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.

BOTUCATU – SP
2008

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

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.

BOTUCATU – SP
2008

*Gostaria de agradecer a **Deus** por estar sempre ao meu lado abençoando-me e iluminando o meu caminho, dando-me força nos momentos difíceis e perdoando os meus erros. Agradeço por todas as oportunidades e vitórias que conquistei. Agradeço também por todos os momentos difíceis, pois muitas coisas aprendi com eles.*

" Grandes foram as lutas, maiores as vitórias.

Sempre estiveste comigo.

Muitas vezes, pensei que este momento nunca chegaria.

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.

Sei que não cheguei ao fim, mas ao início de uma longa caminhada.

Dedico este trabalho...

Aos meus pais, Florisvaldo e Maria de Lourdes, que me deram a vida e sempre acreditaram na minha capacidade, que me apoiaram incondicionalmente e, com muita luta, foram responsáveis por minha educação e valores.

Aos meus irmãos (Plínio e Paulo) e toda minha família, que mesmo à distância torcem por mim.

Ao meu namorado Marcos, que em pouco tempo se mostrou tão presente em minha vida, por todo seu amor, carinho, compreensão e paciência para agüentar minhas crises.

Muito obrigada!

Aos meus orientadores:

Profa. Dra. Ângela Maria Victoriano de Campos Soares

Que me estendeu as mãos quando mais precisei, aceitou me orientar e participou ativamente deste trabalho. Compreendeu minha ausência neste momento final e me ajudou no que foi preciso.
Meus sinceros agradecimentos e o meu profundo respeito!

Prof. Dr. Ramon Kaneno

Que iniciou minha orientação e me transmitiu ao longo de muitos anos não apenas um pouco de sua sabedoria, mas também me serviu de exemplo como um bom profissional e pessoa.
Obrigada por tudo!

Agradecimentos

Agradeço, com todo carinho, a todos que contribuíram para que este trabalho fosse concretizado e dividiram comigo esta caminhada:

Aos meus **doadores de sangue**, se não fosse por vocês este trabalho, com certeza, não teria se concretizado.

A **Profa. Dra. Maria Terezinha Serrão Peraçoli** pelo auxílio nos ensaios de fagocitose e dosagem de citocinas.

A **Profa. Dra. Márcia Guimarães da Silva** pelo auxílio na dosagem de citocinas e por me disponibilizar seu laboratório.

Ao **Prof. Dr. Luiz Claudio Di Stasi** pelo auxílio durante o preparo da fração ATF.

A **Dra. Márjorie A. Golim** pelo auxílio nos experimentos de citometria de fluxo, bem como na análise dos resultados.

Ao **Dr Gordon D. Brown** (Institute of Infectious Disease and Molecular Medicine, University of Cape Town, South Africa) pela doação do anticorpo GE2 (anti-βGR) utilizado neste trabalho.

Aos professores do Depto de Microbiologia e Imunologia, **Dr Silvio Luís de Oliveira, Dr. José Maurício Sforcin, Dra. Maria Terezinha Serrão Peraçoli** e **Dra. Alexandrina Sartori**, por sempre contribuírem na solução de problemas práticos e pelas sugestões na elaboração do trabalho.

Aos funcionários do Depto de Microbiologia e Imunologia, **Luiz Alquati** e **"Lula"**, pelo auxílio nos experimentos e por tornar o ambiente de trabalho sempre harmonioso.

Às secretárias do Depto de Microbiologia e Imunologia, **Sônia** e **Nice** e a secretária da PG em Patologia, **Tânia**, por toda a atenção.

À **Janete,** funcionária da limpeza do Depto de Microbiologia e Imunologia, por tornar sempre limpo e agradável nosso local de trabalho.

Ao **serviço de Biblioteca e Documentação** da UNESP-Botucatu, pela elaboração da ficha catalográfica e revisão das referências.

Aos **funcionários da Seção de pós-graduação** da Faculdade de Medicina de Botucatu-UNESP, pelos serviços e esclarecimentos sempre quando solicitados.

A TODOS os **amigos e amigas (estagiários e pós-graduandos)** do Depto de Microbiologia e Imunologia, por compartilharem comigo momentos agradáveis, por fazerem do nosso ambiente de trabalho um lugar gostoso e tornando meu almoço sempre animado.

As minhas amigas **Maria Augusta, Rosi, Dalva, Eliane e Adriana** pelos momentos agradáveis de descontração.

Aos amigos **Roberta Baroni** e **Rinaldson**, por tudo que vocês tem feito por mim, pela amizade e força nesse momento tão estressante e difícil de minha vida.

As amigas, **Andréa Vanessa Pinto, Graziela Gorete Romagnoli, Lindsey Castoldi, Maria Carolina Gameiro** e **Fabiane Catanho Lopes** (“ex ramonetes”) por toda confiança e amizade mesmo a distância.

Aos amigos **Virginia Richini Pereira** e **Sérgio Pereira** por toda a amizade e carinho quando me receberam em sua casa. Novamente, a você Virgínia por tudo que me fez, por todas as ajudas e favores.

À **Fundação de Amparo à Pesquisa do Estado de São Paulo** (FAPESP) e ao **Conselho Nacional do Desenvolvimento Científico e Tecnológico** (CNPq) pela concessão dos recursos financeiros para a realização deste trabalho e pela concessão de bolsa de doutorado.

" 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.

Referências *

Ahn WS, Kim DJ, Chae GT, Lee JM, Bae SM, Sin JI et al. Natural killer cell activity and quality of life were improved by consumption of a mushroom extract, *Agaricus blazei murril* Kyowa, in gynecological câncer patients undergoing chemoterapy. Int J Gynecol Cancer. 2004; (14):589-94.

Akira S, Uematsu S. Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006; (124):783-801.

Angeli JP, Ribeiro LR, Gonzaga ML, Soares Sde A, Ricardo MP, Tsuboy MS,et al. Protective effects of beta-glucan extracted from Agaricus brasiliensis against chemically induced DNA damage in human lymphocytes. Cell Biol Toxicol. 2006; (22):285-91.

Arizumi K, Shen GL, Shikano S, Xu S, Ritter R 3rd, Kumamoto T. et al. Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. J Biol Chem. 2000; (275): 20157-67.

Bagchi A, Herrup EA, Warren HS, Trigillo J, Shin HS, Valentine C et al. MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. J Immunol. 2007; (178):1164-71.

Biondo C, Midiri A, Messina L, Tomasello F, Garufi G, Catania MR et al. MyD88 and TLR2, but not TLR4, are required for host defense against Cryptococcus neoformans. Eur J Immunol. 2005; (35): 870-8.

* International Committee of Medical Journal Editors. Uniform Requirements for Manuscripts Submitted to Biomedical Journal: sample references. [Homepage on the Internet]. Bethesda: U.S. National Library of Medicine; 2003 [last updated 2003 July 09; cited 2005 Jun 01]. Available from:http://www.nlm.nih.gov/bsd/uniform_requirements.html
National Library of Medicine. List of journals indexed in Index Medicus. Washington, 2003. 240p.

Borchers AT, Stern JS, Hackman RM, Keen CL, Gershwin ME. Mushrooms, tumors, and immunity. Proc Soc Exp Biol Med. 1999; (221): 281-93.

Braedel S, Radsak M, Einsele H, Latge JP, Michan A, Loeffer J et al. Aspergillus fumigatus antigens active innate immune cells via toll-like receptors 2 and 4. Br J Haematol. 2004; (125): 392-9.

Brown GD, Gordon S. Immune recognition. A new receptor for beta-glucans. Nature. 2001; (413):36-7.

Brown GD, Taylor PR, Reid DM, Willment JA, Williams DL, Martinez-Pomares L. et al. Dectin-1 is a major β-glucan receptor on macrophages.J Exp Med. 2002; (296): 407-12.

Brown GD, Herre J, Williams DL, Willment JA, Marshall ASJ Gordon S. Dectin-1 mediates the biological effects of β-glucans. J Exp Med. 2003; (197):1119-24.

Brown GD, Gordon S. Fungal beta-glucans and mammalian immunity. Immunity. 2003;(19):311-5.

Brown GD. Dectin-1: a signaling non-TLR pattern-recognition receptor. Nat Rev Immunol. 2006; (6):33-43.

Calderone R, Diamond R, Senet JM, Warmington J., Filler S, Edwards JE. Host cell-fungal cell interations. J Med Vet Mycol. 1994; (32) 151-64.

Cambi A., Gijzen K, de Vries JM, Torensma R, Joosten B, Adema GJ et al. The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for *Candida albicans* on dendritic cells. Eur J Immunol. 2003; (33): 532-8.

Carmelini CM, Maraschin M, de Mendonça MM, Zucco C, Ferreira AG, Tavares LA. Structural characterization of beta-glucans of Agaricus brasiliensis in different stages of fruiting body maturity and their use in nutraceutical products. Biotechnol Lett. 2005; (27): 1295-9.

Chang R. Functional properties of edible mushrooms. Nutr Rev**.** 1996; (54): S91-3.

Cheung PCK. The hypocholesterolemic effect of extracellular polysaccharide from the submerged fermentation of mushroom. Nutr Res.1996; (16): 1953-7.

Czarnecki R, Grzybek J. Antiinflamatory and vasoprotective activities of polysaccharides isolated from fruiting bodies of higher fungi P.l. polysaccharides from *Trametes gibbosa* (Pers.:Fr.) Fr. (Polyporaceae). Phytother Res. 1995; (9): 123-7.

Czop JK, Puglisi AV, Miorandi DZ, Austen KF. Perturbation of β-glucan receptors on human neutrophils initiates phagocytosis and leukotriene B4 production. J Immunol. 1988; (141):3170-6.

Czop JK, Kay J. Isolation and characterization of β-glucan receptors on human mononuclear phagocytes. J Exp Med. 1991; (173): 1511-20.

Di Renzo L, Yefenof E, Klein E. The function of human NK cells is enhanced by β-glucan, a ligand of CR3 (CD11b/CD18). Eur J Immunol. 1991; (21): 1755-8.

Djeu JY Role of tumor necrosis factor and colony-stimulating factors in phagocyte function against *Candida albicans*. Diagn. Microbiol. Infect. Dis. 1990; (13): 383-6.

Dubordeau M, Athman R, Balloy V, Huerre M, Chignard M, Philpott DJ et al. Aspergillus fumigatus induces innate immune responses in alveolar macrophages through the MAPK pathway independently of TLR2 and TLR4. J Immunol. 2006; (177): 3994-4001.

Ebina T, Fujimiya Y. Antitumor effect of a peptide-glucan preparation extracted from *Agaricus blazei* in a double-grafted tumor system in mice. Biotherapy.1998; (11): 259-65.

Edman JC Micologia Médica. In: Jawetz E, Melnick JL, Adelberg EA, editors. Microbiologia médica. 20.ed. Rio de Janeiro: Guanabara-Koogan S.A.; 1998. p.420-43.

Eira AF, Pinto AVFS, Fontanari LM, Santos SA, Barbisan ALTS, Lorenzo JSF et al. Efeitos da temperatura de preparação sobre a ação antitumoral de extratos de cogumelos comestíveis. In: Reunião anual da SBPC; 2000; Brasília: Anais da 52° Reunião anual da SBPC; 2000.

Faccin LC, Benati F, Rincão VP, Mantovani MS, Soares SA, Gonzaga ML et al. Antiviral activity of aqueous and ethanol extracts and of an isolated polysaccharide from Agaricus brasiliensis against poliovirus type 1. Lett Appl Microbiol. 2007; (45):24-8.

Ferwerda G, Meyer-Wentrup F, Kullberg BJ, Netea MG, Adema GJ. Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. Cell Microbiol. 2008; (10):2058-66.

Fujimiya Y, Suzuki Y, Oshiman K, Kobori H, Moriguchi K, Nakashima H et al. Selective tumoricidal effect of soluble proteoglucan extracted from the basidiomycete, *Agaricus blazei* Murill, mediated via natural killer cell activation and apoptosis. Cancer Immunol Immunother.1998; (46): 147-59.

Fujimiya Y, Suzuki Y, Katakura R, Ebina T. Tumor-specific cytocidal and immunopotentiating effects of relatively low molecular weight products derived from the basidiomycete, *Agaricus blazei* Murill. Anticancer Res. 1999; (19): 113-8.

Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM. Collaborative induction of inflammatory responses by dectin-1 and toll-like receptor 2. J Exp Med. 2003; (197):1107-17.

Grinde B, Hetland G, Johnson E. Effects on gene expression and viral load of medicinal extract from *Agaricus blazei* in patients with chronic hepatitis C infection. Int Immunopharmacol. 2006; (6): 1311-14.

Hikino H, Mizuno T. Hypoglycemic actions of some heteroglycans of *Ganoderma lucidum* fruti bodies. Planta Med. 1989; (55):385.

Ito H, Shimura K, Itoh H, Kawade M. et al. Antitumor effects of a new polysaccharide-protein complex (ATOM) prepared from *Agaricus blazei* (Iwade strain 101) “himematsutake” and its mechanisms in tumor-bearing mice. Anticancer Res. 1997; (17): 277-84.

Kaneno R, Fontanari LM, Santos SA, Di Stasi LC, Rodrigues Filho E, Eira AF. Effects of extracts from Brazilian sun-mushroom (*Agaricus blazei*) on the NK activity and lymphoproliferative responsiveness of Ehrlich tumor-bearing mice. Food Chem Toxicol. 2004; (42):909-16.

Kasai H, HE LM, Kawamura M, Yang PT, Deng XW, Munkanta M, et al. IL-12 production induced by *Agaricus blazei* Fraction H (ABH) involves Toll-like receptor (TLR). Evid Based Complement Alternat Med. 2004; (1):259-67.

Kawagishi H, Inagaki R, Kanao T, Mizuno T, Shimura K, Ito H et al Fractionation and antitumor activity of the water-soluble residue of Agaricus blazei fruitig bodies. Carbohydr Res. 1989; (186):267-73.

Kawagishi H, Kanao T, Inagaki R., Mizuno T, Shimura K, Ito H et al Formolysis of a potent antitumor (1→6) - β-D-glucan-protein complex from *Agaricus blazei* fruiting bodies and antitumor activity of the resulting products. Carbohydr Polym. 1990; (12):393-403.

Kullberg BJ, Van’t Wout JW, Hoogstraten C, Van Furth R. Recombinant interferon-γ enhances resistance to acute disseminated *Candida albicans* infection in mice. J Infect Dis. 1993; (168):436-43.

Le Cabec V, Emorine LJ, Toesca I, Cougoule C, Maridonneau-Parini I. The human macrophage mannose receptor is not a professional phagocytic receptor. J Leukoc Biol. 2005; (77):934-43.

LeibundGut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, et al. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol. 2007; (8):630-8.

Liu GQ, Wang XL. Optimization of critical medium components using response surface methodology for biomass and extracellular polysaccharide production by Agaricus blazei. Appl Microbiol Biotechnol. 2007; (74):78-83.

Los M, Schenk H, Hexel K, Baeuerle PA, Dröge W, Schulze-Osthoff K. IL-2 gene expression and NF-kB activation through CD28 requires reactive oxygen production by 5-lipoxygenase. Embo J. 1995; (4): 3731-40.

Loyola W, Gaziri DA, Gaziri LC., Felipe I. Concanavalin A enhances phagocytosis and killing of *Candida albicans* by mice peritoneal neutrophils and macrophages. FEMS Immunol Med Microbiol. 2002; (33): 201-8.

Mahauthaman R, Howell CJ, Spur BW, Youlten LJ, Clark TJ, Lessof MH et al. The generation and cellular distribution of leukotriene C4 in human eosinophils stimulated by unopsonized zymosan and glucan particles. J Allergy Clin Immunol. 1988; (81): 696-705.

Mambula SS. Toll-like receptor (TLR) signaling in response to Aspergillus fumigatus. J Biol Chem.2002; (277): 3932-6.

Marodi L, Schreiber S, Anderson CD, MacDermott RP, Korchak HM, Johnston Jr RB. Enhancement of macrophage candidacidal activity by interferon-gamma: increased phagocytosis, killing and calcium signal mediated by a decreased number of mannose receptors. J Clin Invest. 1993; (91):2596-601.

Marr KA, Balajee AS, Hawn TR, Ozinsky A, Pham U, Akira S et al. Differential role of MyD88 in macrophage-mediated responses to opportunistc fungal pathogens. Infect Immun. 2003; (71): 5280-6.

Martins PR, Gameiro MC, Castoldi L, Romagnoli GG, Lopes FC, Pinto AV et al. Polysaccharide-rich fraction of *Agaricus brasiliensis* enhances the candidacidal activity of murine macrophages. Mem Inst Oswaldo Cruz. 2008; (103):244-50.

Meier A, Kirsching CJ, Nikolaus T, Wagner H, Heesemann J, Ebel F. Toll-like receptor (TLR) 2 and TLR4 are essential for *Aspergillus*-induced activation of murine macrophages. Cell Microbiol. 2003; (5): 561-70.

Mizuno T, Hagiwara T, Nakamura T. Antitumor activity and some properties of water-soluble polyssacarides from Himematsutake, the fruiting body of *Agaricus blazei* Murril. Agric Biol Chem 1990a; (54): 2889-96.

Mizuno T, Tinagari R, Kanao T. Antitumor activity and some properties of water-soluble polyssacarides from Himematsutake, the fruiting body of *Agaricus blazei* Murril. Agric Biol Chem 1990b; (54): 2897-905.

Mizuno M, Morimoto M, Minato K, Ito H, Tsuchida H. Polysaccharide from *Agaricus blazei* stimulate lymphocyte T-cell subsets in mice. Biosci Biotechnol Biochem. 1998; (62): 434-7.

Moilanen E, Whittle B, Moncada S. Nitric oxide as a factor in inflammation. In: Gallin JI, Snyderman R, editors. Inflammation: basic principles and Clinical correlates. (3 ed.). Philadelphia: Lippincot Williams & Wilkins; 1999. p.787-800.

Mueller A, Raptis J, Rice PJ, Kalbfleisch JH, Stout RD, Ensley HE et al. The influence of glucan polymer structure and solution conformation on binding to (1→3)-β-D-glucan receptors in a human monocyte-like cell line. Glycobiology 2000; (10): 339-46.

Nakamura K, Miyagi K, Koguchi Y. Limited contribution of Toll-like receptor 2 and 4 to the host response to fungal infectious pathogen, *Cryptococcus neoformans.* FEMS Immunol Med Microbiol. 2006; (47):148-54.

Netea MG, Van der Graaf CAA, Vonk AG, Verschueren I, Van der Meer JWM., Kullberg BJ. The role of toll-like receptor (TLR)2 and TLR4 in the host defense against disseminated candidiasis. J Infect Dis.2002; (185):1483-9.

Netea MG, Warris A, Van der Meer JW, Fenton MJ, Verver-Janssen TJ, Jacobs LE, Andresen T, Verweij PE, Kullberg BJ. *Aspergillus fumigatus* evades immune recognition during germination through loss of toll-like receptor-4-mediated signal transduction. J Infect Dis. 2003;(188): 320-6.

Netea MG, Van der Graaf C, Van der Meer JWM, Kullberg BJ. Recognition of fungal pathogens by Toll-like receptors. Eur J Clin Microbiol.2004a; (23):672-6.

Netea MG, Sutmuller R, Hermann C, Van der Graaf CAA, Van der Meer JWM , Van Krieken JH et al. Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. J Immunol. 2004b; (172):3712-8.

Netea MG, Gow NA, Munro CA, Bates S, Collins C, Ferwerda G et al. Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. J Clin Invest. 2006; (116):1642-50.

Ofek I., Goldhar J, Keisari Y, Sharon N. Nonopsonic phagocytosis of microorganisms. Annu Rev Microbiol. 1995; (49): 239-76.

Ooi VEC. Hepatoprotective effect of some edible mushrooms. Phytother Res. 1996; (10): 536-8.

Palm NW & Medzhitov R. Antifungal defense turns 17. Nat Immnol. 2007; (8):549-51.

Park EJ, Ko G, Kim J, Sohn. Antifibrotic effects of a polysaccharide extracted from *Ganoderma lucidum*, glycyrrhizin, and pentoxifyline in rats with cirrhosis induced in biliary obstruction. Biol Pharm Bull. 1997; (20): 417-20.

Paul-Clark MJ, McMaster SK, Belcher E, Sorrentino R, Anandarajah J, Fleet M et al. Differential effects of Gram-positive versus Gram-negative bactéria on NOSII and TNFalpha in macrophages: role of TLRs in synergy between the two. Br J Pharmacol. 2006; (148): 1067-75.

Pick E, Keisare Y. A simple colorimetric method for measurement of hydrogen peroxide produced by cells in culture. J Immunol Methods. 1980; (38): 161-172.

Pietrella D, Corbucci C, Perito S, Bistoni G, Vecchiarelli A. Mannoproteins from Cryptococcus neoformans promote dendritic cell maturation and activation. Infect Immun. 2005;(73):820-7.

Romani L. Immunity to fungal infections. Nat Rev Immunol. 2004; (4): 1-13.

Sakagami H, Aoki T, Simpson A, Tanuma S. Induction of immunopotentiation activity by a protein-bound polysaccharide, PSK (Review). Anticancer Res. 1991; (11): 993-1000.

Sakurai T, Suzuki I, Kinoshita A, Oikawa S, Masuda A, Ohsawa M et al. Effect of intraperitoneally administered β-1,3-glucan, SSG, obtained from *Sclerotinia sclerotiorum* IFO 9395 on the functions of murine alveolar macrophages. Chem Pharm Bull. 1991; (39): 214-17.

Schlesinger PH, Doebber TW, Mandell BF, White R, DeSchryver C, Rodman JS et al. Plasma clearance of glycoproteins with terminal mannose and N-acetylglucosamine by liver non-parenchymal cells. Studies with beta-glucuronidase, N-acetyl-beta-D-glucosaminidase, ribonuclease B and agalacto-orosomucoid. Biochem J. 1978; (176): 103-9.

Shepherd VL, Campbell EJ, Senior RM, Stahl PD. Characterization of the mannose/fucose receptor on human mononuclear phagocytes. J Reticuloendothel Soc. 1982; (32): 423-31.

Shoham S, Huang C, Chen JM, Golenbock DT, Levitz SM.Toll-like receptor 4 mediates intracellular signaling without TNF release in response to Cryptococcus neoformans polysaccharide capsule. J Immunol. 2001; (166): 4620-6.

Smedsrod B, Einarsson M, Pertoft H. Tissue plasminogen activator is endocytosed by mannose and galactose receptors of rat liver cells. Thromb Haemost. 1988; (59): 480-84.

Sorimachi K, Akimoto K, Ikehara Y, Inafuku K, Okubo A, Yamazaki S. Secretion of TNF-α, Il-8 and nitric oxide by macrophages activated with *Agaricus blazei* Murill fractions *in vitro*. Cell Struct Funct. 2001; (26):103-8.

Stahl P, Gordon S. Expression of a mannosyl-fucosyl receptor for endocytosis on cultured primary macrophages and their hybrids. J Cell Biol. 1982; (93):49-56.

Stahl P, Ezekowitz RA. The mannose receptor is a pattern recognition receptor involved in host defense. Curr Opin Immunol. 1998; (10)**:**50-5.

Stevenhagen A., Furth R. Interferon-gamma activates the oxidative killing of *Candida albicans* by human granulocytes. Clin Exp Immunol. 1993; (91):170-5.

Suzuki I, Tanaka H, Kinoshita A, Oikawa S, Osawa M, Yadomae T. Effect of orally administered β-glucan on macrophage function in mice. Int J Immunopharmacol. 1990; (12): 675-84.

Tachado SD, Zhang J, Zhu J, Patel N, Cushion M, Koziel H. Pneumocystis-mediated IL-8 release by macrophages requires coexpression of mannose receptors and TLR2. J Leukoc Biol. 2007; (81):205-11.

Takeda K, Akira S. TLR signaling pathways. Semin Immunol 2004; 16: 3-9.

Van't Wout JW, Linde I, Leijh PCJ, Furth V. Contribution of granulocytes and monocyts to resistance against experimental disseminated *Candida albicans* infections. Eur J Clin Microbiol Infect Dis. 1988; (7): 736-41.

Villamón E, Gozalbo D, Roig P, O'Connor JE, Fradelizi D, Gil ML. Toll-like receptor-2 is essential in murine defensas against *Candida albicans* infections. Microbes Infect. 2004a; (6), p.1-7.

Villamón E, Gozalbo D, Roig P, O'Connor JE, Ferrandiz ML, Fradelizi D et al. Toll-like receptor-2 is dispensable for acquired host immune resistance to *Candida albicans* in a murine model disseminated candidiasis. Microbes Infect. 2004b; (6):542-8.

Wang JE, Warris A, Ellingsen EA, Jorgensen PF, Flo THInvolvement of CD14 and toll-like receptors in activation of human monocytesby Aspergillus fumigatus hyphae. Infect Immun. 2001, (69): 2402–406.

Wasser SP, Diduck MY, Amazonas MLLA, Nevo E, Stamets P, Eira AF. Is a widely cultivated culinary-medicinal royal sun Agaricus (the himematsutake mushroom) indeed *Agaricus blazei* muril? Intern J Medicinal Mush. 2002; (4): 267-90.

Willment JA, Gordon S, Brown GD. Characterization of the human β-glucan receptor and its alternatively spliced isoforms. J Biol Chem. 2001; (276): 43818-23.

Willment JA, Brown GD. C-type lectin receptors in antifungal immunity. Trends Microbiol. 2008; (16):27-32.

Yamamoto Y, Klein TW, Friedman H. Involvement of mannose receptor in cytokine interleukin-1beta (IL-1beta), IL-6, and granulocyte-macrophage colony-stimulating factor responses, but not in chemokine macrophage inflammatory protein 1 beta (MIP-1beta), MIP-2, and KC responses, caused by attachment of *Candida albicans* to macrophages. Infect Immun. 1997; (65):1077-82.

Yauch LE, Mansour MK, Shoham S, Rottman JB, Levitz SM. Involvement of CD14, toll-like receptors 2 and 4, and MyD88 in the host response to the fungal pathogen Cryptococcus neoformans in vivo. Infect Immun. 2004;72: 5373-82.

Zhang J, Zhu J, Imrich A, Cushion M, Kinane TB, Koziel H. Pneumocystis activates human alveolar macrophage NF-kappaB signaling through mannose receptors. Infect Immun. 2004; (72):3147-60.

Zhang J, Tachado SD, Patel N, Zhu J, Imrich A, Manfruelli P et al. Negative regulatory role of mannose receptors on human alveolar macrophage proinflammatory cytokine release in vitro. J Leukoc Biol. 2005; (78):665-74.

Manuscrito

* De acordo com as normas da Revista FEMS Immunology and Medical Microbiology

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

Priscila Raquel Martins[1,2], Márjorie de Assis Golim[3], Ângela Maria Victoriano de Campos Soares[1,2], Maria Terezinha Serrão Peraçoli[1], Gordon D. Brown[4], Ramon Kaneno[1,2]

1. Department of Microbiology and Immunology, Institute of Bioscience of Botucatu-SP, UNESP, Brazil.
2. Department of Phatology, Faculty of Medicine of Botucatu-SP, UNESP, Brazil.
3. Hemocentro Division, Faculty of Medicine of Botucatu-SP, UNESP, Brazil.
4. Institute of Infectious Disease and Molecular Medicine, University of Cape Town, South Africa.

Keywords: *A. brasiliensis,* TLRs, phagocytosis

running title: *A. brasiliensis* derived polyssacharide modulates human monocytes activity

Corresponding author
Ramon Kaneno
Depto de Microbiologia e Imunologia, IBB, UNESP, Rubião Jr, s/n°, CxP. 510, 18618-000, Botucatu-SP, Brasil , fone +55 14-38116058, fax +55-14-38153744
E-mail address: rskaneno@yahoo.com.br

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.

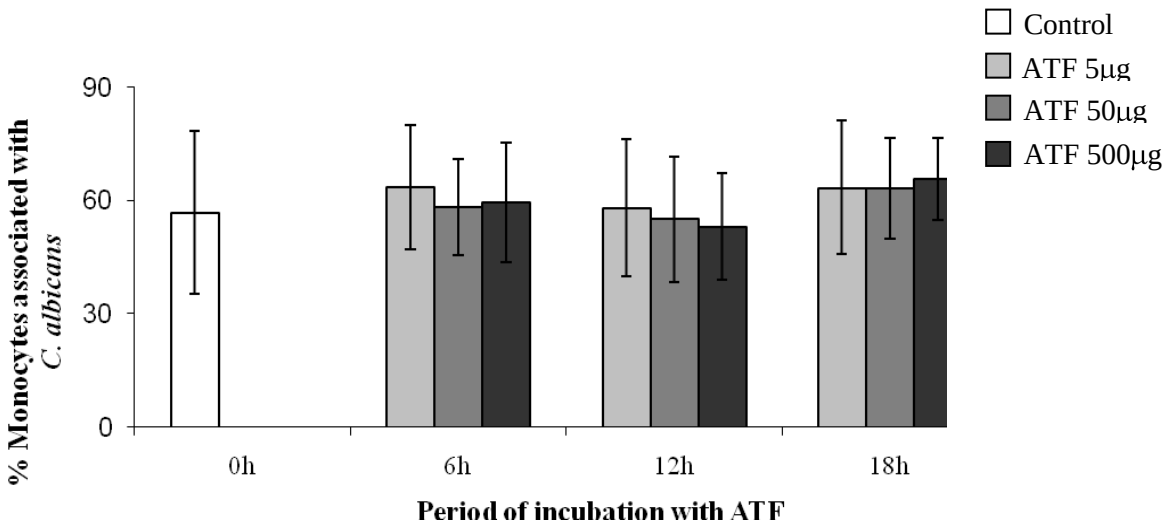

Fig.1. Effect of different ATF concentrations and treatment times on the percentage of $CD14^+$ human cells involved in the adherence and phagocytosis of FITC-*Candida albicans*, determined by flow cytometry. The results are expressed on mean ± standard deviation of cells percentage detected in assays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

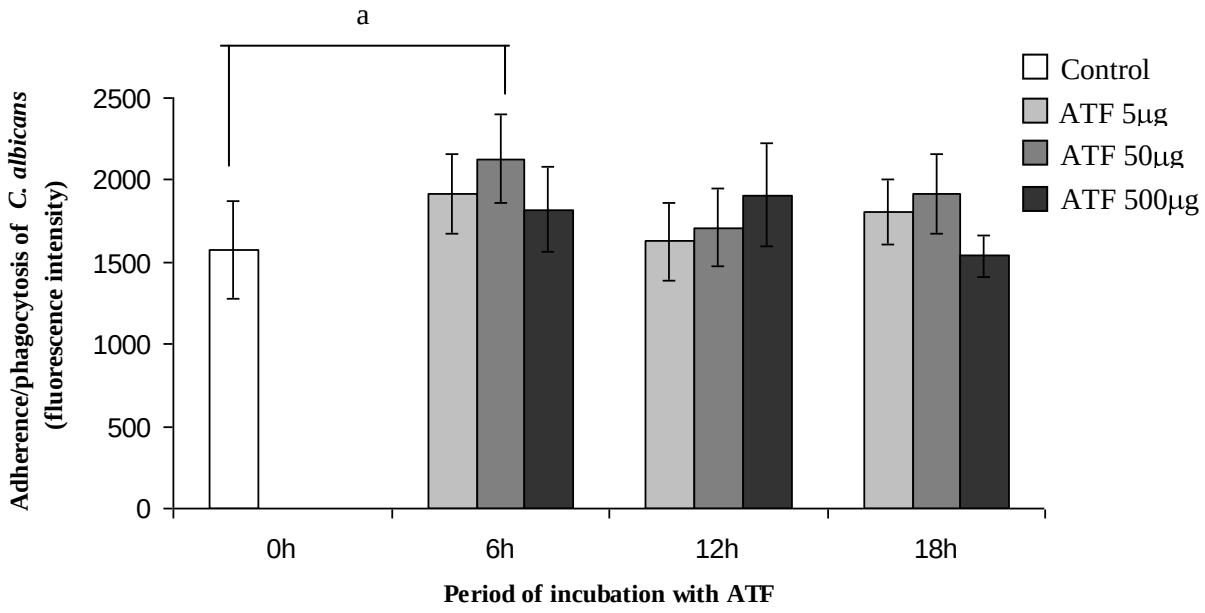

Fig.2. Effect of different ATF concentrations and treatment times on the adherence/phagocytosis of FITC-*Candida albicans*, determined by flow cytometry. The results are expressed on mean ± standard deviation of fluorescence intensity values detected in cells obtained from 7 subjects. a= $p < 0,01$,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

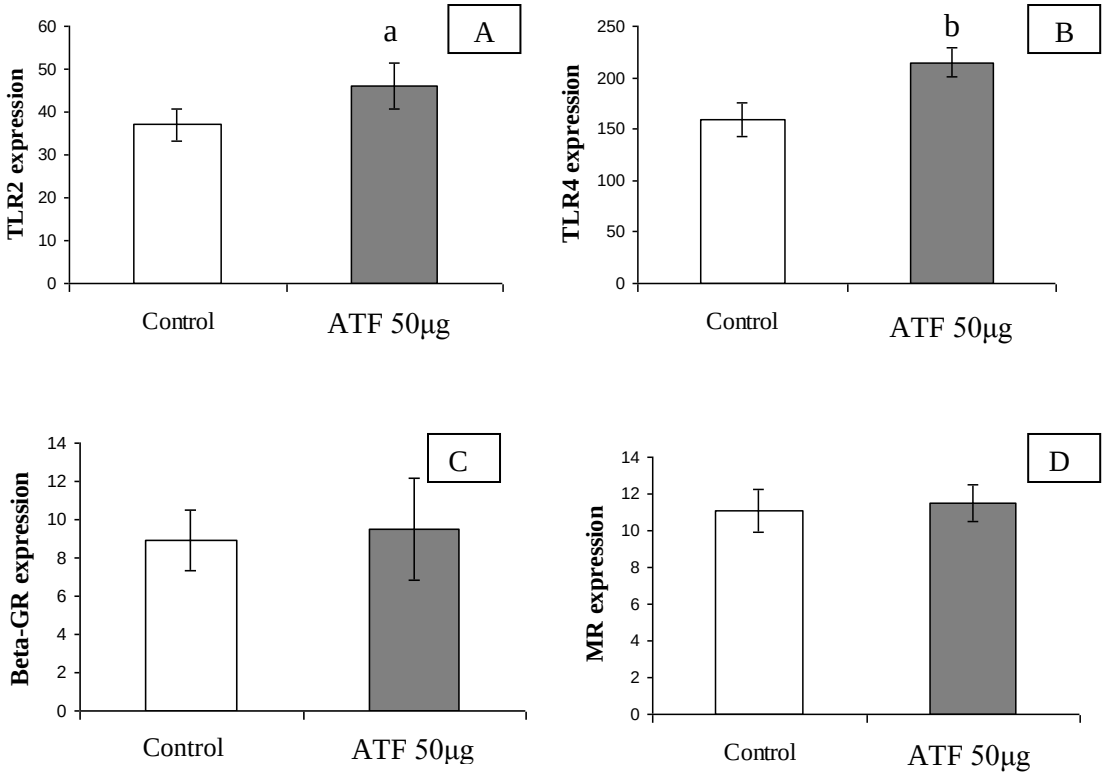

Fig.3. Effect of ATF on TLR2 (A), TLR4 (B), βGR (C) and MR (D) expression by human monocytes. The results are expressed on mean ± standard deviation of fluorescence intensity values detected in cells obtained from 6 subjects. a= p< 0, 05; b= p< 0, 01, paired t test.

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.

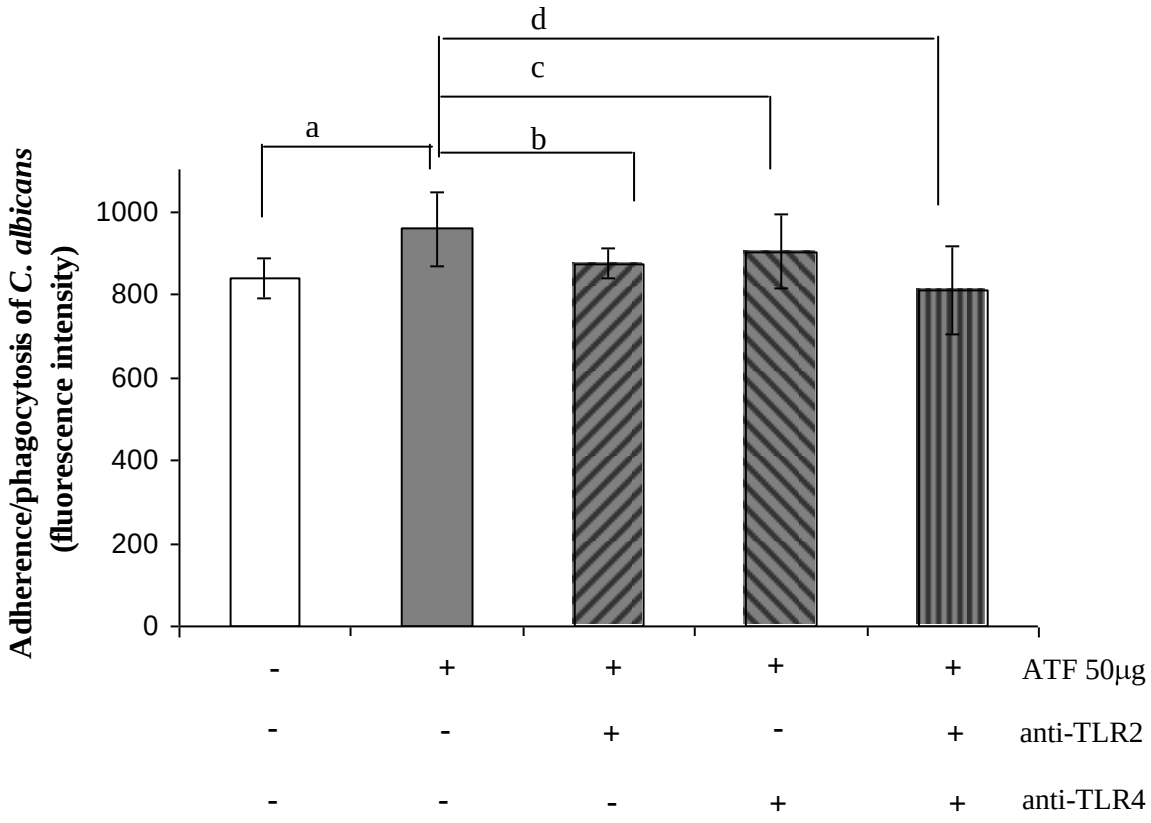

Fig. 4. Role of TLR2 and TLR4 on adherence/phagocytosis of *Candida albicans* induced by ATF. The results are expressed on mean ± standard deviation of fluorescence intensity values detected in cells obtained from 7 subjects. a = p< 0,001; b = p< 0,01; c = p< 0,05; d = p< 0,001, Student-Newman-Keuls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

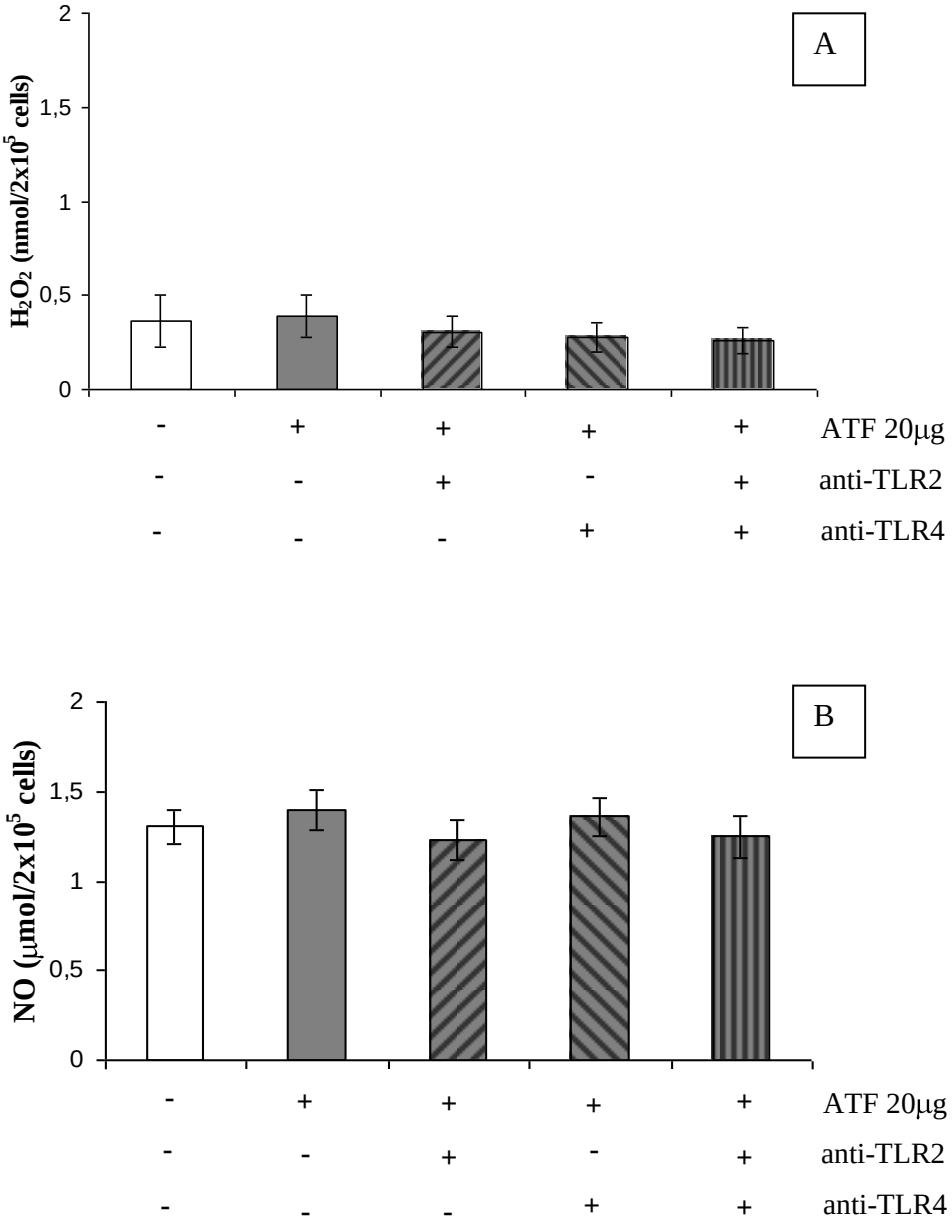

Fig. 5. Effect of ATF on H_2O_2 (A) and NO (B) release by human monocytes submitted or not to TLR2 and TLR4 blockade. The results are expressed on mean ± standard deviation of metabolites levels released by cells obtained from 11 subjects.

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.

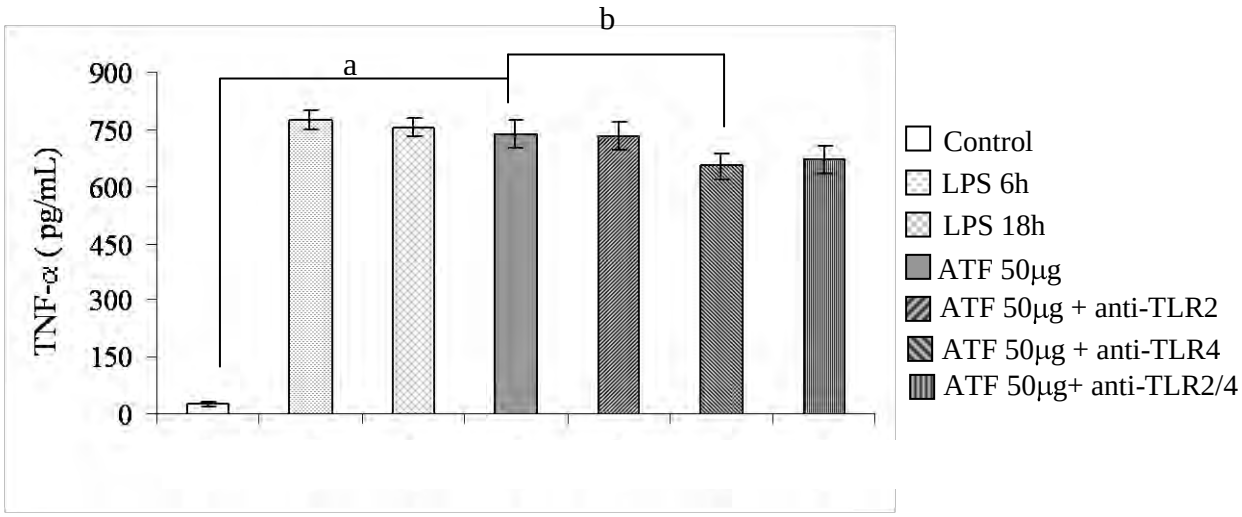

Fig. 6. Effect of ATF on TNF-α production by human monocytes submitted or not to TLR2 and TLR4 blockade. The results are expressed on mean ± standard deviation of cytokines levels released by cells obtained from 15 subjects. a= p< 0,001; b= p< 0, 05, Tukey-Kramer multiple comparisons test.

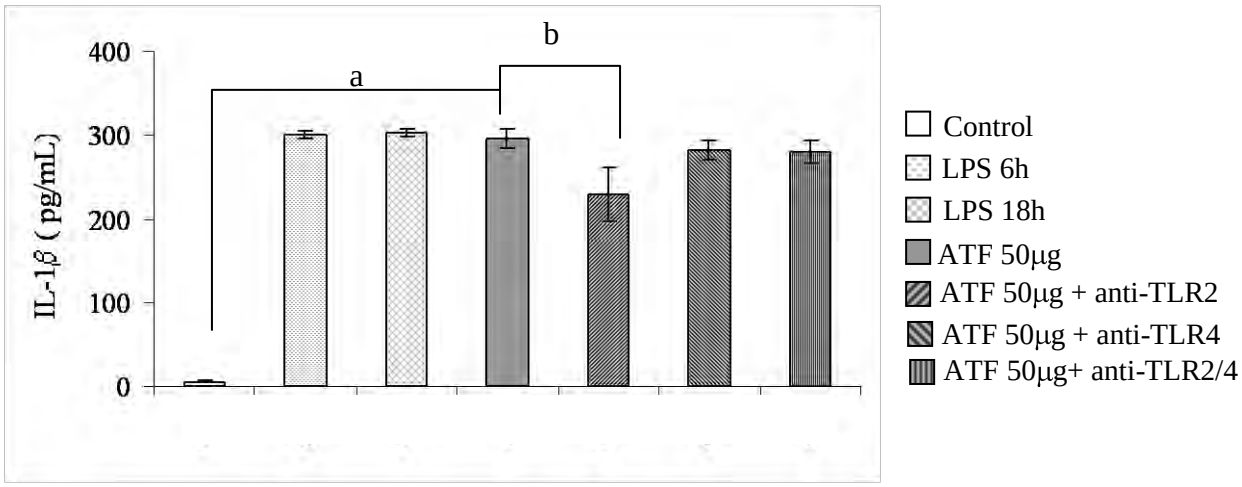

Fig. 7. Effect of ATF on IL-1β by human monocytes submitted or not to TLR2 and TLR4 blockade.The results are expressed on mean ± standard deviation of cytokines levels released by cells obtained from 15 subjects. a= p< 0,001; b= p< 0, 01, Tukey-Kramer multiple comparisons test.

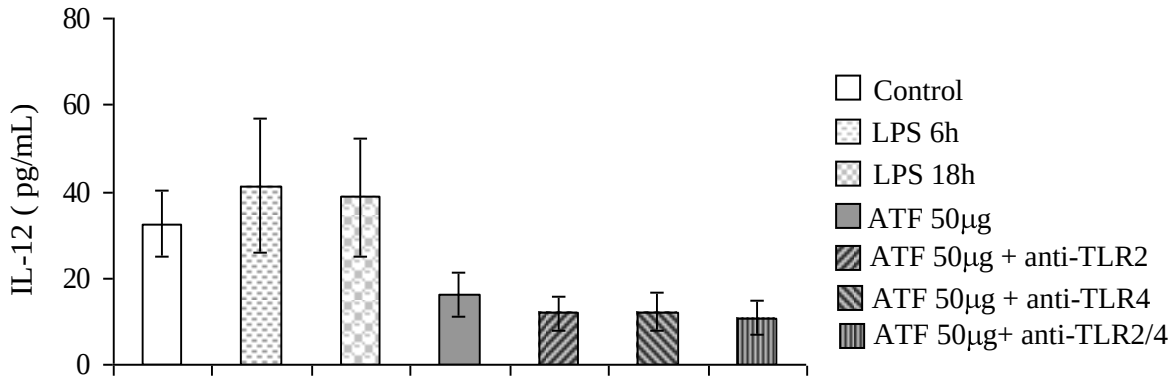

Fig. 8. Effect of ATF on IL-12 production by human monocytes submitted or not to TLR2 and TLR4 blockade. The results are expressed on mean ± standard deviation of cytokines levels released by cells obtained from 15 subjects.

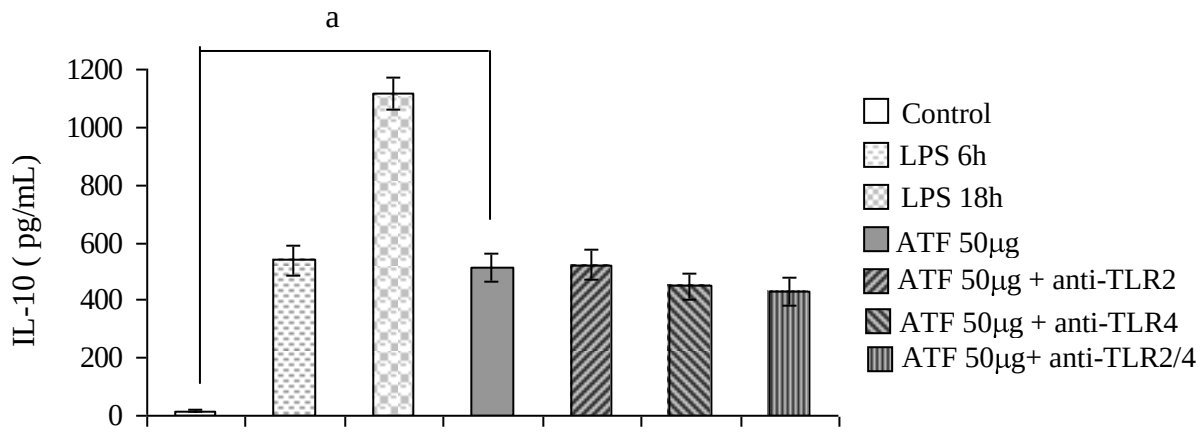

Fig. 9. Effect of ATF on IL-10 production by human monocytes submitted or not to TLR2 and TLR4 blockade. The results are expressed on mean ± standard deviation of cytokines levels released by cells obtained from 15 subjects. a= p< 0,001 Tukey-Kramer multiple comparisons test.

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.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 06/06925-2). P.R. Martins was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References*

Bernardshaw S, Ellertsen LK, Hetland G, Johnson E. (2005). An extract of the mushroom *Agaricus blazei* Murill differentially stimulates production of pro-inflammatory cytokines in human monocytes and human vein endothelial cells in vitro. *Inflammation* 29:147-53.

Borchers AT, Stern JS, Hackman RM, Keen CL, Gershwin ME. (1999). Mushrooms, tumors, and immunity. *Proc Soc Exp Biol Med* 221: 281-93.

Brown GD, Taylor PR, Reid DM, Willment JA, Williams DL, Martinez-Pomares L. et al. (2002). Dectin-1 is a major β-glucan receptor on macrophages.*J Exp Med* 296: 407-12.

Brown GD, Gordon S. (2003). Fungal beta-glucans and mammalian immunity. *Immunity* 19:311-5.

Brown GD, Herre J, Williams DL, Willment JA, Marshall ASJ Gordon S. (2003). Dectin-1 mediates the biological effects of β-glucans. *J Exp Med.* 197:1119-24.

Brown GD. (2006). Dectin-1: a signaling non-TLR pattern-recognition receptor. Nat *Rev Immunol* 6:33-43.

Chaka W, Scharringa J, Verheul AFM, Verhoef J, Strijp AGV, Hoepelman IM. (1995). Quantitative analysis of phagocytosis and killing of *Cryptococcus neoformans* by human peripheral blood mononuclear cells by flow cytometry. *Clin Diagn Lab Immunol.* 2:753-59.

Denis M. (1994) Human monocytes/macrophages: NO or no NO? *J Leukoc Biol*. 55: 682-4.

* De acordo com as normas da Revista FEMS Immunology and Medical Microbiology

De Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. (1991) Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med.* 174:1209-20.

Djeu JY (1990) Role of tumor necrosis factor and colony-stimulating factors in phagocyte function against *Candida albicans*. *Diagn Microbiol Infect Dis* 13: 383-6.

Donders GG. (2002) Lower genital tract infections in diabetic women. *Curr Infect Dis Rep.* 4: 536-9.

Ebina T, Fujimiya Y. (1998) Antitumor effect of a peptide-glucan preparation extracted from *Agaricus blazei* in a double-grafted tumor system in mice. *Biotherapy.* 11: 259-65.

Ezekowitz RA, Sastry K, Bailly P, Warner A. (1990) Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-I cells. *J Exp Med.* 172:1785-94.

Ezekowitz RA, Williams DJ, Koziel H, Armstrong MY, Warner A, Richards FF et al. (1991) Uptake of Pneumocystis carinii mediated by the macrophage mannose receptor. *Nature* 351: 155-8.

Ferwerda G, Meyer-Wentrup F, Kullberg BJ, Netea MG, Adema GJ. (2008) Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. *Cell Microbiol.* 10:2058-66.

Fujimiya Y, Suzuki Y, Oshiman K, Kobori H, Moriguchi K, Nakashima H et al. (1998) Selective tumoricidal effect of soluble proteoglucan extracted from the basidiomycete, *Agaricus blazei* Murill, mediated via natural killer cell activation and apoptosis. *Cancer Immunol Immunother.* 46: 147-59.

Fujimiya Y, Suzuki Y, Katakura R, Ebina T (1999) Tumor-specific cytocidal and immunopotentiating effects of relatively low molecular weight products derived from the basidiomycete, *Agaricus blazei* Murill. *Anticancer Res* 19: 113-8.

Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM (2003) Collaborative induction of inflammatory responses by dectin-1 and toll-like receptor 2. *J Exp Med* 197:1107-17.

Gow NA, Netea MG, Munro CA, Ferwerda G, Bates S, Mora-Montes HM et al. (2007) Immune recognition of *Candida albicans* beta-glucan by dectin-1. *J Infect Dis* 196: 1565-71.

Green LC (1981) Nitrite biossynthesis in man. *Proc Natl Acad Sci* 18: 7764-8.

Heinsbroek SE, Taylor PR, Matinez FO, Martinez-Pomares L, Brown GD, Gordon S. (2008) *PLos Pathog* 11: e1000218.

Ito H, Shimura K, Itoh H, Kawade M. et al. (1997) Antitumor effects of a new polysaccharide-protein complex (ATOM) prepared from *Agaricus blazei* (Iwade strain 101) “himematsutake” and its mechanisms in tumor-bearing mice. *Anticancer Res* 17: 277-84.

Kasai H, HE LM, Kawamura M, Yang PT, Deng XW, Munkanta M, et al. (2004) IL-12 production induced by *Agaricus blazei* Fraction H (ABH) involves Toll-like receptor (TLR). *Evid Based Complement Alternat Med* 1:259-67.

Kim GY, Han MG, Song YS, Shin BC, Shin YI, Lee HJ et al. (2004) Proteoglycan isolated from Phellinus linteus induces toll-like receptors 2- and 4-mediated maturation of murine dendritic cells via activation of ERK, p38, and NF-κB. *Biol Pharm Bull* 27: 1656-62.

Klein RS, Harris CA, Small CB, Moll B, Lesser M, Friedland GH (1984) Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. *N Engl J Med.* 311: 354-8.

Kullberg BJ, Van't Wout JW, Hoogstraten C, Van Furth R. (1993) Recombinant interferon-γ enhances resistance to acute disseminated *Candida albicans* infection in mice. *J Infect Dis* 168:436-43.

Le Cabec V, Emorine LJ, Toesca I, Cougoule C, Maridonneau-Parini I. (2005) The human macrophage mannose receptor is not a professional phagocytic receptor. *J Leukoc Biol*. 77:934-43.

LeibundGut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, et al. (2007) Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat Immunol.* 8:630-8.

Loyola W, Gaziri DA, Gaziri LC., Felipe I. (2002) Concanavalin A enhances phagocytosis and killing of *Candida albicans* by mice peritoneal neutrophils and macrophages. *FEMS Immunol Med Microbiol.* 33: 201-8.

Marodi L, Schreiber S, Anderson CD, MacDermott RP, Korchak HM, Johnston Jr RB. (1993) Enhancement of macrophage candidacidal activity by interferon-gamma: increased phagocytosis, killing and calcium signal mediated by a decreased number of mannose receptors. *J Clin Invest.* 91:2596-601.

Martins PR, Gameiro MC, Castoldi L, Romagnoli GG, Lopes FC, Pinto AV et al. (2008) Polysaccharide-rich fraction of *Agaricus brasiliensis* enhances the candidacidal activity of murine macrophages. *Mem Inst Oswaldo Cruz* 103:244-50.

Mizuno T, Hagiwara T, Nakamura T (1990a) Antitumor activity and some properties of water-soluble polyssacarides from Himematsutake, the fruiting body of *Agaricus blazei* Murril. *Agric Biol Chem* 54: 2889-96.

Mizuno T, Tinagari R, Kanao T (1990b) Antitumor activity and some properties of water-soluble polyssacarides from Himematsutake, the fruiting body of *Agaricus blazei* Murril. *Agric Biol Chem* 54: 2897-905.

Nakajima A, Ishida T, Koga M, Takeuchi T, Mazda O, Takeuchi M. (2002) Effect of hot water extracted from *Agaricus blazei* murril on antibody-producing cells in mice. Inter Immunopharmacol 2:1205-11.

Netea MG, Van der Graaf CAA, Vonk AG, Verschueren I, Van der Meer JWM, Kullberg BJ (2002) The role of toll-like receptor TLR2 and TLR4 in the host defense against disseminated candidiasis. *J Infect Dis* 185:1483-9.

Netea MG, Sutmuller R, Hermann C, Van der Graaf CAA, Van der Meer JWM , Van Krieken JH et al (2004) Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. *J Immunol.* 172:3712-8.

Netea MG, Brown GD, Kullberg BJ, Gow NAR (2008) An integrated model of the recognition of Candida albicans by the innate immune system. *Nat Rev Microbiol* 6: 67–78.

Pick A, Mizel A. (1981) A rapid microassay of the measurements of superoxid and hydrogen peroxide production by macrophages in culture using automatic enzyme immunoassay reader. *J Immunol.* 46:2111-26.

Porcaro I, Vidal M, Jouvert S, Stahl PD, Giaimis J. (2003) Mannose receptor contribution to Candida albicans phagocytosis by murine E-clone J774 macrophages. *J Leukoc Biol* 74: 206–15.

Ridola V, Chachaty E, Raimondo G, Corradini N, Brugieres L, Valteau-Coanet D et al. (2004) Candida infections in children treated with conventional chemotherapy for solid tumors (transplant recipients excluded): The Institut Gustave Roussy Pedriatrics Department experience. *Pediatr Blood Cancer* 42: 332-7.

Romani L. (1999) Immunity to Candida albicans: Th1, Th2 cells and beyond. *Curr Opin Microbiol* 2:363–7.

Romani L , Puccetti P (2007) Controlling pathogenic inflammation to fungi. *Expert Rev Anti infect ther* 5: 1007-17.

Sato M, Sano H, Iwaki D, Kudo K, Konishi M, Takahashi H et al. (2003) Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF-kappa B activationand TNF-alpha secretion are down-regulated by lung collectin surfactant protein A. *J Immunol* 171: 417–25.

Scheemann M, Schoedon G, Hofer S, Blau N, Guerrero L, Schaffner A. (1993) Nitric oxide synthase is not a constituent of the antimicrobial armature of human monocuclear phagocytes. *J Infect Dis* 167: 1358-63.

Sorimachi K, Akimoto K, Ikehara Y, Inafuku K, Okubo A, Yamazaki S. (2001) Secretion of TNF-α, Il-8 and nitric oxide by macrophages activated with *Agaricus blazei* Murill fractions *in vitro*. *Cell Struct Funct* 26:103-8.

Stevenhagen A., Furth R (1993) Interferon-gamma activates the oxidative killing of *Candida albicans* by human granulocytes. *Clin Exp Immunol* 91:170-5.

Szolnoky G, Bata-Csorgo Z, Kenderessy AS, Kiss M, Pivarcsi A, Novák Z et al. (2001) A mannose-binding receptor is expressed on human keratinocytes and mediates killing of *Candida albicans* *J Invest Dermatol* 117:205-13.

Tada H, Nemoto E, Shimauchi H, Watanabe T, Mikami T (2002) Saccharomyces cerevisiae- and Candida albicans-derived mannan induced production of tumor necrosis factor alpha by human monocytes in a CD14- andToll-like receptor 4-dependent manner. *Microbiol Immunol* 46: 503–12.

Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M et al. (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat Immunol* 8: 31–8.

Van't Wout JW, Linde I, Leijh PCJ, Furth V (1988) Contribution of granulocytes and monocyts to resistance against experimental disseminated *Candida albicans* infections. *Eur J Clin Microbiol Infect Dis* 7: 736-41.

Villamón E, Gozalbo D, Roig P, O'Connor JE, Fradelizi D, Gil ML (2004a) Toll-like receptor-2 is essential in murine defensas against *Candida albicans* infections. *Microbes Infect* 6: 1-7.

Villamón E, Gozalbo D, Roig P, O'Connor JE, Ferrandiz ML, Fradelizi D et al. (2004b) Toll-like receptor-2 is dispensable for acquired host immune resistance to *Candida albicans* in a murine model disseminated candidiasis. *Microbes Infect*. 6:542-8.

Wang JE, Warris A, Ellingsen EA, Jorgensen PF, Flo TH (2001) Involvement of CD14 and toll-like receptors in activation of human monocytesby Aspergillus fumigatus hyphae. *Infect Immun* 69: 2402–406.

Wasser SP, Diduck MY, Amazonas MLLA, Nevo E, Stamets P, Eira AF (2002) Is a widely cultivated culinary-medicinal royal sun Agaricus (the himematsutake mushroom) indeed *Agaricus blazei* muril? *Intern J Medicinal Mush* 4: 267-90.

Willment JA, Gordon S, Brown GD. (2001) Characterization of the human β-glucan receptor and its alternatively spliced isoforms. *J Biol Chem* 276: 43818-23.

Willment JA, Brown GD (2008) C-type lectin receptors in antifungal immunity. *Trends Microbiol* 16:27-32.

Yoon YD, Han SB, Kang JS, Lee CW, Park SK, Lee HS et al. (2004) Toll-like receptor 4-dependent activation of macrophages by polysaccharide isolated from the radix of *Platycodon grandiflorum*. *Int Immunopharmacol* 3:1873-82.

ANEXOS

FEMS Immunology & Medical Microbiology

Published on behalf of the Federation of European Microbiological Societies

Edited by:
Patrik M. Bavoil

Print ISSN: 0928-8244
Online ISSN: 1574-695X
Frequency: Nine times a year
Current Volume: 55 / 2009
ISI Journal Citation Reports® Ranking: 2007: 58/94 (Microbiology); 86/119 (Immunology); 35/50 (Infectious Diseases)
Impact Factor: 1.928

Author Guidelines

FEMS publishes five journals in the area of microbiology. All five journals follow the same instructions for manuscript preparation. If one journal has a different procedure, this will be mentioned at the appropriate place.

EDITORIAL POLICY

All submitted papers should be complete in themselves and adequately supported by experimental detail; they should not be preliminary versions of communications to be published elsewhere. Descriptions of new methods are acceptable, and the Editors welcome papers that put forward new hypotheses. However, papers that provide confirmatory evidence or merely extend observations firmly established in one species or field site to another will not be accepted unless there are strong reasons for doing so. Members of the Editorial Boards and other appropriate experts will referee the papers. Editors handling papers will independently make decisions on acceptance, revision or rejection based on the referees' reports. The Chief Editors or Editors will usually reject papers outside the scope of the journal with an immediate decision. Authors who feel that there are substantial grounds for disagreement with an Editor's decision should contact the Chief Editor, whose decision will be final. Authors who wish to withdraw their manuscript (at any stage of the process) should contact their Editor.

AIMS AND SCOPE

FEMS Microbiology Letters

The Editors give priority to concise papers that merit urgent publication by virtue of their originality, general interest and their contribution to new developments in microbiology. All aspects of microbiology, except virology (other than bacteriophages), are covered. Areas of special interest include: molecular biology and genetics; genomics; microbial biochemistry and physiology; structure and development; pathogenicity; medical and veterinary microbiology; environmental microbiology; applied microbiology and microbial biotechnology; systematics and bioinformatics. Papers (Research Letters and MiniReviews) can deal with any type of microorganism: bacteria and bacteriophage, yeasts, filamentous fungi and protozoa, cyanobacteria and eukaryotic algae.

FEMS Microbiology Reviews

This journal publishes reviews dealing with all aspects of microbiology that have not been surveyed recently. They should be devoted to topics of current interest and may be of a speculative and selective nature or they may provide comprehensive, critical and authoritative coverage. Reviews should provide new perspectives and critical, detailed discussions of significant trends in the areas being reviewed. Historical analyses of important subjects will also be accepted. All reviews should address both specialists and the general reader. Whenever possible, reviews should be put into the framework of general microbiology and biology. Manuscripts of lectures delivered at symposia that do not review the related field are not acceptable, nor are unevaluated compilations of the literature.

FEMS Microbiology Ecology

The Editors aim to ensure efficient publication of high-quality papers that are original and provide a significant contribution to the understanding of microbial ecology. The journal contains Research Articles and MiniReviews on fundamental aspects of the ecology of microorganisms in natural soil, aquatic and atmospheric habitats, including extreme environments, and in artificial or managed environments. Research papers on pure cultures and in the areas of plant pathology and medical, food or veterinary microbiology will be published where they provide valuable generic information on microbial ecology. Papers can deal with culturable and non-culturable forms of any type of microorganism: bacteria, archaea, filamentous fungi, yeasts, protozoa, cyanobacteria, algae or viruses.

FEMS Immunology and Medical Microbiology

The editors of *FEMS Immunology and Medical Microbiology* aim to publish outstanding primary Research Articles and MiniReviews reporting on hypothesis-driven studies relating to infection, infection control and their molecular and

cellular correlates. The infection typically involves that of humans or animals by microorganisms of all classes, i.e. viruses, bacteria, fungi or protozoa. The scientific approaches of these studies correspond broadly to the fields of immunology, medical microbiology, cell biology (of infectious diseases), and the biochemistry, molecular biology and genetics of pathogens. These include prominently the overlapping subspecialties of molecular and cellular microbial pathogenesis, host innate and adaptive immune responses to infection, '-omics' of pathogens and/or of the infected host, and modelling of the infection or disease (from biomathematical to in vitro to animal modelling). The Journal will also consider outstanding vaccine-related studies and molecular diagnostic and epidemiology studies that are focused on the infectious agent or the infection process.

FEMS Yeast Research

The Editors aim to ensure efficient publication of high-quality papers that are original and provide a significant contribution to the field of yeast research. The journal contains Research Articles and MiniReviews on fundamental and applied aspects of all areas of yeast research, including yeast physiology, biochemistry, molecular biology, genetics, functional genomics, taxonomy, ecology, medical aspects, diagnostics, food spoilage, industrial applications, fermentation and biotechnology. Scientists using yeast as a model organism are welcome to submit their manuscript, particularly if this article has direct relevance to the yeast community. Papers can deal with any yeast or yeast-like organism. Descriptions of new yeasts will be considered.

MINIREVIEWS (not applicable to *FEMS Microbiology Reviews*)

MiniReviews are concise articles covering topics of current interest or controversial aspects of subjects within the scope of the journal. The style for MiniReviews is the same as for research papers or research letters with the following amendments: the maximum length of the text is about 7000 words (and for MiniReviews in *FEMS Microbiology Letters* about 3500 words); a combined total of six figures and tables is allowed. Colour figures or diagrams are encouraged and will be printed free of charge providing the Editor agrees that the use of colour adds value to the MiniReview. There is no rigid format for MiniReviews but they should generally include an Abstract and a brief Introduction in which the background to the article is presented. The remainder of the text should be arranged under a single, or a maximum two levels of subheading, finishing with a Conclusion or Outlook section.

MiniReviews are normally invited, but prospective authors are encouraged to contact the listed Editors to discuss possible contributions:

a) For *FEMS Microbiology Letters*: Ian Henderson, Simon Silver or Derek Sullivan.

b) For *FEMS Microbiology Ecology*, *FEMS Immunology and Medical Microbiology* and *FEMS Yeast Research*: the Chief Editors.

LETTERS TO THE EDITOR AND SHORT COMMUNICATIONS

Letters to the Editor are brief communications focusing on an article that has been published in the journal within the previous six months. They should focus on some aspect(s) of the paper that is, in the author's opinion, incorrectly stated or interpreted, controversial, misleading or in some other way worthy of comment. All Letters to the Editor must address a scientific issue in an objective fashion, should be fewer than 1000 words, and will be externally refereed. If acceptable for publication, they will be offered to the original authors for comment. Short Communications (not applicable to *FEMS Microbiology Letters*) are similar to a short paper but without the limitations of subdivisions into Introductions, Methods, etc. They should include the title page and the abstract and not exceed 1600 words. References should be kept to a minimum, one table or illustration is acceptable. Please choose the manuscript type 'Letter to the Editor' or 'Other' when uploading through the online submission system.

SUBMISSION PROCEDURES

All FEMS journals

Manuscripts should be submitted through Manuscript Central® http://mc.manuscriptcentral.com/fems. Instructions for the submission procedure can be found under 'FEMS Submission Instructions', reached via the 'Instructions and Forms' button at the top right of all Manuscript Central pages.

FEMS Microbiology Reviews

Manuscripts reach *FEMS Microbiology Reviews* by one of the following routes. Reviews may be solicited from international leading investigators by one of the Editors; alternatively proposals for reviews may be sent to the Chief Editor or one of the Editors with appropriate interests. Editors' contact details and fields of interest are listed in each issue. Authors are encouraged to contact Editors directly by e-mail.

Such proposals should contain:

a) an outline (1-3 pages);

b) a short statement describing the aim, scope and relevance of the review, and an indication of why the review is timely;

c) information on whether there has been any review covering this or a related field in the past few years, and, if so, the specific importance of the proposed review;

d) a statement as to when the completed review might be expected;

e) full contact details of four experts in the field who are familiar with the topic;

f) a list of recent key references showing the contributions to the field made by the author(s).

The proposals will be evaluated and authors may be invited to submit the review, if the material is satisfactory and of general interest.

Revision

Manuscripts may be returned to authors for modification of the scientific content and/or for shortening and language corrections. Revised versions must be submitted online through Manuscript Central. You will need to go to the list ''Manuscripts with Decisions'' (under My Manuscripts) and from there you need to click ''create a revision'' at the right-hand side (under Actions). At this stage, we require a source file of your text and tables (.doc or .rtf format, but not .pdf). You must clearly indicate in the designated place and/or cover letter the changes that have been made. Figures should be uploaded in separate files and at sufficient resolution (see section on Preparation of data). All obsolete files of the previous version should be deleted from the revised submission. If a paper that is returned to the authors for amendment is not resubmitted in revised form within one month (*FEMS Microbiology Letters*) or two months (other journals) it will be regarded as withdrawn. Any revised version received subsequently will be treated as a newly submitted manuscript and the date of receipt will be altered accordingly.

PREPARATION OF MANUSCRIPTS

Language

Manuscripts should be in English (consistent with either British or American spelling). Authors who are unsure of correct English usage should have their manuscripts checked by someone proficient in the language. You are strongly advised to ensure that the English is of a publishable standard prior to submission. Manuscripts that are deficient in this respect may be returned to the author without peer review.

Pre-submission English-language editing

Authors for whom English is a second language may choose to have their manuscript professionally edited before submission to improve the English. A list of independent suppliers of editing services can be found at http://www.blackwellpublishing.com/bauthor/english_language.asp. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

Layout of manuscripts

FEMS strongly recommends that you compile your manuscript in MS Word and save it as a .doc file, using the following layout.

a) Title page, followed by the abstract, main text in one single column and references.

b) Tables, each on a separate page.

c) Figure legends.

d) Figures, putting each figure on a separate page and ensuring that the figure is at least the size it will be in the printed document. Include the figure number (e.g. Fig. 1) and legend well outside the boundary of the space occupied by the figure. If you wish to upload separate figure files, Manuscript Central will combine your manuscript main body and figure files into one online .pdf file. Please ensure that you upload the figures only once, i.e. either embedded at the end of the text document or as separate files.

e) Include page and line numbering (continuous).

f) The right-hand margin justification should be switched off. Artificial word breaks at the end of lines must be avoided.

g) If you do not use MS Word then save in MS Word format in the word processor that you use. Rich text (.rtf) format may also be used.

h) Use standard fonts (Arial, Times New Roman, Symbol, Helvetica, Times). In your Word document, on the Tools menu, click Options, select the Embed TrueType fonts check box and then click the Save tab.

Length

One journal page is about three manuscript pages, each table is about 0.3 of a printed page and each figure is about 0.25 of a printed page.

FEMS Microbiology Letters

Priority will be given to short papers. The majority of papers will occupy only four to six pages of the journal. The text (including abstract but excluding the title page, references in text and as list, and figure legends) should not exceed 3000 words. References should be kept to a minimum and a combined total of six figures and tables are permitted. If the paper exceeds these guidelines, the manuscript will be returned for condensation without review unless the authors have provided compelling reasons for the exceptional length.

FEMS Microbiology Reviews

The length of the review should be at least eight pages upon publication in the journal. High quality colour figures, diagrams or photographs are encouraged and will be printed free of charge providing the Editor agrees that the use of colour adds value to the Review.

Other FEMS journals

There is no maximum length for papers, but the length should be justified by the content and authors are urged to be concise. Excessively long reference lists should be avoided. Repetition of information in the text and illustrations should not occur. Very short papers (Short Communications) may be published in these journals only if they offer a significant, though small, increase in knowledge or understanding of the field.

Title, authors, keywords and running title

The manuscript should not form part of a numbered series but should be headed by a concise, informative title. Authors are reminded that titles are widely used in information-retrieval systems. The title should be followed by the name(s) of the author(s) (with first or middle names in full and including all initials) and by the name(s) and address(es) of the institute(s) where the work was performed. For multiple authors with different affiliations, please indicate the relevant affiliations. The name, full postal address, telephone and fax numbers, and e-mail address of one corresponding author should be provided in a footnote. FEMS journals have one corresponding author. Shared or equal contribution of authors should be mentioned at the end of the article. A list of three to six keywords must be included on the first page. Authors are requested to consult the subject indices of the individual journals or the list of subject headings from Index Medicus for preferred synonyms and standard abbreviations. Plural terms should be avoided. The title is not used for preparing the index, so important words and phrases may appear in both the title and keywords. General terms, such as 'Enzyme', 'Membrane', 'Transport', etc., should not be used unless qualified, e.g. 'Enzyme activation', 'Membrane phosphorylation', 'Ion transport'.

Please supply a short running title of up to 60 characters (including spaces).

General organisation of manuscripts

Materials and methods and *Results* are normally written in the past tense and the present tense is occasionally used in the *Introduction* and *Discussion*.

a) *Abstract*. This should be a single paragraph of fewer than 200 words and must be intelligible without reference to the full paper. Ideally, references are not cited.

b) Abbreviations should be avoided, but if necessary, they must be defined the first time they are used in the main text. Do not abbreviate genus in the title, keywords, or at first use in the Abstract and Introduction.

c) *Introduction*. This should state the aims and objectives, but should not contain a summary of the results.

d) *Materials and methods*. Sufficient detail must be provided to allow the work to be repeated. Suppliers of materials and a brief address should be mentioned if this might affect the results.

e) *Results* (the presentation of data is described below).

f) *Discussion*. This should not simply recapitulate the *Results*. Combined *Results and Discussion* sections are encouraged when appropriate.

g) *Acknowledgements* can be made to funding agencies, colleagues who assisted with the work or the preparation of the manuscript, and those who contributed materials or provided unpublished data.

h) *References*.

FEMS Microbiology Reviews

The review should contain the items listed above, excepting that the *Materials and methods* and *Results* sections will not be relevant. The *Discussion* section is preferably replaced by *Concluding remarks*, which do not repeat the *Introduction* or main sections but may, for example, point to future directions.

Preparation of Supporting Information

Electronic Supporting Information may be provided to support and enhance your manuscript with, e.g. supporting applications, movies, animation sequences, high-resolution images, background datasets or sound clips. Supporting files will be published, subject to editorial approval, online alongside the electronic version of your article. Authors should submit the Supporting Information at the same time as the manuscript, but in separate file(s). Select 'Supplemental files', 'Supporting Document' or 'MultiMedia' for the file designation when uploading through the online submission system. Upload a separate .doc file listing concise and descriptive captions for each file uploaded as Supporting Information. Please indicate that you have uploaded these files in your cover letter and state clearly whether they are intended for eventual online publication as Supporting Information, or are for peer review purposes only.

Presentation of data

Do not tabulate or illustrate points that can be adequately and concisely described in the text. Do not repeat information in both tables and figures. Figures and tables, along with their legend (and/or footnote), should be understandable in their own right without having to refer to the main text. Tables should be supplied in Word or Excel format, and must be editable (not pasted in as a picture).

(a) *Tables*. Explanatory footnotes should be related to the legend or table using superscript, lower-case letters. All abbreviations should be defined after the footnotes below the table.

(b) *Line art*.

- Figures should be supplied at twice their final size with wide margins.

- A single column figure is 80 mm, two-thirds page width is 114mm and two-column width is 168 mm.

- All lines should be drawn at 1.5 point (0.5 mm wide), broken line styles may be used to differentiate multiple plot lines if desired.

- Letters and numbers should be 16 point (capitals 4mm high) non-serif.

- Symbols in the figure itself should be 3mm in diameter. Lines drawn to accompany the points should not go through hollow symbols.

- Grid lines should not be used.

- Numbers used as axis labels should have minimum significant figures; amounts less than unity must carry a preceding zero (e.g. 0.5 not .5).

- Larger composite figures may be designed to occupy two columns when this can achieve an overall saving in space. The character, line and symbol sizes should be adjusted accordingly to achieve the same sizes on the printed page.

(c) *Half-tone and colour figures*. Magnification should be indicated where appropriate by inclusion of a bar marker. Photographs of electropherograms, etc., in which there is poor contrast may be better replaced by line drawings, but in this case the photographs should be submitted for scrutiny by the Editor. If photographs have been digitally processed to enhance their quality, this should be stated. **Colour illustrations will be published free of charge provided that the colour is deemed essential for interpretation of the figure.** Please note that colour figures will appear in colour in the online article, regardless of whether colour was deemed essential for the print copy. Suggestions for cover illustrations for the journal are also welcome.

(d) *Electronic submission of figures.* High-quality figures are required when the final version of the manuscript is uploaded through Manuscript Central, and should be prepared using the following guidelines.

- Please use high-quality graphics programs such as Adobe Photoshop or Adobe Illustrator.

- Figures should be at the desired size for the printed article, i.e. 80mm wide for single column, 114mm for two-thirds page width and 168mm for double column.

- For halftones, the resolution should be a minimum of 300 dpi; combination artwork at a minimum of 500 dpi; and for line figures preferably 1000 to 1200 dpi.

- Combination artwork (artwork containing half-tone and line art elements, e.g. electrophoresis gels or Southern blots with lane and fragment sizes labeled) must be in EPS. If the combination artwork is scanned, the preferred format is .tif with a resolution of at least 500 dpi using LZW compression.

- Colour artwork should be saved as CMYK, not RGB.

- The following figure formats are acceptable (as long as they are created with the instructions given above):

- .tif and .eps (please be sure to embed all fonts used)

- Illustrator (.ai) or Photoshop files (.psd)

- One file must be submitted for each figure.

- Save files with LZW compression.

You will find further helpful and simplified guidelines by clicking the 'Preferred FEMS format for revised manuscripts' icon, reached via the 'Instructions and Forms' button at the top right of all Manuscript Central pages. Detailed information can be found at http://www.blackwellpublishing.com/bauthor/illustration.asp. In principle, the electronic files will be used for producing the final publication, but the Publisher may request a set of highquality printouts of your figures for production purposes.

(e) *Figure legends.* Legends should consist of a preliminary sentence constituting a title, followed by a brief description of the way the particular experiment was carried out, and any other necessary description of symbols or lines. All abbreviations must be defined.

Reproducibility of results and statistical tests (except for *FEMS Microbiology Reviews*)

Authors should state how many times experiments were repeated and whether mean or representative results are shown. Variability should be indicated statistically wherever possible as part of, but not in place of, a proper statistical analysis. If results are expressed as percentages, the absolute value corresponding to 100% must be stated. Avoid values with unjustified numbers of significant figures; in most cases three significant figures is consistent with the accuracy attained in microbiological experiments.

Results of statistical tests should be presented wherever possible as evidence for conclusions reached. Such information must be presented concisely to illuminate the results, but not to dominate them. The tests used should be briefly described in the Materials and methods section. Details of the diagnostic checks made for the assumptions of the statistical tests and for the validity of any transformations used should be stated clearly. Further information can be found in the following references: (a) Sokal, R.R. and Rohlf, F.J. (1981) *Biometry*. W.H. Freeman, San Francisco; (b) Fry, J.C. (1993) *Biological Data Analysis: A Practical Approach*. IRL Press, Oxford.

Nomenclature, abbreviations and units

Authors should follow internationally accepted rules and conventions. Authors should provide evidence for the thorough identification of new isolates and use the most recent acceptable name.

Prokaryotes. The spelling of bacterial names should follow the list of Prokaryotic Names with Standing in Nomenclature http://www.bacterio.cict.fr/. If there is a reason to use a name that does not have standing in nomenclature, the name should be printed in roman type and enclosed in quotation marks and an appropriate statement concerning the nomenclatural status of the name should be made in the text (for an example, see Int. J. Syst. Bacteriol. (1980) 30: 547-556).

Fungi. The authors should use recently accepted binomials controlled by the International Code of Botanical Nomenclature (http://www.bgbm.fu-berlin.de/iapt/nomenclature/code/SaintLouis/0000St.Luistitle.htm). Scientific names of yeasts can be found in: *The Yeasts: a Taxonomic Study*, 4th ed. (C. P. Kurtzman and J.W. Fell, ed., Elsevier B.V., Amsterdam, The Netherlands, 1998). Taxonomic texts should cite nomenclatural authorities at the first time a name is mentioned. For abbreviation of authors' names, see http://www.indexfungorum.org/AuthorsOfFungalNames.htm. All bacterial taxa should be italicized.

Viruses. Names used for viruses should be those approved by the International Committee on Taxonomy of Viruses (ICTV) http://www.ncbi.nlm.nih.gov/ICTVdb/. If desired, synonyms may be added parenthetically when the name is first mentioned. Approved generic (or group) and family names may also be used.

Enzymes. For enzymes, use the recommended (trivial) name assigned by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology as described in Enzyme Nomenclature http://www.chem.qmw.ac.uk/iubmb/ enzyme/.

Genes. Genetic nomenclature should essentially follow the recommendations of Demerec *et al*. (*Genetics* (1966) 54: 61-76), and those given in the instructions to authors of the *Journal of Bacteriology* and *Molecular and Cellular Biology* (January issues). Biochemical compounds. Consult the *European Journal of Biochemistry* or the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (http://www.chem.qmw.ac.uk/iubmb/).

Abbreviations. Abbreviations should only be used as an aid to the reader and their use should be strictly limited. Define each abbreviation and introduce it in parentheses the first time it is used: e.g. 'cultures were grown in Eagle minimal essential medium (MEM)'. Eliminate abbreviations that are not used at least six times in the manuscript. In addition to

abbreviations to the international system of units of measurements, other common units (e.g., bp, kb, Da), chemical symbols for the elements, and the standard biochemical abbreviations (see *Eur. J. Biochem*.) should be used without definition. Standard chemical symbols and trivial names or their symbols (folate, Ala, Leu, etc.) may be used for terms that appear in full in the neighbouring text. Abbreviations other than those recommended by the IUPAC-IUB (Biochemical Nomenclature and related Documents, 1978) should be used only when a case can be made for necessity, such as in tables and figures. A short guide on the use of common abbreviations can be found on the Author page on the FEMS website (http://www.fems-microbiology.org/website/nl/page125.asp).

Reporting numerical data. The international system of units (SI) should be used; mL is acceptable in place of cm^3 for liquid measures. The form for units is mg mL^{-1} and not mg/mL, parentheses should be used to improve clarity, e.g. mL $(g\ drywt\ soil)^{-1}\ h^{-1}$. The prefixes k, m, m, n, and p should be used in combination with the standard units for reporting length, weight, volume and molarity for 10^3, 10^{-6}, 10^{-9}, and 10^{-12}, respectively. Use mg mL^{-1} or mg g^{-1} instead of the ambiguous ppm. Units of temperature are presented as follows: 37 1C or 324 K.

References

Reference citations in the text follow the name and date system. References should be inserted in parentheses in date order, as follows: (Brown, 1996; Brown & Smith, 1997; Smith et al., 1998). The reference list itself must be in alphabetical order according to the first-named author, then by number of authors, then chronologically within the one-author group, alphabetically within the two-author group and chronologically within the three or more author group. The title of the article must be included. For papers with ten or fewer authors, all authors must be listed. For papers with eleven or more authors, the first three names should be listed, followed by 'et al.'. Standard abbreviations of journal titles should be used, as in the Index Medicus. The following formats should be followed:

O'Donnell CM & Edwards C (1992) Nitrosating activity in *Escherichia coli. FEMS Microbiol Lett* 95: 87-94.

Dinter Z & van Morein B (1990) *Virus Infections in Ruminants.* Elsevier, Amsterdam.

McCarthy AJ (1989) Thermomonospora. *Bergey's Manual of Systematic Bacteriology, Vol. 4* (Williams ST, Sharpe ME & Holt JG, eds), pp. 2552-2572. Williams and Wilkins, Baltimore, MD.

Tang CR (2001) Cloning of a new ice nucleation active gene for insect pest control. PhD Thesis, Chinese Academy of Agricultural Sciences, Beijing.

Reference should not be made to work 'in press' unless it has been accepted for publication; a DOI number should then be provided. Unpublished results and personal communications may be mentioned within the text itself provided that (a) the names and initials of all the persons involved are listed, and (b) they have all granted permission for the citation. In the case of an online journal publication the DOI number of the reference should be used.

Nucleotide and amino acid sequences

Any new nucleotide or amino acid sequences must be deposited in an appropriate data bank. Authors are encouraged to use the EMBL Data Library but can also use other archives, such as GenBank. **An accession number must be obtained before submission to the Editors and this fact should be mentioned in the covering letter.** Authors should include the accession number in the appropriate figure legend. Authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources, should type this information in **bold, underlined text**. Letters in the accession number should always be capitalised (e.g. GenBank accession nos. **AI631510, AI631511, AI632198** and **BF223228**). Authors are advised to check accession numbers very carefully. **An error in a letter or number can result in a dead link.** In the final version of the printed article, the accession number text will not appear bold or underlined. In the final version of the electronic copy, the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article.

COPYRIGHT AND ONLINEOPEN

Upon acceptance of an article, the corresponding author should sign either a Copyright Assignment formor OnlineOpen form. OnlineOpen is a pay-to-publish service from Wiley-Blackwell that offers authors whose papers are accepted for publication the opportunity to pay up-front for their manuscript to become open access (i.e. free for all to view and download) via the Wiley InterScience website. Each OnlineOpen article will be subject to a one-off fee to be met by or on behalf of the Author in advance of publication. Upon online publication, the article (both full-text and PDF versions) will be available to all for viewing and download free of charge. The print version of the article will also be branded as OnlineOpen and will draw attention to the fact that the paper can be downloaded for free via the Wiley InterScience service. Any authors wishing to use the OnlineOpen service will be required to complete the relevant combined payment and copyright licence form available from the links at the end of these notes.

Once complete the Copyright assignment form or OnlineOpen form should be sent to the Editorial Office along with the rest of the manuscript materials as soon as possible after the acceptance. Prior to acceptance there is no requirement to inform an Editorial Office that you intend to publish your paper OnlineOpen.

The copyright statement for OnlineOpen authors will read:

©[date] The Author(s) Journal compilation

PROOFS AND AUTHOR SERVICES

Author Services enables authors to track their article - once it has been accepted - through the production process to publication online and in print. Authors can check the status of their articles online and choose to receive automated e-mails at key stages of production. The author will receive an e-mail with a unique link that enables them to register and have their article automatically added to the system. Please ensure that a complete e-mail address is provided when submitting the manuscript. Visit http://www.blackwellpublishing.com/bauthor for more details on online production tracking and for a wealth of resources including FAQs and tips on article preparation, submission and more.

When proofs have been produced, the corresponding author will receive an e-mail alert from the Publisher containing a link to a web site. It is therefore essential that the e-mail address of the corresponding author is working and current. The proof can be downloaded as a PDF file from this site Acrobat Reader will be required in order to read this file. This software can be downloaded (free of charge) from the following web site: http://www.adobe.com/products/acrobat/readstep2.html. This will enable the file to be opened, read on screen or printed out. Further instructions will be sent with the proof regarding how to indicate and communicate any changes to the Publisher. Hard copy proofs will be posted if no e-mail address is available. Queries will be addressed to the corresponding author. Excessive changes made by the author in the proofs, excluding typesetting errors, may be charged for separately. The Editors reserve the right to make minor alterations to the text without altering the scientific content.

For *FEMS Microbiology Letters*, the Publisher reserves the right to proceed with publication if corrections are not communicated within three days.

OFFPRINTS

A PDF offprint of the online published article will be provided free of charge to the corresponding author, and may be distributed subject to the Publisher's terms and conditions. Paper offprints of the printed published article may be purchased if ordered via the method stipulated on the instructions that will accompany the proofs. Printed offprints are posted to the correspondence address given for the paper unless a different address is specified when ordered. Note that it is not uncommon for printed offprints to take up to eight weeks to arrive after publication of the journal.

ETHICAL AND RELATED ASPECTS

The Editors expect that new and variant organisms, viruses and vectors described in FEMS journals will be made available, under written request and for their own use, to all qualified members of the scientific community. If delays in strain or vector distribution are anticipated or if they are available from sources other than the authors, this should be indicated. The Editors encourage authors to deposit important strains in publicly accessible culture collections and to refer to the collections and strain numbers in the text. In the case of materials that have been distributed by individuals, authors should indicate the laboratory strain designations and name and address of the donor as well as the original culture collection identification number, if any.

Papers describing experimental work with humans must include a statement that the Ethical Committee of the institution in which the work was done has approved it, and that the subjects gave informed consent to the work. Experiments with animals or with genetically manipulated organisms must have been undertaken in accordance with the legal requirements of the relevant local or national authority. Procedures must be such that experimental animals do not suffer unnecessarily. Submission of a manuscript implies that the work described has not been published before (except in the form of an abstract or as part of a published lecture, review or academic thesis, in which case reference should be made in a footnote to the title) and that it is not under consideration for publication elsewhere. **The corresponding author must ensure that its publication has been approved by all co-authors and tacitly or explicitly by the responsible authorities in the laboratories where the work was carried out and that all persons entitled to authorship have been named.** If accepted, the article must not be published elsewhere in the same form in either the same or another language, without the consent of the Editor and Publisher. Each named author must be responsible for at least the part describing his or her contribution and must have seen the entire final text before submission and any substantial subsequent revisions. Authorship should be dealt with carefully and all authors should agree with it at the submission stage. FEMS journals do not permit adding or removing of authors or rearranging their order after acceptance. The Editors must be notified in writing by the corresponding author of any deviation from these rules. Should any author become aware of a breach of ethics he/she should contact the Chief Editor of the journal who will endeavour to retract the article. Articles published in FEMS journals represent the scientific findings and opinions of the authors.Whilst the Editors and Publisher make every effort to ensure the accuracy of all published matter, they can accept no responsibility or liability, collectively or individually, for any erroneous, misleading or unintentionally damaging statements, which may appear in the journal. Authors must draw attention in the Materials and methods to any chemical or biological hazards that may be involved in the experiments described.

PERMISSION TO REPRODUCE MATERIAL

Individuals wishing to reproduce material (not exceeding 250 words of text) from articles published in FEMS journals for non-commercial purposes may do so providing the original publication is acknowledged accordingly and the authors' approval is obtained, and in this case no special permission is needed from the Publisher. Authors may also include the article in a thesis without special permission. In all other cases, permissions may be sought directly from the Journal Rights Department: e-mail journalsrights@wiley.com.

QUICK LINKS

- Manuscript submission: http://mc.manuscriptcentral.com/fems; and instructions can be reached via the 'Instructions and Forms' button at the top right of all Manuscript Central pages
- FEMS journals: http://www.fems-microbiology.org
- Editorial Office: e-mail admin.journals@fems-microbiology.org
- Production Office: e-mail fems@wiley.com
- Electronic Graphics: http://www.blackwellpublishing.com/bauthor/illustration.asp
- Copyright assignment forms (CAF) and OnlineOpen forms (OOF):