do que animais normais e este fato esteve associado com o aumento

da quimiotaxia e capacidade candidacida dos macrófagos. Os autores não encontraram diferenças quanto à produção de TNF-α, IL-1 e IL-6, porém, a produção de IL-10 foi fortemente prejudicada nos animais TLR2$^{-/-}$, fato que esteve associado com a diminuição de células T regulatórias CD4^{+}CD25^{+}(Treg). Frente a esses dados, os autores concluíram que a *C. albicans* escapa das defesas do hospedeiro através de sinais mediados pelo TLR2.

Esses resultados conflitantes podem ser atribuídos ao uso de diferentes protocolos experimentais, mas eles nos dão contribuições importantes para o entendimento da imunopatologia do processo infeccioso.

As diferentes formas da *Candida* podem levar a ativação de receptores diferentes, assim, na forma de hifas é reconhecida somente pelo TLR2 induzindo, preferencialmente, a produção de citocinas antinflamatórias enquanto que na forma de blastoconídeos interagem com TLR4, dectin-1 e TLR2, resultando em um padrão de ativação celular complexo (Romani, 2004; Netea et al., 2006).

Como observado com a *C. albicans*, o papel do TLR na infecção com *Cryptococcus neoformans* também não está totalmente esclarecido. O principal componente da cápsula polissacarídica do *Cryptococcus neoformans,* a glucuronoxilomanana, circula no sangue e no fluido cerebroespinhal do hospedeiro infectado. Esse polissacarídeo leva a ativação de células transfectadas com CD14 e TLR4, mas esta interação resulta em uma ativação incompleta das células e não produção de TNF-α (Shoham et al., 2001). *In vivo*, MyD88 e o TLR2, mas não o TLR4, mostraram ser importantes na indução de uma resposta protetora contra *Cryptococcus neoformans* (Yauch et al., 2004; Biondo et al., 2005). Contudo, outros trabalhos sugerem que o TLR2 e TLR4 não contribuem com a resposta do hospedeiro contra esse patógeno (Nakamura et al., 2006).

O envolvimento do TLR2, TLR4 e MyD88 durante a infecção por outros fungos oportunistas, como *Aspergilus* fumigatus, também tem sido estudado (Wang et al., 2001; Marr et al., 2003; Meier et al., 2003; Netea et al., 2003; Braedel et al., 2004; Dubordeau et al., 2006). Assim como descrito para *C. albicans*, a germinação de conídio para hifa foi proposto como um mecanismo de escape desse fungo. Assim conídios são reconhecidos pelo TLR2 e TLR4, resultando na produção de citocinas proinflamatórias, enquanto que as hifas estimulam a produção de IL-1 usando mecanismos dependentes de TLR2 (Netea et al., 2003).

Outros PRRs também poderiam estar envolvidos no reconhecimento da *C. albicans*. Dentre eles podemos destacar as lectinas do tipo C (*C-type lectin receptors*, CLRs) que são uma grande família de moléculas ligadoras de carboidrato cálcio-dependentes expressas em macrófagos, células dendríticas e outros leucócitos (Willment & Brown, 2008). Vários CLRs como o receptor de manose (MR) e o "Dectin-1" estão envolvidos na imunidade antifúngica e seus papéis não tem sido totalmente esclarecidos (Willment & Brown, 2008).

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 (Willment & Brown, 2008). O MR foi primeiramente identificado em células de Kupffer de ratos como um sistema de captura específico de glicoproteínas fucosilada e manosilada/N-acetilglucosamina terminal (Schlesinger et al., 1978). Estudos têm demonstrado sua expressão em macrófagos peritoneais (Stahl & Gordon, 1982) e alveolares (Stahl & Ezekowitz, 1998), bem como fagócitos mononucleares humanos (Shepherd et al., 1982). Estudos têm sugerido que o principal papel do MR é o *clearance* endocítico de glicoproteínas derivadas do hospedeiro (Smedsrod et al., 1988) e que ele pode mediar a fagocitose de microrganismo não-opsonizado interagindo com polissacarídeos da parede celular, bem como manana fúngica, cápsula bacteriana, lipopolissacáride e lipoarabinomanana (Ofek et al., 1995).

Embora trabalhos sugiram o envolvimento do MR na fagocitose de vários fungos, há evidências sugerindo que o MR medeie primariamente a ligação destes organismos e não a sua ingestão (Le Cabec et al., 2005).

O envolvimento do MR na fagocitose da *C. albicans* e resposta antifúngica têm sido demonstrados por alguns autores. Nesse sentido, Loyola et al. (2002) demonstraram que o aumento da atividade candidacida de macrófagos peritoneais murinos ativados por Con-A, estava associado à maior expressão do MR. Da mesma forma, observamos em experimentos prévios que o aumento da atividade candidacida de macrófagos peritoneais murinos estava associado ao aumento da expressão desses receptores e produção de H_2O_2 (Martins et al., 2008). Nos estudos de Cambi et al. (2003) os autores sugerem que o MR e o CD209, um receptor de ligação a *C. albicans* identificado como DC-SIGN, são os principais receptores de ligação com a *C. albicans.*

Na resposta ao fungo, o MR pode induzir a ativação do NF-κB e a produção de várias citocinas, incluindo a IL-12, IL-8, IL-1β, IL-6 e fator estimulador de colônias granulócitos-macrófagos (GM-CSF) (Zhang et al., 2004; Pietrella et al., 2005; Tachado et al., 2007). Yamamoto et al. (1997) sugerem o envolvimento de MR na produção de citocinas em resposta a *C. albicans*. Entretanto, com certos fungos, como o *Pneumocystis*, o MR talvez exerça um papel imunossupressor, inibindo a produção de citocinas inflamatórias como o TNF (Zhang et al., 2005).

Finalmente, outro receptor que pode contribuir com a fagocitose é o "dectin-1", expresso amplamente em fagócitos como macrófagos e células dendríticas e contribuem com o desenvolvimento de uma resposta imune em resposta ao reconhecimento de β-glucanas (Brown & Gordon, 2003).

O "dectin-1" é um tipo de receptor transmembrânico do tipo II que possui um domínio de reconhecimento de carboidratos, uma haste, uma região transmembrânica e um domínio citoplasmático intracelular que contem um motivo citoplasmático semelhantes-ITAM (Arizumi et al., 2000).

Em resposta a β-glucanas, o dectin-1 é capaz de mediar sinalização intracelular através desses motivos ITAM (Brown et al., 2006) levando a uma variedade de respostas celulares incluindo fagocitose, explosão respiratória, ativação e regulação de fosfolipase A_2 (PLA_2) e cicloxigenase 2 (COX2), além da produção de várias citocinas e quimiocinas como TNF, MIP-2, IL-2, IL-10, IL-6 e IL-23 (Brown et al., 2006; Leibundgut-Landmann et al., 2007).

A ativação do dectin-1 pela *C. albicans* ou com o curdlan (β-1,3-glucana, ligante do dectin-1) induz preferencialmente a produção de TGF-β e IL-6 e, subsequentemente leva a ativação de linfócitos Th17. Essas células secretam IL-17, que induz a produção de quimiocinas no sítio da infecção, levando ao recrutamento de neutrófios e uma importante defesa contra patógenos extracelulares, incluindo a *C. albicans* (Leibundgut-Landmann et al., 2007; Palm & Medzhitoo, 2007).

A sinalização pelo "dectin-1" é suficiente para muitas respostas, entretanto outras como a produção de citocinas proinflamatórias e quimiocinas requerem a colaboração da sinalização pelos TLRs (Brown et al., 2006). Em colaboração com o TLR2, por exemplo, pode desencadear a produção de TNF-α e IL-12 (Brown, 2006).

Em camundongos, o "dectin-1" foi identificado como sendo o principal receptor para β-glucanas em macrófagos, mediando a resposta proinflamatória em

colaboração com os TLRs (Brown et al, 2002; Brown et al, 2003). Brown & Gordon (2001) demonstraram que este receptor desencadeia a fagocitose de partículas contendo β-glucanas quando ectopicamente expressos em células normalmente não fagocíticas. Esses autores observaram ainda que o "dectin-1" e os TLRs em macrófagos e células dendríticas tem ação sinérgica na mediação da produção de citocinas como a IL-12 e o TNF-α promovendo uma resposta Th1. Os autores sugerem que o "dectin-1" desencadeia a fagocitose e estimula a produção de ROIS contribuindo com a morte do microrganismo enquanto que o TLR induz uma sinalização através do NF-κB que leva a produção de citocinas inflamatórias e essa resposta é aumentada pelo dectin-1.

A ligação cruzada entre "dectin-1", TLR2 e TLR4 pode exercer um papel importante na resposta imune contra a *C. albicans*. Foi demonstrado que β-glucanas presentes na parede da *Candida* está protegida do "dectin-1" pela presença de um componente externo da parede celular e que, com o crescimento fúngico e a separação celular, acabam expondo quantidades suficientes de β-glucanas desencadeando a ativação do "dectin-1" em macrófagos. Durante o crescimento filamentoso da *C. albicans*, as β-glucanas não estão expostas e o "dectin1", portanto, não é ativado, permitindo que o patógeno escape da resposta imune (Gantner et al., 2005).

O sinergismo entre "dectin1", TLR2 e TLR4 para indução de citocinas pode ser importante nas infecções por *Candida*, uma vez que, cada camada da parede celular da *C. albicans* pode desencadear a expressão receptores específicos. A ligação cruzada entre esses receptores levarão a uma resposta imune muito favorável para o hospedeiro (Ferwerda et al., 2008).

O homólogo humano do "dectin-1" é chamado de receptor de β-glucana (βGR) e apresenta duas isoformas principais βGR-A e βGR-B, as quais diferem entre si quanto à presença e ausência da região de haste, respectivamente; ambas têm demonstrado o envolvimento no reconhecimento de β-glucanas (Willment et al., 2001). A atividade do βGR tem sido descrita em vários leucócitos humanos como monócitos (Czop & Kay, 1991), macrófagos (Mueller et al., 2000), eosinófilos (Mahauthaman et al., 1988), neutrófilos (Czop et al., 1988) e células NK (Di Renzo et al., 1991). Além do reconhecimento de β-glucanas, o "dectin-1" também reconhece um ligante endógeno de células T e age como uma molécula co-estimulatória

induzindo a proliferação de $CD4^+$ e $CD8^+$ *in vitro* (Arizumi et al., 2000; Willment et al., 2001).

A ativação de PRRs orquestram o desenvolvimento de resposta imune inata e adaptativa, as quais são necessárias para a proteção do hospedeiro contra infecções. Contudo, se a ativação desses receptores de imunidade inata for excessiva, altos níveis de mediadores inflamatórios, como o IFN-γ, TNF-α e NO, são produzidos e podem exercer efeitos deletérios ao hospedeiro.

Dada a importância que essa variedade de receptores pode assumir no reconhecimento de patógenos e ativação de fagócitos, é possível pensar que a administração de agentes imunomoduladores capazes de estimular a expressão de receptores de imunidade inata pelas células fagocíticas pode ser potencialmente útil, uma vez que podem atuar como adjuvantes no tratamento de infecções incluindo a *C. albicans*. Desse modo, numerosos estudos têm indicado que produtos naturais possuem propriedades imunopotenciadoras, entre estes, destacam-se os cogumelos comestíveis e medicinais cujo consumo é particularmente difundido entre os povos orientais, gerando considerável interesse em possíveis agentes farmacológicos antitumorais.

Os cogumelos comestíveis possuem importante valor nutricional e diferentes componentes bioativos cujo conteúdo e propriedades medicinais dependem da maneira pela qual são preparados para consumo (Chang, 1996) isto é, concentração, fase de coleta, diluente utilizado, método de extração, entre outros fatores (Eira et al., 2000). A principal substância bioativa de cogumelos são polissacarídeos obtidos do corpo de frutificação (Mizuno et al., 1990a; Mizuno et al., 1990b; Fujimiya et al., 1998) e a atividade imunomoduladora é atribuída principalmente às β-glucanas (Mizuno et al., 1990a.; Ito et al., 1997), uma vez que esses polissacarídeos têm sido usados experimentalmente e terapeuticamente como imunomoduladores, potencializando a resposta do hospedeiro frente a tumores e uma variedade de infecções (Brown & Gordon, 2003).

O *Agaricus blazei* ss. Heinem. (*A. blazei*) é um cogumelo comestível popularmente conhecido como cogumelo do sol®, cogumelo princesa, cogumelo Piedade, himmematsutake e Royal agaricus. Faz parte de uma divisão do Reino Fungi, denominada Basidiomicota, representada por organismos saprófitas que necessitam de matéria orgânica encontrada na natureza para o seu desenvolvimento. Este cogumelo tem excelente valor nutricional, contendo

proteínas, gorduras (ácidos graxos), fibras, açúcares, minerais como P, Fe, Ca, Zn, Cu, Mn, e vitaminas (B1, B2, C, K, D, niacina entre outras) (Mizuno et al., 1990a). Originário da Mata Atlântica, da região sul do Estado de São Paulo, nos anos 70 foi levado para o Japão, onde se iniciaram as pesquisas sobre suas prováveis propriedades medicinais.

Com base nas diferenças morfológicas do corpo de frutificação, encontradas entre a espécie cultivada no Brasil e a amostra originalmente descrita como *A. blazei*, Wasser *et al.*, (2002), propuseram sua classificação como espécie distinta, denominada *A. brasiliensis*, nomenclatura já adotada por nosso grupo e por outros pesquisadores (Carmelini et al., 2005; Angeli et al., 2006; Faccin et al., 2007).

O *A. blazei* apresenta propriedade medicinal destacando sua atividade antitumoral (Mizuno et al., 1990a, Mizuno et al., 1990b) e imunoestimulatória (Ito et al., 1997, Fujimiya et al., 1998, Liu et al., 2007). O extrato desse cogumelo modula a resposta imune, ativando células NK (Fujimiya et al., 1998; Fujimiya et al.,1999; Kaneno et al., 2004; Liu et al., 2007) linfócitos (Mizuno et al., 1998; Liu et al., 2007) e macrófagos (Sorimachi et al., 2001; Kasai et al., 2004). As principais substâncias bioativas do *A. blazei* são polissacarídeos obtidos do corpo de frutificação (Mizuno et al. 1990a, Mizuno et al. 1990b, Fujimiya et al. 1998, Ebina & Fujimiya 1998) e sua atividade imunomodulatória é atribuído principalmente as β-glucanas (Mizuno et al. 1990a, Mizuno et al. 1990b, Ito et al. 1997), as quais são encontradas também em outras espécies de cogumelos como *Lentinus edodes* e *Ganoderma lucidum* (Borchers et al. 1999).

Nas investigações iniciais sobre as propriedades do cogumelo *A. blazei,* Kawagishi et al. (1989) demonstraram que o extrato etanólico de *A. blazei* possui capacidade de retardar o crescimento do sarcoma 180 inoculado subcutaneamente em camundongos. No ano seguinte, estes mesmos autores (Kawagishi et al., 1990) realizaram extrações seqüenciais com etanol, oxalato de amônio e hidróxido de sódio e através de cromatografia em gel, obtiveram várias frações do produto que foram testadas quanto à atividade antitumoral no mesmo modelo. Os melhores resultados foram apresentados por uma fração de baixo peso molecular denominada FIII-2-b, constituída principalmente de cadeias simples de (1→6) β-glucopiranosil (43,3%) e de proteínas (50,2%), com conteúdos elevados de alanina e leucina, e baixo teor de metionina, histidina e tirosina. Posteriormente, Fujimiya et al. (1998) verificaram que a fração rica em polissacarídeos (fração ATF), obtida por extração

com oxalato de amônio, confere proteção contra o desenvolvimento de fibrossarcoma induzido por metilcolantreno (Meth A), quando administrada “in situ”. Estes pesquisadores observaram ainda que o tratamento confere proteção contra o desenvolvimento do tumor inoculado subsequentemente em local distante do sítio primário, simulando a ocorrência de metástase.

A extração da fração ATF foi previamente padronizada em nosso laboratório de acordo com o método descrito por Fujimiya et al. (1998) e a análise por ressonância magnética nuclear (RNM) demonstrou que a nossa fração é similar àquela apresentada pelo primeiro grupo que demonstrou as propriedades antitumorais do *A. blazei* (Kawagishi et al.,1989; 1990). As concentrações de proteína (13,4%) e carboidrato (86,6%) indicam que a ATF é rica em carboidrato assim como demonstrado por Ebina & Fujimiya (1998). Fujimiya et al. (1998) demonstraram que a atividade antitumoral e imunomodulatória da ATF foi devido a presença de (1→4)-α-D-glucana e ramificações de (1→6)-β-D-glucana. A espectroscopia de correlação heteronuclear (HMQC) da ATF extraída em nosso laboratório indica que o principal componente presente é (1→6)-β-glucana.

Além de sua atividade antitumoral estudos têm demonstrado que polissacarídeos de cogumelos têm propriedades hepatoprotetora (Ooi, 1996), antifibrótica (Park et al., 1997), antinflamatória (Czarnecki & Grzybek, 1995), hipoglicêmica (Hikino & Mizuno, 1989), hipocolesterolêmica (Cheung, 1996), antiviral (Faccin et al., 2007) e antimicrobiana (Sakagami et al., 1991). Com relação a essa última propriedade, Suzuki et al. (1990) demonstraram que a fração β-1,3-glucana obtida do filtrado da cultura da *Sclerotinia sclerotiorum* (SSG), quando administrada oralmente, aumenta a atividade candidacida de macrófagos peritoneais murinos devido ao aumento de fagocitose, liberação de H_2O_2 e IL-1. Sakurai et al. (1991) demonstraram que essa mesma fração quando administrada intraperitonealmente também aumenta a quantidade de IL-1, aumentando a produção de fatores estimuladores de colônias (CSF) que, por sua vez, promovem a proliferação de macrófagos alveolares e o aumento da atividade candidacida, sugerindo que esse polissacarídeo pode ser efetivo na terapia contra infecções microbianas.

Kasai et al. (2004) observaram que o composto ABH derivado de hemicelulose do cogumelo *A. blazei* induz a produção de IL-12 por monócitos humanos via TLR4-CD14, além de aumentar a atividade citotóxica de células NK

contra células tumorais. Os autores sugerem que este composto ABH pode desencadear uma resposta imune eficaz na defesa do hospedeiro contra o câncer ou doenças infecciosas. Embora haja poucos trabalhos reportando os efeitos de *A. blazei* em humanos (Ahn et al., 2004; Grinde et al., 2006; Liu et al., 2007), muitas pessoas habitualmente consomem esse cogumelo na forma de chás ou pílulas.

Sustentando a hipótese de que *A. blazei* poderia desencadear uma resposta imune eficaz na defesa do hospedeiro contra doenças infecciosas, observamos previamente que macrófagos peritoneais de camundongos tratados com um extrato rico em peptidoglucanas obtido do cogumelo *A. blazei* (fração oxalato solúvel ácido tratada - ATF), apresentam maior capacidade candidacida quando comparados com macrófagos de animais normais inoculados com PBS. A essa observação, soma-se o fato de que as células desses animais apresentaram maior produção de H_2O_2 e expressão de MR com maior intensidade que os controles (Martins et al., 2008).

Os trabalhos citados acima mostram o grande potencial imunomodulador de substâncias extraídas dos cogumelos comestíveis e medicinais. Dentro desse contexto, trabalhos devem ser desenvolvidos no sentido de aprofundar o nosso conhecimento sobre os seus mecanismos de ação. Esses estudos são necessários no sentido de cada vez mais reconhecer o uso dessas substâncias como imunoterápicos.

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Manuscrito

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Increased phagocytosis of *Candida albicans* induced by *Agaricus brasiliensis*-derived polysaccharides involves TLR2, TLR4 and cytokines modulation

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Abstract

Agaricus brasiliensis is a mushroom whose medicinal properties include antitumoral and immunomodulatory activities. The main bioactive substances of this mushroom are polysaccharides obtained from the fruiting bodies, being their immunomodulatory activities attributed mainly to β-glucans. In this paper we aimed to study the role of a polysaccharide–rich fraction (ATF) of this mushroom on innate immunity receptors expression such as β-glucan and mannose receptors (βGR and MR), toll-like receptors (TLRs:TLR2 and TLR4) , phagocytosis of *Candida albicans*, cytokine production (TNF-α, IL-1β, IL-12 and IL-10) and H_2O_2 and NO release by human monocytes. ATF significantly increased *Candida* adherence/ phagocytosis by modulating TLR2 and TLR4 expression, since this polysaccharide had no effect on βGR and MR as well as on H_2O_2 and NO production. Moreover, this polysaccharide increased IL-1β and TNF-α production, being this effect also related to its capacity to increase TLR2 and TLR4 respectively. IL-10 levels were also increased. However an association between this effect and toll like receptors expression was not detected. In summary, our results provide evidence about the role of this extract on host resistance against some infectious agents through modulation of some phagocytic cells activities, including those of human monocytes.

1. Introduction

Candida albicans (*C. albicans*) is a very common dimorphic fungus mostly confined to the gastrointestinal, genitourinary tracts and the skin of healthy individuals. However, in immunosuppressed individual such as diabetes (Donders,2002), cancer (Ridola et al, 2004) and HIV (Klein et al, 1984) patients it can cause severe infection. A coordinate action of both innate and adaptive cell-mediated immune mechanisms are critical in preventing this commensal organism from establishing an disseminated infection, a process in which phagocytic cells activation is of crucial importance (Romani,1999), After binding of *C. albicans*, by these cells the release of proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and IFN-γ is the first step (Van't woutt et al., 1988; Marodi et al., 1993) in the activation of anticandidal innate immune responses. These cytokines activate neutrophils and macrophages to phagocytose the fungus and release oxygen and nitrogen radicals that are the main molecules with toxicity to these microorganisms. (Djeu, 1990; Kullberg et al., 1993).

The activation of innate immunity and stimulation of proinflammatory cytokines involves recognition of microbial components know as pathogen-associated molecular patterns (PAMPs) by a limited number of germline-encoded proteins, the pattern recognition receptors (PRRs). Over recent years, a growing number of opsonic and non-opsonic PRRs that recognize fungal PAMPs have been identified (Willment & Brown, 2008). These include various Toll-like receptors (TLRs) including TLR2, TLR4, TLR6 and TLR9, that have been suggested to play an crucial role in the cytokine response to fungal cells (Wang et al., 2001; Netea et al., 2002;Tada et al., 2002; Sato et al., 2003; Villamon et al., 2004a).

Other receptors have the capacity to recognize mannan component of yeast cell walls. One of these is the mannose receptor (MR) (Ezekowitz et al., 1990; Ezekowitz et al., 1991; Loyola et al., 2002; Porcaro et al., 2003). Although works have suggested the envolvement of the MR in phagocytosis, there are evidences suggesting that the this receptor mediates the linking of these organisms but is unable to mediate phagocytosis upon binding (Le Cabec et al., 2005). Although MR does not seem to be involved in uptake of *C. albicans* recent studies showed that it is required for cytokine production upon recognition (Heinsbroek et al., 2008).

Finally, another receptor that can contribute with phagocytosis is “dectin-1” that acts as a major receptor for fungal 1-3 β-glucans (Brown et al., 2002) and shows to mediate the proinflamatory response in collaboration with the TLR (Brown et al., 2002; Brown et al., 2003; Gantner et al., 2003). Dectin-1 is capable to mediate intracellular signals leading to phagocytosis, respiratory burst, activation and regulation of phospholipase A2 (PLA2) and cyclooxygenase 2 (COX2) and cytokines production such TNF, MIP-2,IL-2, IL-10, IL-6 and IL-23 (Brown et al., 2006; Leibundgut-Landmann et al., 2007).The human homologue of Dectin-1, is the β-glucan receptor (βGR), that differs from the murine receptor because it can be alternatively spliced into two major and a number of minor isoforms. The two major isoforms - βGRA and βGRB- have been shown to be functional for β-glucan recognition (Willment et al., 2001). Given the importance that this variety of receptors can assume in the pathogens recognition and activation of phagocytes, it is reasonable to hypothetisize that the administration of immunomodulatory agents able to stimulate the expression of these receptors can be potentially useful as adjuvant in the treatment of infections such as *C. albicans*.

Agaricus blazei Murrill (*A. blazei*) whose Brazilian variety was suggested as a new specie to be named *Agaricus brasiliensis* sp.nov., (*A. brasiliensis*) (Wasser et al., 2002) is a medicinal mushroom whose characteristics include a wide range of medicinal properties including antitumoral (Mizuno et al. 1990a, Mizuno et al., 1990b) and immunostimulatory activities (Ito et al. 1997, Fujimiya et al. 1998). The main bioactive substances of this mushroom are polysaccharides obtained from the fruiting bodies (Mizuno et al. 1990a, Mizuno et al. 1990b, Fujimiya et al. 1998, Ebina and Fujimiya 1998), and its immunomodulatory activity is attributed mainly to β-glucans (Mizuno et al. 1990a, Mizuno et al. 1990b, Ito et al. 1997), A polysaccharide-rich fraction obtained by acid treatment of the ammonium oxalate-soluble extract of A. blazei (ATF) was shown to be able to cause tumor infiltration by NK cells; and it inhibits in vitro tumor cell growth by inducing apoptosis (Fujimiya et al. 1998, 1999). Sorimachi et al. (2001) have observed that extracts from *A. blazei* are able to activate macrophage functions. Previous results on our lab have shown that ATF was able to inhibit the growth of Ehrlich tumor and partially inhibit the production of IL-10 by spleen cells of tumor-bearing mice (unpublished observations). Besides the role on host resistance against tumors recent results on our lab have suggested that ATF

can increase host resistance against some infectious agents such as *C. albicans*, through the stimulation of microbicidal activity of macrophages. Mice treatment with this fraction results in peritoneal macrophages increased fungicidal activity that was associated with higher levels of H_2O_2, and a significative increase in mannose receptor expression (Martins et al., 2008). In the present work we had interest in extending our studies on modulatory role of ATF on phagocytes function against fungi, evaluating its effect on innate immunity receptors expression such as βGR, MR, TLR2 and TLR4, phagocytosis of *C. albicans*, cytokine production (TNF-α, IL-1β, IL-12 and IL-10) and H_2O_2 and NO release by human monocytes.

2. Materials and Methods

2.1. Donors

This study included healthy donors from the University Hospital of the Botucatu Medical School, São Paulo State University, Brazil (age range 25-50 years). Approval from the institutional Ethics Committee was obtained, as well as informed consent from all the blood donors.

2.2. Acid-treated ammonium oxalate-soluble fraction (ATF)

The oxalate-soluble, acid-treated fraction (ATF) of *A. brasiliensis* was obtained according to the method described by Fujimyia et al. (1998). Briefly, 800g of a dried and powdered sample of *A. brasiliensis* were mixed with 80% ethanol and boiled for 15hr in a closed system. After this period, the supernatant was discarded and the process was repeated twice. After the last extraction with ethanol, the pellet was mixed with distilled water, boiled for 15 hr (3 times) and then mixed with 5% ammonium oxalate and extracted twice at boiling point for 10hr. The supernatants were pooled and filtered (Millipore cod. 2502500) to remove insoluble particles and the supernatant was dialyzed for 72 hr against distilled water. The efficiency of dialysis for the removal of ammonium residues was followed by analysis of the dialysis liquid using the Nessler reactive. The oxalate-soluble solution was acidified

with 1 mol l^{-1} HCl for 24 hr at room temperature, followed by neutralization with 1 mol l^{-1} NaOH (final pH = 7.0) and the final solution (ATF) was lyophilized and stored at –20 ºC. Before use, ATF was rehydrated with PBS and the sample was autoclaved to obtain a sterile solution (previous experiments have shown that autoclaved samples were more efficient against tumor growth than filtered ones).

The presence of oxalate was checked by heating ATF samples with 500 μl of 0.1N potassium permanganate for 1 min. at 100 ºC. The final solution of ATF was compared with a standard curve (4.0; 2.0; 0.5; 0.25 and 0.125%) prepared with 1N HCl (Borches et al., 1999), showing that the residual concentration of oxalate was lower than 0.125%. Endotoxin was analyzed by a Lymulus amebocyte lysate test (E-toxate kit - Sigma ET0200), and this extract presented less than 0.06 EU/ml.

2.3. Isolation of human peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from heparinized peripheral blood of healthy adult donors by density gradient centrifugation on Histopaque®-1077 (Sigma-Aldrich, Inc., St. Louis, Mo., USA). The cell fraction containing PBMCs was washed twice with RPMI – 640 tissue culture medium (Sigma-Aldrich). After, cells were suspended in a complete tissue culture medium (CTCM) consisting of RPMI-1640 tissue culture medium supplemented with 2mM of L-glutamine (Sigma-Aldrich), 40μg ml^{-1} of gentamicin (Gibco Laboratories, Grand Island, N.Y., USA) and 10% heat-inactivated autologous human serum. The cellular concentration was adjusted based on monocytes count that were identified by the method of neutral red uptake (incubation with 0.02% neutral red for 10min). Then, PBMCs were adjusted to $2x10^6$monocytes / ml^{-1} for H_2O_2 and NO release assays or $1x10^6$ monocytes ml^{-1} for the others assays.

2.4. *C. albicans* suspension

Yeast cells of *C. albicans*, sample H-428/03, originally isolated from a patient of the University Hospital of the Botucatu Medical School, São Paulo State University, Brazil and maintained at –70 ºC, were defrosted and grown in Sabouraud-Dextrose-Agar medium (Oxoid, Ltd.), at 35 ºC for 24 hr. The cells were collected and washed with sterile pyrogen-free salt-solution and resuspended at a concentration of

$5x10^6$ yeasts ml^{-1}. The viability of yeast cells were evaluated by phase microscopy (>95 % of viable cells).

2.5. Fluorescein isothiocyanate (FITC) *C. albicans* labelling

Yeasts labelling with FITC was determined by a method of Chaka et al. (1995) and Szolnoky et al. (2001) with slight modifications. Yeasts obtained as described in 2.4 section was incubated with FITC (100μg ml^{-1}) (Sigma Chemical Co.) in a 0.1M carbonate-bicarbonate buffer (pH 9.0) for 30min and at 37°C with occasional agitation. After, yeast suspension was centrifuged for 10min (1000g), the supernatant was removed and yeasts washed in PBS (2000g). They were then ressuspended in 10 ml of 0.1M carbonate-bicarbonate buffer containing 4% bovine serum albumin and incubated at 37^o C, for 15min. After, the suspension were centrifuged and washed twice in PBS to remove BSA bound to FITC. Labeled yeasts were suspended at $5x10^6$ yeasts ml^{-1}in RPMI-1640 tissue culture medium.

2.6. *C. albicans* adherence / phagocytosis

This assay was performed by flow cytometry analysis. For this, 500μl of PBMCs suspension ($1x10^6$ monocytes ml^{-1}) were distributed into poliesterene tubes for cytometric analysis (BD Labware) following incubation with CTCM alone or CTCM plus 5.0μg, 50μg and 500μg of ATF for 6h, 12h or 18h at 37 °C. After incubation, cells were washed and challenged with FITC-*C.albicans* (ratio yeast:monocyte = 5:1) for 30 min at 37°C under 5% CO_2 (PBMC - FITC-*C. albicans*). In order to distinguish monocytes from other cells, the suspension PBMC - FITC-*C. albicans* cells were incubated with anti-CD14 PerCP-Cy™5.5-conjugated monoclonal antibody (mAb) (BD-Pharmingen). The adherence and phagocytosis of FITC-*C.albicans* by anti CD14 labelled monocytes were analyzed with a FACS Calibur flow cytometer (Becton Dickinson). Data (an average of 10,000 events per sample) were analyzed with the Cell Quest 3.1 Software.

2.7. Expression of surface receptors (βGR, MR, TLR2 and TLR4)

This assay was performed by flow cytometry analysis. PBMCs ($1x10^6$ monocytes ml^{-1}) were distributed (500µl) into poliesterene tubes for cytometric analysis (BD Labware) following incubation for 6 h at 37°C under 5% CO_2 with CTCM alone, or CTCM plus 50µg ATF. Cells were washed and incubated with anti-CD14 PerCP-Cy™5.5-conjugated mAb (BD-Pharmingen) and with the mAbs specific for the different receptors: FITC-conjugated anti-MR (BioLegend), FITC-conjugated anti-TLR2 (BioLegend), PE-conjugated anti-TLR4 (BioLegend), according to the instructions of the manufacturer. The β-glucan receptor (βGR)expression was evaluated by incubation with a primary mAb (GE2) that recognizes both βGRA e βGRB isoforms (genteelly provided by Dr Gordon D. Brown, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, South Africa) followed by incubation with a FITC-labeled secondary antibody (Biolegend). After incubation at 15 min in room temperature, the cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson). Data (an average of 10,000 events per sample) were analyzed with the Cell Quest Software.

2.8. Hydrogen peroxide (H_2O_2) release

The H_2O_2 release by monocytes was determined according to Pick & Mizel (1981). Briefly, PBMC were plated on 96-well flat-bottomed microculture plates in a final concentration of $2x10^6$ cells ml^{-1} (100 µl $well^{-1}$) following incubation for 2 h at 37°C under 5% CO_2. Adherent cells were cultured for 6 hr with CTCM alone or CTCM plus 20µg ATF. Cells were washed and cultured for 24 hr without any stimulus. The supernatants were removed and 100µl of 1% phenol red solution, containing 140 mM NaCl, 10 mM K_2HPO_4, 5.5 mM dextrose and 5.5 mM horseradish peroxidase was added to the adherent cells and incubated for 1 hr at 37 °C in a humidified chamber (5% CO_2 in air). The reaction was stopped by addition of 10µl of 1N NaOH and the absorbance was measured at 620nm, using an automatic enzyme immunoassay reader.

2.9. Oxide nitric (NO) release

NO release by monocytes was determined based on Griess' reaction (1981). Supernatants of adherent cells (obtained as describe on 2.8) were collected after 24 hr and mixed with 100 μl of Griess reagent (N-1-naphthyl-ethyl-enediamine 0,1% + sulfanilamide 1% in H_3PO_4 5%). After 10 minutes, absorbance was measured at 540nm, using an automatic enzyme immunoassay reader.

2.10. Cytokine production

Isolated PBMCs ($1x10^6$ monocytes $ml^{-1)}$ were distributed into 24-wells tissue culture plates (500μl) following incubation for 2 h at 37°C under 5% CO_2. Adherent cells were cultured for 6 hr with CTCM alone, LPS (positive control) or 50μg ATF. After incubation, supernatants were collected and the concentrations of TNF-α, IL-1β, IL-12p70 and IL-10 were measured using sandwich enzyme-linked immunosorbent assays (ELISA), with antibodies from R&D Systems (Minneapolis, MN) and BioLegend (San Diego, CA), according to the instructions of the manufacturer.

2.11. Receptors blockade assays

In some experiments the effect of TLR2 and TLR4 on some cells functions was evaluated. For this, PBMC ($1x10^6$ monocytes ml^{-1}) were incubated during 1 h with mAbs anti-TLR2 (TLR2.1, BioLegend) or anti-TLR4 (HTA125, BioLegend), treated for 6 hr with CTCM alone, or 50 or 20 μg of ATF followed by assays related to indicated monocyte function.

2.12. Statistical Analysis

Data were analyzed using software Graphpad Instat, San Diego, USA. Results were compared by different statistical methods according to the characteristics of the data and they are indicated in the legend of each figure. Significance level set at $p<0.05$.

3. Results

3.1. Effect of ATF on adherence/phagocytosis of FITC-labelled *Candida albicans* by human monocytes.

As phagocytosis of FITC-labelled *Candida albicans* by human monocytes, was evaluated using flow cytometry analysis, two parameters could be analysed: the percentage of $CD14^+$ cells involved in adherence and uptake of monocytes (fig.1) as well as a semi quantification of the number of adhered and phagocytized yeasts, by calculating the fluorescence intensity of positive cells (fig. 2).

As can be analyzed, ATF independently of concentration and treatment time did not affect the number of monocytes involved in *Candida* phagocytosis. However, treatment with 50µg of this polysaccharide during 6h results in a significative increase in the capacity of these cells to adhere and phagocytize the fungus.

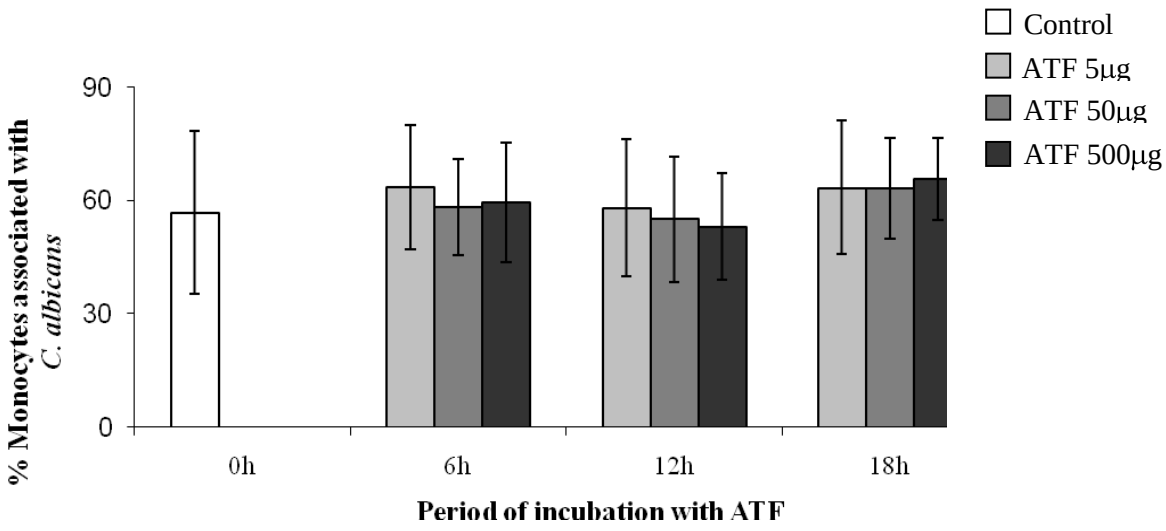

Fig.1. Effect of different ATF concentrations and treatment times on the percentage of $CD14^+$ human cells involved in the adherence and phagocytosis of FITC-*Candida albicans*, determined by flow cytometry. The results are expressed on mean ± standard deviation of cells percentage detected in assays performed with 7 subjects.

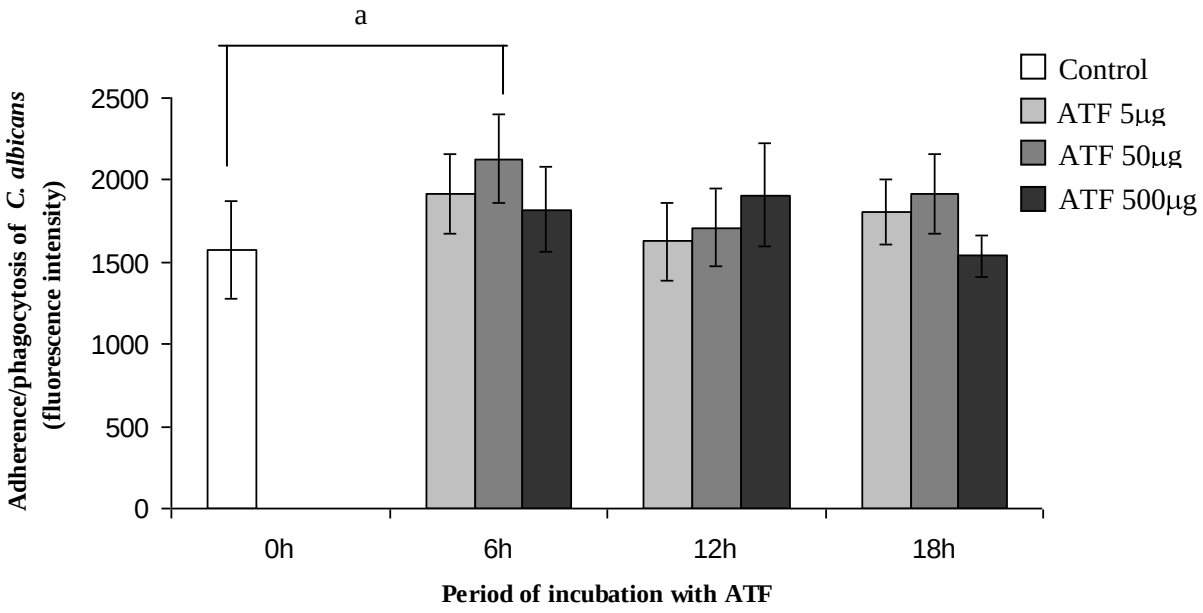

Fig.2. Effect of different ATF concentrations and treatment times on the adherence/phagocytosis of FITC-*Candida albicans*, determined by flow cytometry. The results are expressed on mean ± standard deviation of fluorescence intensity values detected in cells obtained from 7 subjects. a= $p < 0,01$, Student-Newman-Keuls multiple comparisons test.

3.2. Effect of ATF on TLR2, TLR4, βGR and MR expression.

In view of the above results, we asked if the increase in adherence / phagocytosis of FITC-*Candida albicans* induced by ATF was due to its capacity to modulating some important receptors involved in innate immunity. In the figure 3 the results of TLR2 (3A), TLR4 (3B), βGR (3C) and MR (3D) expression can be analyzed. Also based on the above results we choose for the subsequent experiments the ATF concentration of 50ug and the time treatment of 6h. In these conditions, ATF significantly increased TLR2 and TLR4 expression. However, this fraction had no effect on the expression of MR and βGR.

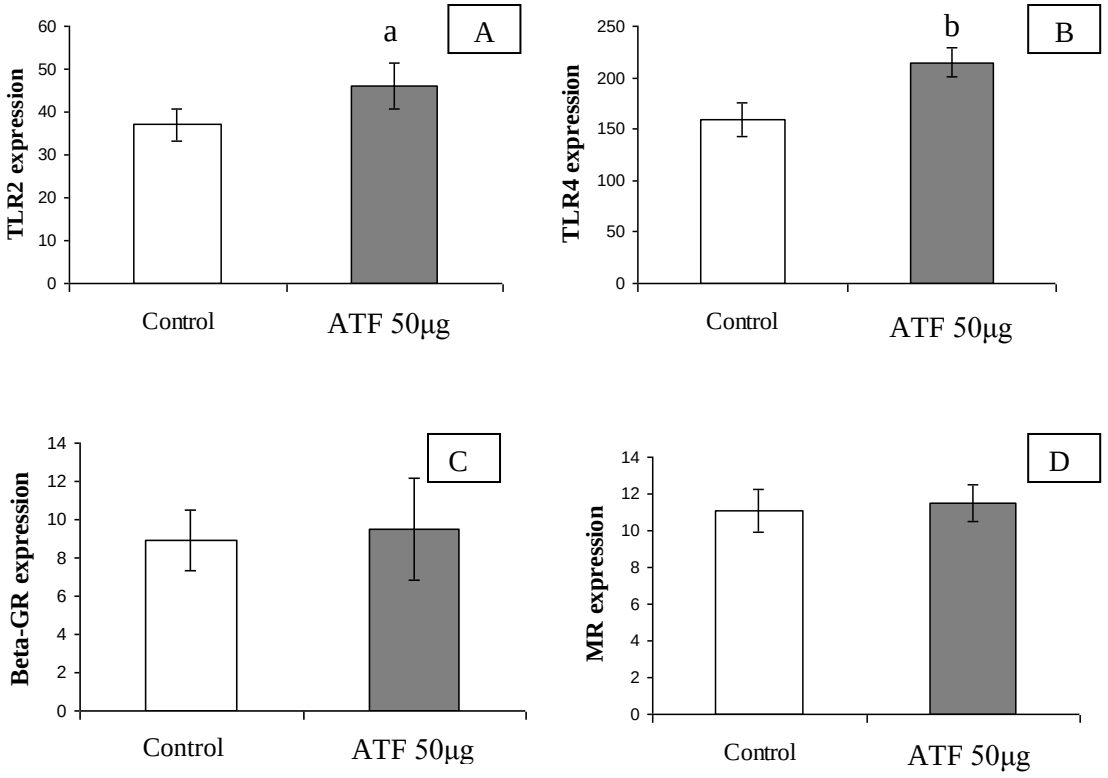

Fig.3. Effect of ATF on TLR2 (A), TLR4 (B), βGR (C) and MR (D) expression by human monocytes. The results are expressed on mean ± standard deviation of fluorescence intensity values detected in cells obtained from 6 subjects. a= p< 0, 05; b= p< 0, 01, paired t test.

3.3. Role of TLR2 and TLR4 on increased *Candida albicans* adherence/ phagocytosis induced by ATF.

In a next set of experiments we asked if increased on *Candida* adherence/ phagocytosis by ATF was related to its effect on TLR2 and TLR4 expression (fig. 4). As expected ATF increased fungus phagocytosis. However, this effect is significantly reduced when TLR2 or TLR4 expression is blocked. Of note, this reduction is yet more evident when simultaneous blockade of both receptors was tested. However, these results are not statistically different from those obtained with individual TLR2 or TLR4 blockade. Together, the results suggest that ATF increases *Candida* phagocytosis by modulating TLR2 and TLR4 expression.

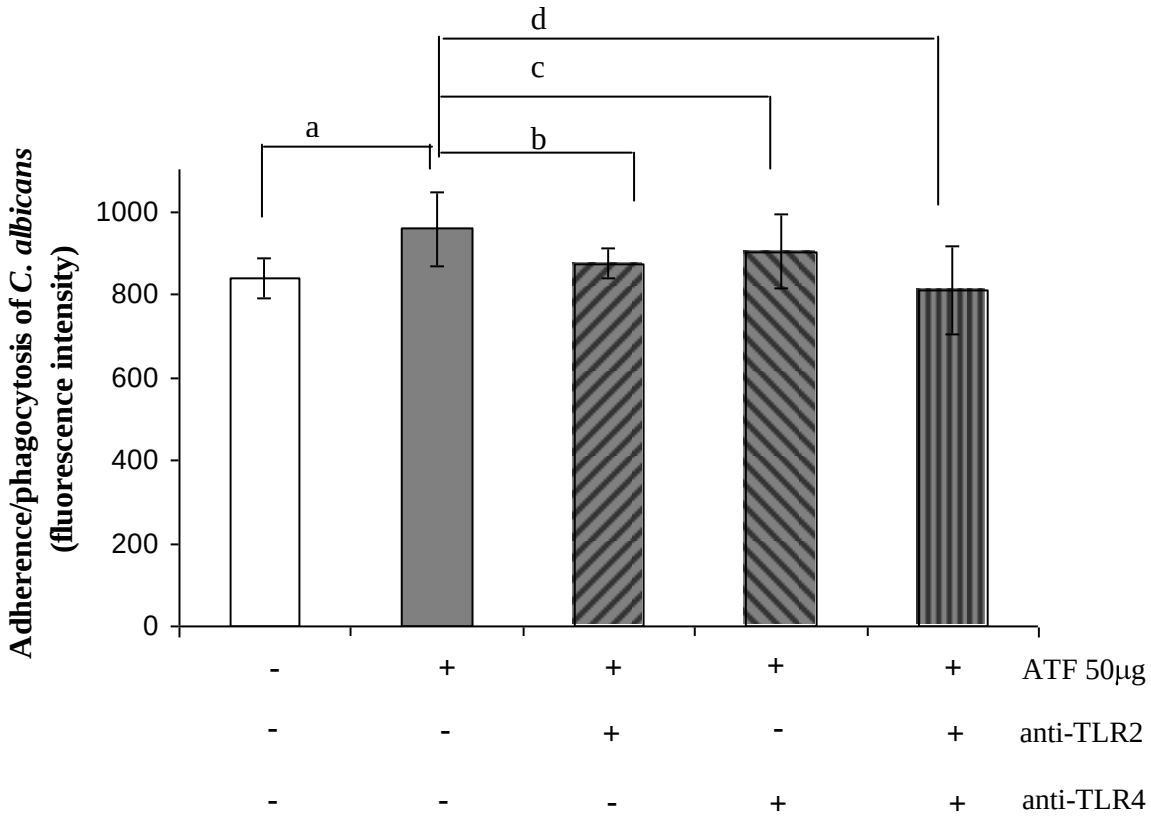

Fig. 4. Role of TLR2 and TLR4 on adherence/phagocytosis of *Candida albicans* induced by ATF. The results are expressed on mean ± standard deviation of fluorescence intensity values detected in cells obtained from 7 subjects. a = p< 0,001; b = p< 0,01; c = p< 0,05; d = p< 0,001, Student-Newman-Keuls multiple comparisons test.

3.4. Effect of ATF on H_2O_2 and NO release

Since ATF increases adherence/phagocytosis of *Candida* modulating TLR2 and TLR4 expression, we had interest in evaluating if this process could also result in oxygen and nitrogen metabolites increase, the molecules involved in *Candida* killing by phagocytic cells. For adjusting its concentration to cells concentration used in this experiment ($2x10^5$ cells/ culture well), ATF was used at 20µg. As can be analyzed in figure 5A (H_2O_2) and 5B (NO), monocytes released basal levels of H_2O_2 and NO, and ATF has no significative effect on this process.

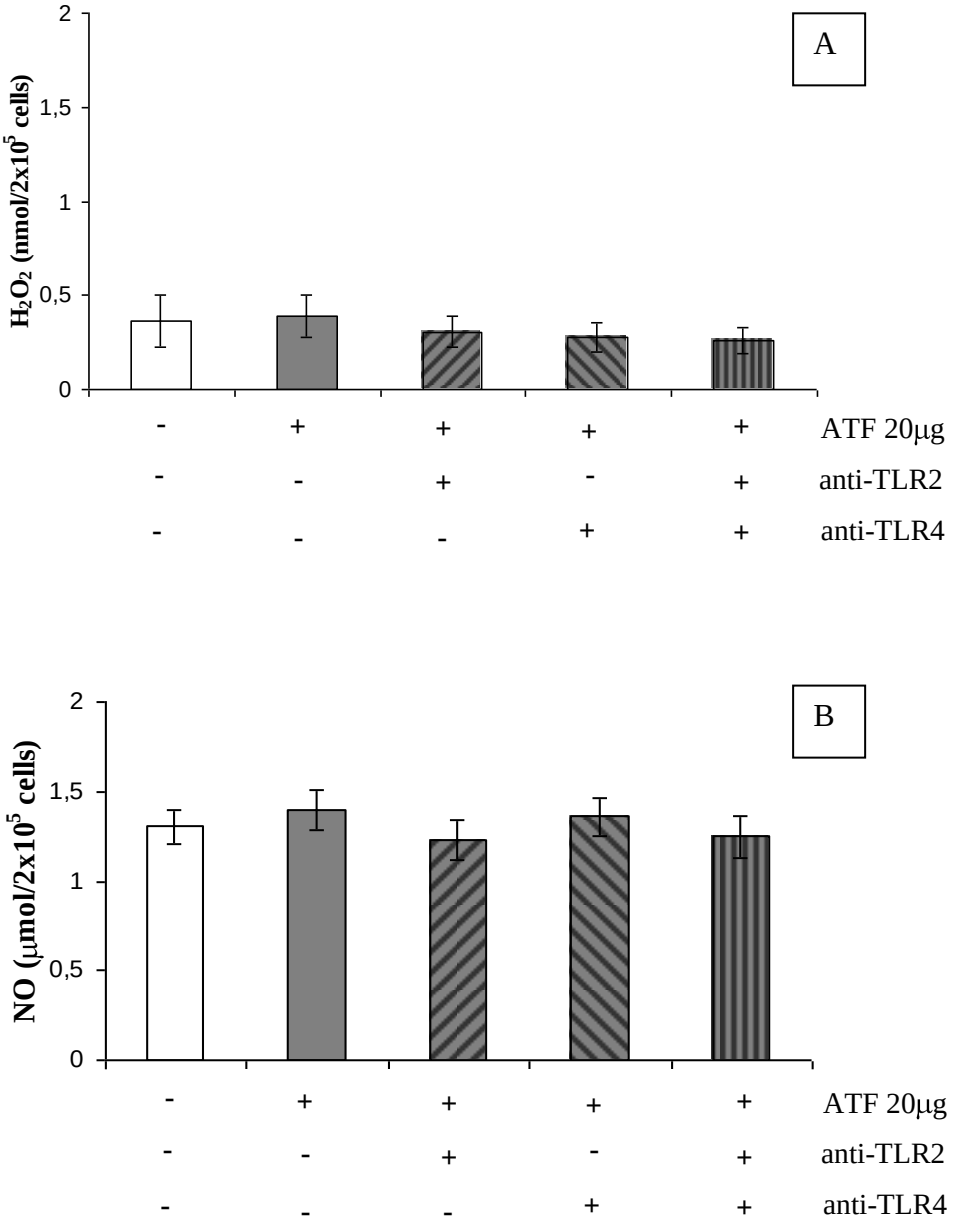

Fig. 5. Effect of ATF on H_2O_2 (A) and NO (B) release by human monocytes submitted or not to TLR2 and TLR4 blockade. The results are expressed on mean ± standard deviation of metabolites levels released by cells obtained from 11 subjects.

3.5. Effect of ATF on cytokines production

We also have interest in evaluating if ATF modulation on TLR2 and TLR4 could result in alterations in the production of some important cytokines of innate immunity such as TNF-α, IL-1, IL-12 and IL-10. The results are shown in figures 6 to 9. LPS was used as a positive stimulus for monocytes cytokines production. As can be analyzed in figure 6, non activated monocytes release basal TNF-α levels that significantly increased after LPS activation. ATF treatment induces cells to produce TNF-α in a similar manner to that detected with LPS. This effect was not altered when TLR2 was blocked before ATF treatment. On the contrary, blocking TLR4 a significative decrease in cytokine levels was detected. The results suggested that ATF increase TNF-α production by human monocytes by modulating TLR4 expression. A similar response profile was detected in the experiments of IL-1β dosage (fig. 7). However, a significative decrease in cytokine levels was detected with TLR2 blockade, suggesting that ATF effect on the production of this cytokine is mediated by this receptor.

The analysis of the results about effect of ATF on IL-12 production revels a response profile different from that obtained for TNF-α and IL-1β (fig. 8). Non activated cells release basal levels of this cytokine that was significantly increased after LPS activation. However, with ATF treatment IL-12 levels tended to be lower than those detected for non activated and LPS activated cells (data statistically no significant). The results suggest a possible negative modulator effect of ATF on IL-12 production.

The results regarding effect of ATF on IL-10 production are shown in Fig 9. ATF treatment increases IL-10 production. However inhibition of either TLR2 or TLR4 did not result in alterations in this cytokine production, demonstrating that ATF effect on IL-10 production is independently of its modulation on TLR2 or TLR4 expression.

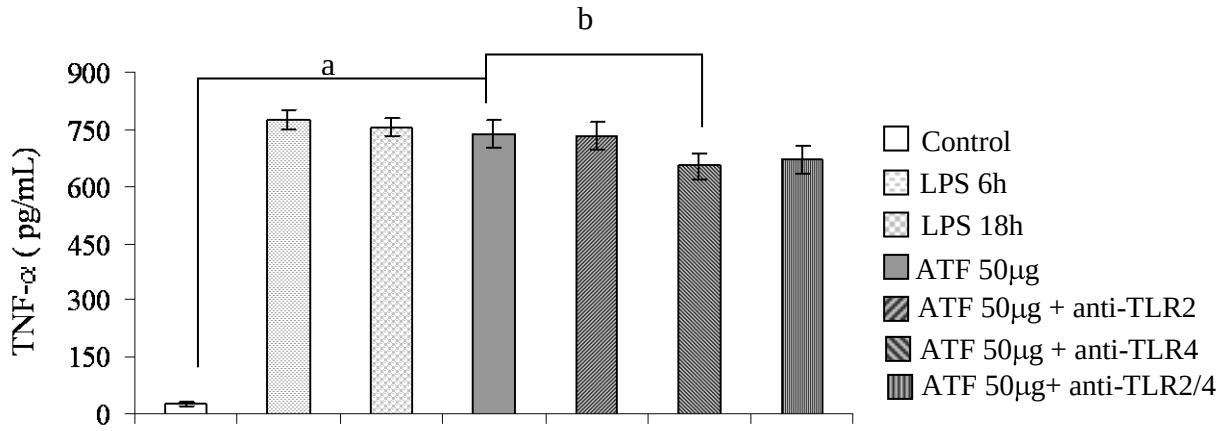

Fig. 6. Effect of ATF on TNF-α production by human monocytes submitted or not to TLR2 and TLR4 blockade. The results are expressed on mean ± standard deviation of cytokines levels released by cells obtained from 15 subjects. a= p< 0,001; b= p< 0, 05, Tukey-Kramer multiple comparisons test.

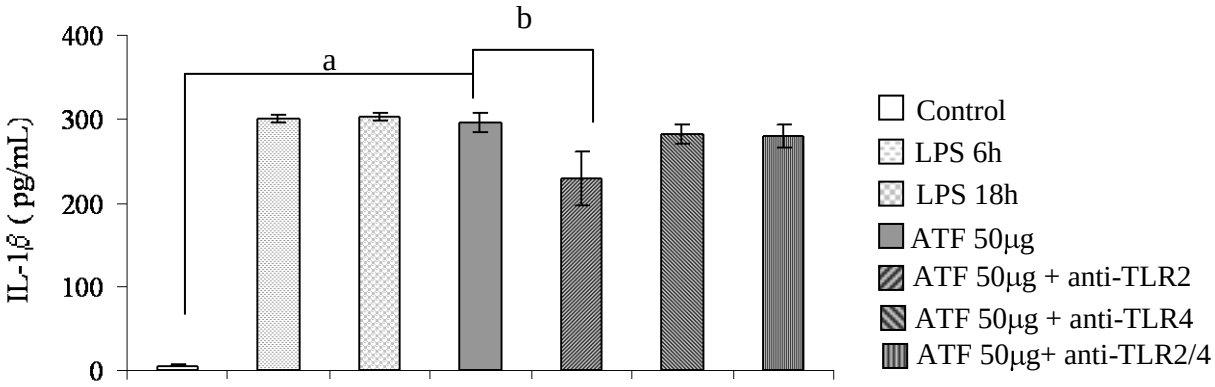

Fig. 7. Effect of ATF on IL-1β by human monocytes submitted or not to TLR2 and TLR4 blockade.The results are expressed on mean ± standard deviation of cytokines levels released by cells obtained from 15 subjects. a= p< 0,001; b= p< 0, 01, Tukey-Kramer multiple comparisons test.

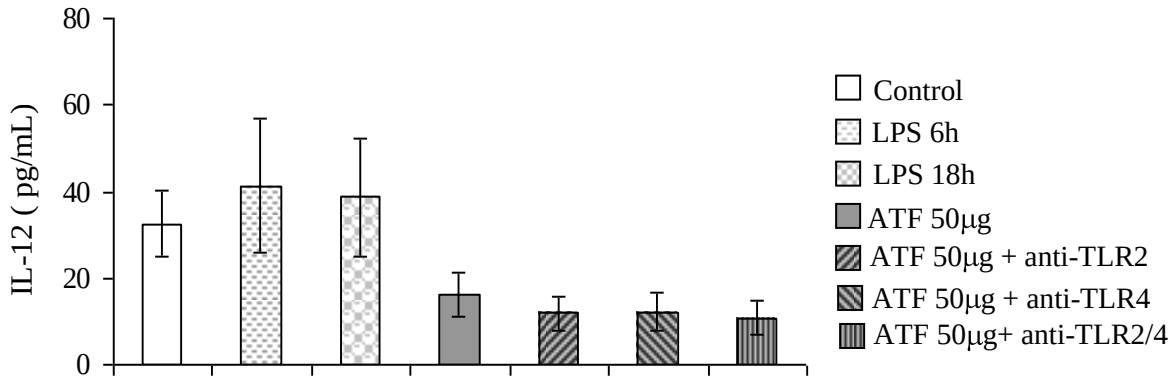

Fig. 8. Effect of ATF on IL-12 production by human monocytes submitted or not to TLR2 and TLR4 blockade. The results are expressed on mean ± standard deviation of cytokines levels released by cells obtained from 15 subjects.

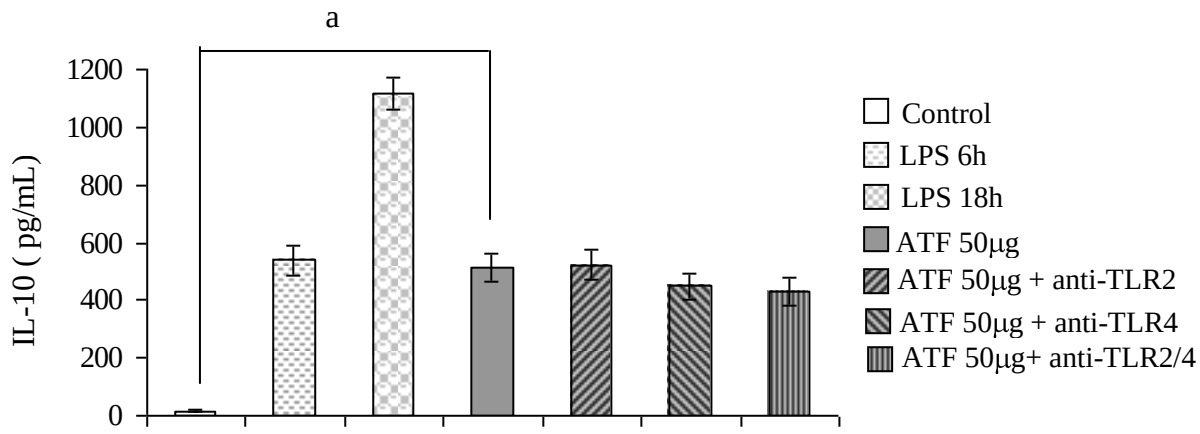

Fig. 9. Effect of ATF on IL-10 production by human monocytes submitted or not to TLR2 and TLR4 blockade. The results are expressed on mean ± standard deviation of cytokines levels released by cells obtained from 15 subjects. a= p< 0,001 Tukey-Kramer multiple comparisons test.

4. Discussion

Candida albicans cell wall mannans and glucans are considered as pathogen-associated molecular patterns (PAMPs) that can be recognized by pattern recognition receptors (PPR) of the immune system, mainly those of phagocytic cells. This process mediates subsequent binding, phagocytosis and stimulation of an inflammatory response (Netea et al., 2008; Willment et al., 2008). In this context the use of substances with immunomodulatory activities on phagocytic cells could be potentially helpful in control this mycosis. Mushroom polysaccharides are important candidates for these actions. In this paper we tested the effect, on human cells activities in response to *Candida*, of a polysaccharide fraction obtained from *Agaricus brasiliensis,* a medicinal mushroom, whose immunomodulatories activities have been demonstrated in last years (Ito et al., 1997; Fujimiya et al., 1999; Sorimachi et al., 2001).

We detected that ATF increases the capacity of these cells to adhere or phagocytosize this fungus. This finding lead us to investigate if this effect could be mediate by ATF modulation on some PRRs, such as TLR2, TLR4, βGR, and MR. We detected that this fraction increases TLR2 and TLR4 expression, with however no effect on βGR and MR. In relation to MR the data are no expected, because some studies have demonstrated the role of this receptor in the modulation of phagocytic cells in response to *Candida*. In this context experiments have led to the suggestion that MR could be involved in *Candida* phagocytosis by murine macrophages (Loyola et al., 2002; Porcaro et al., 2003). Moreover, despite some more recent studies have been demonstrated that this receptor is not involved in *Candida* uptake, its role in cytokines production such as MCP-1 and TNF-α was well established (Le Cabec et al., 2005; Heinsbroek et al., 2008). Finally, previous studies in our laboratories have been demonstrated that ATF treatment *in vivo* increases phagocytosis and killing of *Candida* by murine macrophages. This effect was associated to a higher MR expression. One possible explanation by the lack of ATF effect on MR expression in our study might be the use of different cell populations. In this study human monocytes were tested in contrast to murine macrophages used in previous experiments. In this respect, Ferwerda et al, 2008 demonstrated that macrophages

express higher MR levels when compared to monocytes. Thus, unlike detected for murine cells, this receptor seems not to be significantly involved in the recognition of mannan containing particules by human monocytes.

Lack of ATF effect on βGR, a human homolog of murine dectin-1, was also no expected in our studies, since dectin-1 is the main receptor involved in uptake of *C. albicans* (Brown et al., 2002; Brown et al., 2003; Taylor et al., 2007; Heinsbroek et al., 2008) as well as signals oxygen reactive production and release of cytokines such as TNF-a, IL-2, IL-6, IL-10, and IL-23 (Brown., 2006; Gow et al., 2007; LeibundGut-Landmann et al., 2007).

Unlike the results regarding MR and BGR, ATF significantly increased TLR2 and TLR4 expression. Moreover, experiments blocking these receptors proved that they mediated increase of *Candida* adherence/phagocytosis by this polysaccharide. Modulation of TLRs by substances derived from mushrooms and plants has been described in the literature. Kasai et al., 2004 reported that the substance ABH extracted from *Agaricus blazei* induces IL-12 production by humans monocytes by modulating TLR4 expression. Polysaccharides obtained from the mushroom *Phellinus linteus*, actuated in TLR2 and TLR4 increasing functional and phenotypic maturation of dendritic cells (Kim et al., 2004). Likewise, polysaccharides extracted from the root of the plant *Platycodon grandiflorum* activated macrophages by TLR4/NF-kB modulation (Yoon et al., 2004).

Despite modulating *Candida* adherence/phagocythosis through an increase in TLR2 and TLR4 expression, ATF did not alter H_2O_2 and NO production, the molecules involved in *Candida* killing. Fail of ATF on increasing NO levels might represent an intrinsic inability of human monocytes to release this metabolite, independently of the stimulus, since this capacity is a point of discordance in the literature (Scheemann et al., 1993; Denis, 1994).

However in relation to H_2O_2, the results are unexpected, since it is well established that it is the main monocyte effector molecule against *Candida* (Stevenhan & Furth, 1993). Moreover, the results are not in agreement with previous studies on our lab showing that ATF treatment *in vivo* resulted in an increase in fungicidal activity of murine macrophages that was associated to higher H_2O_2 levels. On the other hand, our results are in agreement with some studies showing that increase in oxygen intermediate reatives production by macrophages stimulated with zymosan is TLR2 independent (Gantner et al., 2003). In addition, Villamón et al.

(2004a) showed that macrophages from TLR2 knockout mice release similar levels of oxygen reative metabolites when compared to normal mice, in response to zymosan and *Candida*. Likewise TLR2, TLR4 appears not be involved in superoxide and NO generation by macrophages in response to this fungus (Netea et al., 2002). Together, these results indicate that TLR2 and TLR4 are not involved in the direct stimulation of host candidacidal mechanisms.

Our studies on cytokines production reveal that ATF increases TNF-α, IL-1 and IL-10 production by human monocytes. A tendency in inhibiting IL-12 release was also detected. Corroborating with our data Sorimachi et al. (2001) observed that *in vitro* treatment of rat bone marrow derived macrophages with an aqueous extracts from *Agaricus blazei* resulted in a significative increase in TNF-α and IL-8 levels. An other extract of the same fungus, when orally administered, induces murine peritoneal macrophages to release higher IL-1β and IL- 6 levels (Nakajima et al., 2002). This same extract increased IL-8, IL-6, TNF-α and IL-1β production by human mononuclear cells, with however, no effect on IL-10 and IL-12 (Bernardshaw et al., 2005).

In this study, increase in TNF-α levels by ATF was related to is modulation on TLR4, while for IL-1β, a TLR2 involvement was detected. In relation to TNF-α our data are discordant of the literature, since studies using C3H/HeJ, defective for TLR4, demonstrated that this receptor was not involved in TNF-α, IL-1-α, and IL-1β production (Netea et al., 2002). The authors discuss that the importance of this receptor for host resistance against *Candida* was not related with its capacity to modulate these pro-inflammatory cytokines, but by its effect on some chemokines such as KC and MIP-2. In the same study, the authors using human mononuclear cells sustained the non involvement of TLR4 in TNF-α and IL-1 production. In contrast, the results supported the effect of TLR2 on proinflammatory cytokines production by these cells (Netea et al., 2002). These data are in accordance only with our data referring IL-1β. However, it is important to emphasize that the role of TLR2 on cytokines production and resistance to *C. albicans* has been a point of discordance in the literature. Some studies showed that TLR2 defective mice infected with *C. albicans* are less resistent to infection, mainly due to cells incapacity to release cytokines such as TNF-α and MIP-2 (Villamón et al., 2004a) or TNF-α, IL-12 and IFN-γ (Villamon et al., 2004b). On the contrary, other studies (Netea et al.,

2004) reported that TLR defective mice are more resistance to infection, due to an increase in chemotaxis and candidacidal capacities of macrophages. Differences in TNF-α, IL-1 and IL-6 levels were not detected. On the other hand IL-10 production was strongly inhibited. The authors concluded that TLR2 mediate *Candida* scape mechanisms from host defense. Different experimental designs might explain these discordant results.

In our study ATF induces a significative increase in IL-10 production. However, this effect was not associated with either TLR2 or TLR4. It is possible that other unidentified receptors are involved in this process. Together, our data on cytokine production strongly suggest that ATF is able to modulate host response activating both, pro and antiinflammatory mechanisms. The balance between pro- and antiinflammatory responses is essential for successful host–fungal interactions (Romani & Puccetti, 2007). Although inflammation is crucial for protective response to fungi, after elimination of the invading microorganism subsequent antiinflammatory signals are need for host protection against deleterious effects of overwhelming response (De Waal Malefyt et al., 1991).

In summary, our data demonstrated that ATF increases TNF-α and IL-1 production by human monocytes, modulating TLR4 and TLR2 expression. These proinflammatory cytokines activated these cells increasing their capacity to phagocytosize *Candida albicans*. On the other hand, ATF by a mechanism independent of TLR2 and TLR4 increased IL-10 production, an antiinflammatory cytokine, that might have a role on controlling this activation process.

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ANEXOS

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