UNIVERSIDADE ESTADUAL PAULISTA

"JÚLIO DE MESQUITA FILHO"

FACULDADE DE MEDICINA VETERINÁRIA

CÂMPUS DE ARAÇATUBA

TLR 2, TLR 4, ROS, ÓXIDO NÍTRICO, P38 E IKK EM CÉLULAS MONONUCLEARES DE CÃES COM LEISHMANIOSE VISCERAL TRATADAS COM O IMUNOMODULADOR P-MAPA

Larissa Martins Melo

Enfermeira

ARAÇATUBA – SP

2014

UNIVERSIDADE ESTADUAL PAULISTA

"JÚLIO DE MESQUITA FILHO"

FACULDADE DE MEDICINA VETERINÁRIA

CÂMPUS DE ARAÇATUBA

TLR 2, TLR 4, ROS, ÓXIDO NÍTRICO, P38 E IKK EM CÉLULAS MONONUCLEARES DE CÃES COM LEISHMANIOSE VISCERAL TRATADAS COM O IMUNOMODULADOR P-MAPA

Larissa Martins Melo

Orientadora: Profª. Adj. Valéria Marçal Felix de Lima

Dissertação apresentada à Faculdade de Medicina Veterinária – Unesp, Campus de Araçatuba, como parte das exigências para a obtenção do título de Mestre em Ciência Animal (Área de Medicina Veterinária Preventiva e Produção Animal).

ARAÇATUBA – SP

2014

Dados Internacionais de Catalogação na Publicação (CIP)
Ficha catalográfica elaborada pela Biblioteca da FMVA / UNESP

Melo, Larissa Martins
M528t TLR 2, TLR 4, ROS, óxido nítrico, P38 e IKK em células mononucleares de cães com leishmaniose visceral tratadas com o imunomodulador P-MAPA / Larissa Martins Melo. -- Araçatuba : [s.n.], 2014.
72 f. : il. + 1 CD-ROM

Dissertação (Mestrado) – Universidade Estadual Paulista, Faculdade de Medicina Veterinária de Araçatuba.
Orientador: Profa. Dra. Valéria Marçal Felix de Lima

1. Leishmania sp. 2. P-MAPA. 3. Cão. 4. Fatores imunológicos. 5. TLR. I. Título.

CDD 636.70896

unesp

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba
Seção Técnica de Graduação e Pós-Graduação

CERTIFICADO DE APROVAÇÃO

TÍTULO: TLR 2, TLR 4, ROS, óxido nítrico, p38 e IKK em células mononucleares de cães com leishmaniose visceral tratadas com o imunomodulador P-MAPA.

AUTORA: LARISSA MARTINS MELO

ORIENTADORA: Dra. VALÉRIA MARÇAL FELIX DE LIMA

Aprovada como parte das exigências para obtenção do Título de MESTRA em CIÊNCIA ANIMAL (MEDICINA VETERINÁRIA PREVENTIVA E PRODUÇÃO ANIMAL) pela Comissão Examinadora.

Dra. APARECIDA DE FATIMA MICHELIN

Dra. JULIANA REGINA PEIRÓ

Dra. VALÉRIA MARÇAL FELIX DE LIMA

DATA DA REALIZAÇÃO: 19 de setembro de 2014.

Presidente da Comissão Examinadora
Dra. VALÉRIA MARÇAL FELIX DE LIMA
- Orientadora -

DADOS CURRICULARES DO AUTOR

LARISSA MARTINS MELO – Araçatuba, 30 de maio de 1988, formada em enfermagem no ano de 2009 pelo Unisalesiano de Araçatuba. Fiz iniciação científica durante a graduação, participei de vários congressos apresentando trabalhos. Publiquei o trabalho de conclusão de curso em uma revista cientifica. Iniciei o mestrado em 2013 no programa de Ciência Animal com foco em Imunologia. Trabalhei com o imunomodulador P-MAPA no tratamento de leishmaniose canina, participei de congressos para apresentação dos resultados. Nesse período publiquei quatro artigos sendo um como primeira autora e os demais como colaboradora.

"Porque sou eu que conheço os planos que tenho para vocês', diz o Senhor, 'planos de fazê-los prosperar e não de causar dano, planos de dar a vocês esperança e um futuro."

Jeremias 29:11

AGRADECIMENTOS

Agradeço a minha família e aos meus amigos, que me ajudaram nos momentos difíceis e que sabem importância desse trabalho na minha vida. Amo vocês!!!

A minha orientadora Valéria Marçal Félix de Lima que me propiciou essa oportunidade, compartilhando momentos de aprendizados, alegrias, festas, viagens e tristezas, me incentivando, me ouvindo nas horas das dúvidas, me fazendo ter empolgação pelo trabalho, mesmo após algum tropeço. Obrigada por tudo!

A Juliana Perosso, uma amiga, que sempre me ajudou, no laboratório, escutando desabafos e sempre me incentivou. Você é demais!!

A Leticia Sanches, amiga de faculdade e mestrado, inteligente, dedicada, sempre sendo companheira e amiga. Te adoro!!

Aos meus eternos amigos Mariana Macedo e Marcos Somenzari, obrigada por me proporcionarem adoráveis momentos, viagens e risadas.

Aos colegas do Laboratório de Imunologia, sempre companheiros e inteligentes, principalmente, o meu amigo querido Breno Fernando, companheiro, que me ensinou muito e sempre disposto a me ajudar.

A turma do Laboratório Clínico pela ajuda e companheirismo. Aos colegas: Guilherme Melo, Alex Nakamura, Augusto Schweigert, Milena Sato, Camila Homem que sempre me ajudaram e foram companheiros.

A FARMABRASILIS por fornecer o imunomodulaor P-MAPA e ao Dr Odilon Nunes por ter desenvolvido este imunomodulador.

A minha banca de qualificação, Profª Juliana Peiró e Profª Kátia Bresciani que me servem de exemplo profissional pela dedicação e comprometimento, obrigada pelas considerações dadas ao meu trabalho.

Agradeço a todos os professores que de alguma forma me ajudaram e me deram apoio nessa minha jornada. Obrigada!

Ao programa de Pós-graduação em Ciência Animal e a Faculdade de Medicina Veterinária Campus de Araçatuba pela oportunidade, por toda a infraestrutura oferecida e por ser tão bem recebida por todos.

SUMÁRIO

TLR 2, TLR 4, ROS, ÓXIDO NÍTRICO, P38 E IKK EM CÉLULAS MONONUCLEARES DE CÃES COM LEISHMANIOSE VISCERAL TRATADAS COM O IMUNOMODULADOR P-MAPA

RESUMO – A Leishmaniose visceral (LV) no Brasil representa um grave problema de saúde pública; portanto, é importante estudar novas alternativas para o tratamento de cães infectados. O imunomodulador agregado de magnésio-amônio fosfolinoleato-palmitoleato anidrido proteína (P-MAPA) melhora a imunocompetência quando o sistema imunológico está comprometido, mas sua dependência de receptores Toll-like (TLRs) e os mecanismos envolvidos na resposta imune ainda não estão claros. Estudou-se a ação *in vitro* de P-MAPA sobre a expressão de TLR2 e TLR4, espécies reativas de oxigénio (ROS), óxido nítrico (NO) e p38 ativada por mitógeno proteína quinase (p38MAPK) e fosforilação de IKK em células mononucleares do sangue periférico (PBMC) e macrófagos de cães saudáveis e infectados. O PBMC ou macrófagos foram isolados e cultivados com diferentes concentrações de P-MAPA (20,100 e 200 µg/mL) em uma atmosfera húmida, a 37°C com 5% de CO_2. A observação revelou que nos macrófagos de cães infectados com *Leishmania* sp. mostraram uma diminuição em TLR2 em comparação com cães saudáveis e em indução com P-MAPA. ROS foram aumentados em PBMC de cães infectados com *Leishmania* sp. em comparação com cães saudáveis e P-MAPA melhorou a produção de ROS. A produção de NO foi aumentada no sobrenadante da cultura a partir de macrófagos estimulados por P-MAPA em ambos os grupos de cães saudáveis e infectados com *Leishmania* sp. O tratamento de macrófagos de cães saudáveis com imunomodulador P-MAPA induziu p38 MAPK e fosforilação de IKK, sugerindo a transdução de sinal por esta via. Estas descobertas sugerem que a P-MAPA tem potencial como uma droga terapêutica no tratamento de leishmaniose visceral canina.

Palavras-chave: Leishmania sp., P-MAPA, cão, imunomodulador, TLR.

TLR 2, TLR 4, ROS, NITRIC OXIDE, AND P38 IKK CELL MONONUCLEAR LEISHMANIASIS VISCERAL DOGS TREATED WITH IMMUNOMODULATOR P-MAPA

SUMMARY - Visceral leishmaniasis (VL) in Brazil represents a serious public health problem; therefore, it is important to study new alternatives to treat infected dogs. The immunomodulator protein aggregate magnesium-ammonium phospholinoleate-palmitoleate anhydride (P-MAPA) improves immunocompetence when the immune system is impaired, but its dependence on Toll-like receptors (TLRs) and the mechanisms involved in the immune response remain unclear. The *in vitro* action of P-MAPA on the expression of TLR2 and TLR4, reactive oxygen species (ROS), nitric oxide (NO) and p38 mitogen-activated protein kinase (p38MAPK) and IKK phosphorylation were studied in mononuclear cells from peripheral blood and macrophages from healthy and *Leishmania*-infected dogs. The PBMC or macrophages were isolated and cultured with different concentrations of P-MAPA (20,100 and 200 µg/mL) in a humid environment at 37°C with 5% CO_2. Observation revealed that *Leishmania*-infected dogs showed a decrease in TLR2 in macrophages compared with healthy dogs and in induction with P-MAPA. ROS were increased in PBMCs from *Leishmania* sp.-infected dogs compared with healthy dogs and P-MAPA improved ROS production. NO production was increased in culture supernatant from macrophages stimulated by P-MAPA in both healthy and *Leishmania*-infected dogs. Treatment of macrophages from healthy dogs with immunomodulatory P-MAPA induced p38 MAPK and IKK phosphorylation, suggesting signal transduction by this pathway. These findings suggest that P-MAPA has potential as a therapeutic drug in the treatment of canine visceral leishmaniasis.

Keywords: *Leishmania* sp., P-MAPA, dog, immunomodulatory, TLR.

Capítulo 1

CAPÍTULO 1 - LEISHMANIOSES

As leishmanioses estão relacionadas principalmente aos países subdesenvolvidos, existindo casos também em países em desenvolvimento e desenvolvidos. É uma das doenças mais negligenciadas do mundo, tendo estimativa que 350 milhões de pessoas estão em risco de contrair um dos tipos de leishmaniose e a perspectiva de ocorrência de novos casos é de 2 milhões/ano, e mais de 12 milhões estejam infectadas, um número subestimado pois só há notificação compulsória em 32 países. Dos 88 países afetados, 72 são classificados como países em desenvolvimento e 13 como países menos desenvolvidos, sendo que 90% dos casos de leishmaniose visceral (LV) ocorrem em Bangladesh, Índia, Nepal, Brasil e Sudão (WHO, 2010).

A Organização Mundial da Saúde (OMS) classificou as leishmanioses na categoria 1, doenças emergentes e sem controle, e as medidas de prevenção devem ser focadas no controle do vetor, dos animais reservatórios e na pesquisa de vacinas eficientes (ROBERTS, 2006). A expansão da urbanização, a imunossupressão e a desnutrição do indivíduo são fatores que ajudam na alta expansão e disseminação da doença pelas regiões do mundo (DESJEUX, 2004).

No Brasil, apesar das tentativas de controle, os casos da doença na forma visceral humana, nos últimos 20 anos, aumentaram e atingiram centros urbanos (BRASIL, 2006). O programa de controle brasileiro, nos últimos 40 anos, é composto da distribuição gratuita do tratamento específico nas unidades públicas de saúde, o controle de reservatórios domésticos, através da identificação e eutanásia dos cães positivos, e o controle de vetores (COSTA; VIERA, 2001). Em torno de 200 a 300 brasileiros morrem anualmente decorrente da LV (COSTA, 2008).

Leishmanioses são causadas por protozoários do gênero *Leishmania*, da ordem Kinetoplastida e família Trypanosomatidae, que pode ser dividido em dois subgêneros: *Leishmania* e *Viannia* (ALENCAR et al., 1991).

A transmissão ocorre através do flebotomíneo não infectado que adquire o parasito ao se alimentar do sangue de um mamífero infectado, através da ingestão de amastigotas livres ou intramacrofágicos no tecido subcutâneo (BASANO; CAMARGO, 2004; SILVA, 2008). As formas intracelulares são liberadas no trato digestivo do inseto, que se diferenciam para formas promastigotas procíclicas, multiplicando-se por divisão binária. Estas formas procíclicas diferenciam-se para formas infectantes metacíclicas e migram então para a probóscide do inseto, de onde poderão infectar um novo hospedeiro mamífero (SILVA, 2008).

A forma infectante para os hospedeiros vertebrados são as promastigotas metacíclicas, que se alojam no intestino do flebotomíneo fêmea. As promastigotas metacíclicas são transmitidas aos hospedeiros durante o repasto sanguíneo e se dirigem para os órgãos linfóides secundários, principalmente no fígado, baço, medula óssea e linfonodos, infectando células do sistema fagocítico mononuclear (SFM), como os monócitos, histiócitos e macrófagos, onde se transformam em amastigotas. As amastigotas se multiplicam por fissão binária até romperem a célula hospedeira, disseminam-se pelas vias hematogênica e linfática, iniciando uma reação inflamatória e proporcionando a atração de outros macrófagos (REY, 2001).

Dependendo da espécie de *Leishmania* e da resposta imune do hospedeiro, a infecção pode determinar diferentes formas clínicas, atingindo a pele e/ou a mucosa na leishmaniose tegumentar ou afetando órgãos internos ricos em células do SFM como o baço, o fígado e a medula óssea na leishmaniose visceral. As espécies que induzem à forma visceral pertencem ao subgênero *Leishmania* e incluem *Leishmania (Leishmania) donovani* e *L. (L.) infantum* no Velho Mundo e *L. (L.) chagasi* nas Américas (DESJEUX, 2004).

LEISHMANIOSE EM CÃES

Os cães, considerados os principais reservatórios da *Leishmania* sp. fora do ambiente silvestre, são importantes na manutenção do ciclo epidemiológico da doença, pois a LV é mais prevalente na população canina que na humana, sendo a infecção no homem normalmente precedida por casos caninos, que apresentam maior quantidade de parasitos na pele favorecendo a infecção dos vetores (SANTA ROSA; OLIVEIRA, 1997).

A leishmaniose canina é geralmente crônica, podendo ser assintomática ou caracterizada por um ou mais dos nove principais sinais clínicos, dos quais podem ser citados: hiporexia, emagrecimento, linfoadenopatia local ou generalizada, lesões cutâneas e oculares, epistaxe, claudicação, anemia, insuficiência renal e diarreia (SOLANO-GALLEGO et al., 2009). A evolução clínica pode ser grave e aguda, levando o animal a óbito em poucas semanas. Em alguns casos, a infecção pode ser latente, evoluindo para a cura espontânea (GENARO, 1993). Aproximadamente 50% de todos os animais soropositivos infectados não mostram nenhum sinal clínico da doença, porém são capazes de infectar os flebotomínios, tendo importante implicação epidemiológica (COURTENAY et al., 2002).

O evolução clínica da doença está relacionado a competência imunológica do hospedeiro para direcionar a resposta para o tipo humoral ou celular. De maneira geral, a população de linfócitos Th (CD4 +) divide-se em duas subpopulações: Th1 e Th2, as quais são definidas com base no seu padrão de citocinas (KOUTINAS; KOUTINAS, 2014). A subpopulação Th1 secreta IL-2 e IFN-γ induzindo, preferencialmente, a ativação de macrófagos e reposta mediada por células; enquanto que a subpopulação Th2 secreta, preferencialmente, IL-4, IL-5 e IL-10, favorecendo a resposta humoral (BARBOSA et al., 2011). O balanço entre estas citocinas determina o padrão de resposta imune. Na infecção por *Leishmania* sp., as células Th1 produzem citocinas que ativam os macrófagos. Esses quando ativados, por sua vez, estimulam a imunidade celular e dessa forma podem eliminar a infecção. Em contraste, quando a infecção está associada com a indução de linfócitos Th2, ocorre a proliferação de linfócitos B e a produção de anticorpos. Na leishmaniose visceral canina a intensa produção

de imunoglobulinas é deletéria e não protetora. Dessa forma, desenvolve-se a infecção. Os imunocomplexos na circulação podem ligar o complemento às células sanguíneas levando ao desenvolvimento de fenômenos patológicos, tais como, trombocitopenia e anemia imunomediadas (SLAPPENDEL, 1988).

A supressão de imunidade celular constitui o aspecto mais importante na patogênese e progressão da doença canina. Observa-se uma ausência da reposta linfoproliferativa *in vitro* a antígenos de *Leishmania* sp. e reação de hipersensibilidade do tipo retardado negativa para leishmanina (SACKS et al., 1987; CARVALHO et al., 1989). Em cães infectados com *Leishmania infantum*, observa-se uma redução no número de linfócitos T $CD4^+$, proliferação do parasita em macrófagos e sua disseminação para vários órgãos, incluindo estômago, intestino e pulmão (BOURDOISEAU et al., 1997, HERVÁS et al., 1996).

Em cães infectados com *Leishmania chagasi* sinais da supressão imunológica são a redução do número de linfócitos T no sangue periférico e do baço (LIMA et al., 2012) e a negatividade do teste intradérmico a antígenos do parasita (DOS-SANTOS et al., 2008). O desenvolvimento de drogas imunomodulatórias para o tratamento da leishmaniose canina está baseada principalmente na progressão da doença, uma vez que a supressão da imunidade celular constitui o aspecto mais importante. Devido à forte relação entre a supressão imunológica e o desenvolvimento da doença, a atividade imunomodulatória de várias substâncias vem sendo estudada no tratamento da leishmaniose.

A vacina FML (fucose-manose ligante) foi utilizada como imunomodulador em cães experimentalmente infectados com *L*. *donovani* e naturalmente infectados com *L*. *chagasi*, mostrando ser eficaz na imunoterapia contra a leishmaniose visceral em cães assintomáticos. As contagens de linfócitos T CD4 e CD21 em PBMC foram normais em cães submetidos à imunoterapia, sugerindo a sua condição de não infecciosas. Todos os cães apresentaram também aumentou significativo de percentuais de linfócitos CD8 (BORJA-CABRERA et al., 2004).

Outro estudo utilizando como imunomodulador a domperidona, um antagonista do receptor de dopamina D2, em cães naturalmente infectados por *Leishmania infantum* mostrou eficácia no controle da doença e redução dos sinais clínicos e anticorpos anti-*Leishmania*, obtendo negatividade em alguns cães, além de um aumento significativo na imunidade celular (GOMEZ-OCHOA et al., 2009).

O arsenal terapêutico utilizado no tratamento da leishmaniose canina (alopurinol, antimoniais, Anfotericina B, entre outras) consegue muitas vezes uma melhora clínica nos cães. Entretanto, não previne a recaída da doença, e muitas vezes não elimina o parasita dos cães infectados. Além disso, estas drogas utilizadas podem ter alta toxicidade (nefrotoxicidade, vômitos, diarreia entre outros sinais clínicos), custo elevado e podem ser ineficazes em alguns casos (BANETH; SHAW, 2002; ALVAR et al., 2004). Estes fatores indicam que novos medicamentos devem ser avaliados para o tratamento da leishmaniose em longo prazo para se avaliar seus benefícios terapêuticos e seus efeitos colaterais.

O composto extracelular purificado, isolado a partir de *Aspergillus oryzae,* denominado agregado polimérico de fosfolinoleato-palmitoleato de magnésio e amônio protéico (P-MAPA), tem potente ação imunomodulatória (JUSTO et al., 2003).

Em diversos modelos experimentais de tumores a administração de MAPA mostrou resultados promissores levando à regressão tumoral (DURAN et al., 1993; DURAN et al., 1999; FÁVARO, 2012).

Em camundongos portadores de tumor ascítico de Ehrlich, a administração de P-MAPA aumentou os níveis de IL-2, a produção de IFN-gama nas células do baço, e a atividade das células NK e redução dos níveis de IL-10 (JUSTO et al., 2003), sugerindo a estimulação da imunidade celular. Em camundongos infectados por *Listeria monocytogenes* a administração do P-MAPA estimulou a mielopoiese e produção de IFN-gama e IL-2 por células de baço, observou-se 40% de cura e aumento da sobrevida (DE MELO et al., 2001).

Em cães seu efeito foi avaliado na infecção natural por *Parvovirus*, tratamento que levou à recuperação de 95% do grupo estudado (DURÁN et al., 1989; DURÁN et al., 1987) e no tratamento de leishmaniose visceral, em que se observou o restabelecimento da imunocompetência do animal, com a redução da carga parasitária na pele, sinais clínicos e IL-10 e aumento dos níveis de IL-2, IFN-γ e CD8+ T (SANTIAGO et al., 2013).

O P-MAPA quando administrado em ratos (DURÁN et al., 2009), primatas (SOUZA-BRITO et al., 1991), humanos (BELUCCI et al., 1991) e cão (SANTIAGO et al., 2013) não apresenta efeito tóxico.

Como citado, diferentes estudos têm mostrado que o P-MAPA induz imunidade celular, porém o seu mecanismo de ação não está esclarecido. O composto por se tratar de um derivado de parede do fungo *Aspergillus oryzae* atua via receptor Toll, como mostrado em um estudo realizado com células humanas HEK 293 (células humanas embrionárias do rim) apresentando efeito estimulador em TLR 2 (FÁVARO, 2012). Esses receptores estão presentes em macrófagos, para o reconhecimento dos fungos. Nos fungos os principais receptores incluem o Toll-like (BURGENER; JUNGI, 2007; MOGENSEN, 2009).

Como mostrado nos estudos os receptores Toll-like estão presentes nos macrófagos sendo uma de suas funções o reconhecimento dos fungos (BURGENER; JUNGI, 2007; MOGENSEN, 2009) e na leishmaniose esta via é uma das primeiras respostas do sistema de defesa contra a LV (AMORIM et al., 2011), por isso escolhido esta via de sinalização para o estudo.

A sinalização via receptores de Toll envolve glicoproteínas de cadeia única que reconhecem estruturas conservadas na superfície dos patógenos. A ligação dos patógenos a tais receptores presentes na superfície celular, estimula a célula do hospedeiro a desencadear uma resposta antimicrobiana. Assim, são ativadas múltiplas sinalizações intracelulares, incluindo moléculas adaptadoras, quinases, tais como MAPkp38, e fatores de transcrição. A transdução de sinal resulta na expressão de genes e sínteses de várias moléculas, incluindo citocinas, quimiocinas, moléculas de adesão e imunorreceptores, os quais

regulam a resposta inata e ao mesmo tempo representa uma importante ligação com a resposta imune adaptativa (MOGENSEN, 2009).

Em um estudo *in vitro* utilizando PBMC de cães naturalmente infectados com *L. infantum* e que apresentavam pelo menos um sinal clínico, foi demonstrado que animais com menor carga parasitológica (por imunoistoquímica) e incapaz de infectar flebotomíneos apresentaram uma maior expressão de receptores TLR 2 comparado com os animais com maior carga parasitológica (por imunoistoquímica) e capazes de infectar flebotomíneos evidenciando a participação desses receptores na LV (AMORIM et al., 2011).

Em cães infectados com *L. infantum* foi demonstrado que a baixa carga parasitária causou um aumento na expressão de receptores TLR2, TLR9, interleucina IL-10 e da citocina TNF-α no jejuno e cólon. Não houve correlação entre as alterações patológicas e imunológicas, mas mostraram o aumento da expressão do receptor TLR2 pela infecção por *L. infantum* (FIGUEIREDO et al., 2013).

A expressão gênica de TLR 2 foi quantificada no sistema nervoso central (cérebro e plexo coróide) e em órgãos periféricos linfóides (baço e linfonodo poplíteo) de cães naturalmente infectados por *L. chagasi*, onde observou-se um aumento na regulação de TLR 2, mostrando a participação de TLR 2 na resposta imune contra o parasita (MELO et al., 2013)

Ainda, os sinais de TLR estimulam o sistema de produção de radicais livres fagócito-oxidase na membrana do fagolisossomo, levando à redução do oxigênio molecular em radicais intermediários do oxigênio (ROI), como radicais superóxido que tem a forma reduzida do fostafato de dinucleotídeo de nicotinamida e adenina (NADPH), agindo como um cofator. O peróxido, pela ação enzimática, produz peróxido de hidrogênio o qual é usado pela enzima mieloperoxidase para converter íons halídeos, que são normalmente não reativos, em ácido hipohalosos reativos sendo tóxicos para os micro-organismos (ABBAS; LICHTMAN, 2005).

No que se refere aos radicais livres, a sinalização de Toll pode também levar à produção de intermediários reativos do nitrogênio, principalmente óxido

nítrico, pela ação da enzima óxido nítrico sintase induzida (iNOS). A iNOS é uma enzima citosólica que está ausente em macrófagos em repouso, mas pode ser induzida em resposta ao lipopolissacarídeos (LPS) e a outros produtos microbianos que ativam os Tolls, especialmente em combinação com o IFN-ɣ. A iNOS catalisa a conversão da arginina em citrulina, e o óxido nítrico livremente difusível é liberado. Dentro dos fagolisossomos, o óxido nítrico pode se combinar com peróxido ou superóxido de hidrogênio, gerados pelo fagócito-oxidase, para produzir radicais de peroxinitrito altamente reativos que podem eliminar micro-organismos (ABBAS; LICHTMAN, 2005).

Em um estudo *in vitro* demonstrou-se que há uma correlação positiva entre a capacidade na produção de ROS em monócitos de cães e a capacidade para eliminar o protozoário da *Leishmania* sp. (PANARO et al., 1998). Contudo, verificou-se que a *L. infantum* possui uma enzima mitocondrial e citosólica que mostra atividade peroxidase (CASTRO et al., 2002). A superexpressão desta enzima em *Leishmania* tem ação protetora contra o estresse oxidativo produzido pelo hospedeiro (DOLAI et al., 2008).

Quanto a produção de NO, Amorim et al. (2011) demonstraram a participação na *L. infantum* quando comprovou, *in vitro*, a maior produção de NO em cães com menor carga parasitária cutânea em comparação com cães com maior carga parasitária demonstrado por imunoistoquímica.

A produção de NO também foi mostrada pela ativação de células 030-D (linha celular de macrófagos canino), por incubação com um sobrenadante derivado de uma linha de células T específicas para *Leishmania*. Além disso, observou o aumento da atividade anti-leishmaniose nestas células após a ativação (PINELLI et al., 2000). Assim, a produção de NO por macrófagos está correlacionada com a indução de atividade anti-*Leishmania*. (PINELLI et al., 2000; VOULDOUKIS et al., 1995).

Estudos prévios do grupo mostraram que a administração do P-MAPA induz melhora clínica dos cães sintomáticos para leishmaniose; e drogas com ação semelhante como o antimonial pentavalente induzem a ativação a proteína quinase p38 e o aumento da produção das espécies reativas de oxigênio e óxido

nítrico. Assim células mononucleares de sangue periférico de cão com LV foram estimuladas *in vitro* com P-MAPA para se investigar o efeito da droga sobre a expressão superficial de receptores TLR2 e TLR4, sobre a produção de espécies reativas de oxigênio e de óxido nítrico, e fosforilação de p38 MAPK e IKK nestas células.

REFERÊNCIAS

ABBAS, A.K.; LICHTTMAN, A.H.; POBER, J.S. Cellular and molecular Immunology. **Philadelphia: W.B. Saunders.** 5.ed., p. 295-296, 2005.

ABREU-SILVA, A. L.; LIMA, T. B.; MACEDO, A. A.; MORAES JÚNIOR, F. J.; DIAS, E. L.; BATISTA, Z. S.; CALABRESE, J. L. P. M.; REBÊLO, J. M. M.; GUERRA, R. M. S. N. C. Soroprevalência, aspectos clínicos e bioquímicos da infecção por *Leishmania* em cães naturalmente infectados e fauna de flebotomíneos em uma área endêmica na ilha de São Luís, Maranhão, Brasil. **Rev. Bras. Parasitol. Vet.**, v. 17, p. 197-203, 2008.

ALENCAR, J. E.; DIETZE, R.; VERONESI, R. Leishmaniose visceral (calazar). **VERONESI, R. Doenças infecciosas e parasitárias**, v. 8, p. 706-17, 1991.

ALVAR, J.; CAÑAVATE, C.; MOLINA, R.; MORENO, J.; NIETO, J. Canine leishmaniasis. **Adv. Parasitol.**, v. 57, p. 1-88, 2004.

AMORIM, I. F. G.; DA SILVA, S. M.; FIGUEIREDO, M. M.; MOURA, E. P.; DE CASTRO, R. S.; DE SOUZA LIMA, T. K.; GONTIJO, N.F; MICHALICK, M.S.M; GOLLOB, K.J.; TAFURI, W. L. Toll receptors type-2 and CR3 expression of

canine monocytes and its correlation with immunohistochemistry and xenodiagnosis in visceral leishmaniasis. **PloS one**, v. 6, n. 11, p. e27679, 2011.

BANETH, G; SHAW, S.E. Chemotherapy of canine leishmaniosis. **Vet. Parasitol.**, v. 106, n. 4, p. 315-324, 2002.

BARBOSA, M. A. G.; ALEXANDRE-PIRES, G.; SOARES-CLEMENTE, M.; MARQUES, C.; RODRIGUES, O. R.; DE BRITO, T. V.; FONSECA, I.; ALVES, L.C.; SANTOS-GOMES, G. M. Cytokine Gene Expression in the Tissues of Dogs Infected by *Leishmania infantum*. **J. Comp. Pathol.**, v. 145, n. 4, p. 336-344, 2011.

BASANO, S. D. A.; AND CAMARGO, L. M. A.. American cutaneous leishmaniasis: history, epidemiology and prospects for control. **Ver. Bras. Epidemiol.**, v. 7, n. 3, p. 328-337, 2004.

BELUCCI, S.; NUNES, O.D.S; CIAMPI, D.B.; DURÁN, N. SB-73 a new immunomodulating compound in the treatment of AIDS patient. **Seventh International Conference on AIDS, Florence, Italy**, p. 16-21, 1991.

BRASIL, 2006. Secretária de Vigilância em Saúde, Ministério da Saúde. Manual de vigilância e controle da leishmaniose visceral. **Brasília: Secretaria de Vigilância em Saúde**, Ministério da Saúde, 2006.

BORJA-CABRERA, G.P.; MENDES, A. C.; SOUZA, E.P.; OKADA, L.Y. H.; TRIVELLATO, F.A.A.; KAWASAKI, J.K.A.; COSTA, C.A; REIS, A.B.; GENARO, O.; BATISTA, L.M.M.; PALATNIK, M.; PALATNIK-DE-SOUSA, C.B. Effective immunotherapy against canine visceral leishmaniasis with the FML-vaccine. **Vaccine**, v. 22, n. 17, p. 2234-2243, 2004.

BOURDOISEAU, G.; BONNEFONT, C.; MAGNOL, J.P.; SAINT ANDRE, I.; CHABANNE L. Lymphocyte subset abnormalities in canine leishmaniasis. **Vet. Immunol. Immunopathol.**, n.56, p. 3-4 e 345-51, 1997.

BURGENER, I.A.; JUNGI, T.W.. Antibodies specific for human or murine Toll-like receptors detect canine leukocytes by flow cytometry. **Vet. Immunol. Immunopathol.**, v. 124, n. 1, p. 184-191, 2008.

CARVALHO, E. M.; BACELLAR, O.; BARRAL, A.; BADARO, R.; JOHNSON JR, W. D. Antigen-specific immunosuppression in visceral leishmaniasis is cell mediated. **J. Clin. Invest.**, v. 83, n. 3, p. 860, 1989.

CASTRO, H.; SOUSA, C.; SANTOS, M.; CORDEIRO-DA-SILVA, A.; FLOHÉ, L.; TOMÁS, A. M. Specificity and kinetics of a mitochondrial peroxiredoxin of *Leishmania infantum*. **Free Radic. Biol. Med.,** n.33, p.1563-74, 2002.

COSTA, C.H.N.; VIEIRA, J.B.F. Changes in the control program of visceral leishmaniasis in Brazil. **Rev. Soc. Bras. Med. Trop.**, v. 34, n. 2, p. 223-228, 2001.

COSTA, J. M. Epidemiologia das leishmanioses no Brasil. **Gazeta médica da Bahia**, v. 74, n. 1, 2008.

COURTENAY, O.; QUINNELL, R.J.; GARCEZ, L.M.; SHAW, J.J.; DYE, C. Infectiousness in a cohort of Brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. **J. Infect. Dis.** nov. 1, 2002.

DE MELO, A.; JUSTO, G. Z.; QUEIROZ, M.L.S. Stimulation of myelopoiesis in *Listeria monocytogenes*-infected mice by an aggregated polymer isolated from *Aspergillus oryzae*. **Hum. Exp. Toxicol.**, vol. 20, n1, p. 38-45, 2001.

DESJEUX, P. Leishmaniasis: current situation and new perspectives. **Comp. Immunol. Microbiol. Infect. Dis.** n. 27, p. 305-318, 2004.

DOLAI, S; YADAV, R.K.; PAL, S; ADAK, S. Leishmania major ascorbate peroxidase over expression protects cells against reactive oxygen species-mediated cardiolipin oxidation. **Free Radic. Biol. Med.,** n.45, p.1520-9, 2008.

DOS-SANTOS, W.L.; JESUS, E.E.; PARANHOS-SILVA, M.; PEREIRA, A.M.; SANTOS, J.C.; BALEEIRO, C.O.; NASCIMENTO, E.G.; MOREIRA, E.D.; OLIVEIRA, G.G.; PONTES-DE-CARVALHO, L.C. Associations among immunological, parasitological and clinical parameters in canine visceral leishmaniasis: Emaciation, spleen parasitism, specific test reaction. **Vet. Immunol. Immunopathol**., v. 123, n. 3, p. 251-259, 2008.

DURÁN, N.; GOWEN, B.B.; COSTA, F.T.M.; JUSTO, G.Z.; BROCCHI, M.; NUNES, O.S.; NUNES, I.S. A biotechnological product and its potential as a new immunomodulator for treatment of animal phlebovirus infection: Punta Toro virus. **Antiviral Res**., v. 83, n. 2, p. 143-147, 2009.

DURÁN, N.; HAUN, M.; HOFFMANN, M.E.; CIAMPI, D.B.; NUNES, O.D.S. Biological activity and citotoxicity of a new metalolipoprotein (SB-73). **Resumos da XVI Reunião Annual da Sociedade Brasileira de Bioquímica, Caxambu, MG, Brasil**, p. 22-25, abril de 1987.

DURÁN, N.; HAUN, M.; PEREIRA-DA-SILVA, L.; PISANI, R. Comparison of the antiviral activity and toxicity of a protein magnesium ammonium phospholinoleate anhydride polymer with other antiviral drugs. **Braz. J. Med. Biol. Res.** v. 23, n. 12, p. 1303-1313, 1989.

DURÁN, N.; JUSTO, G.Z.; QUEIROZ, M.L.S.; VIEIRA-MATOS, A.N.; RETTORI, O. New perspectives in immunomodulatory therapy of tumor induced by an

extracellular aggregated polymer isolated from Aspergillus oryzae. Int**. J. Mol. Med.,** n. 4, p. 49, 1999.

DURÁN, N.; SOUZA-BRITO, A.R.M.; HAUN, M.; DE OLIVEIRA, J.A.; HETEM SAAVEDRA, I.; JUSTO, G.Z. SB-73-immunostimulant. **Drugs Fut.**, n. 18, p. 327, 1993.

FÁVARO, W.J.; NUNES, O.S.; SEIVA, F.R.; NUNES, I.S.; WOOLHISER, L.K.; DURÁN, N.; LENAERTS, A. J. Effects of P-MAPA Immunomodulator on Toll-Like Receptors and p53: Potential Therapeutic Strategies for Infectious Diseases and Cancer. **Infect. Agent. Cancer,** v. 7, n. 1, p. 14, 2012.

FIGUEIREDO, M. M.; AMORIM, I. F.; PINTO, A. J.; BARBOSA, V. S.; DE JESUS PINHEIRO, L.; DEOTI, B.; FARIA, A.M.C.; TAFURI, W. L. Expression of Toll-like receptors 2 and 9 in cells of dog jejunum and colon naturally infected with Leishmania infantum. **BMC immunology.**, v. 14, n. 1, p. 22, 2013.

GENARO, O. **Docente da UFMG.** Leishmaniose visceral canina experimental. 1993. Tese (Doutorado) – Universidade Federal de Minas Gerais, 1993.

GOMEZ-OCHOA, P; CASTILLO, JA; GASCÓN, M; ZARATE, JJ; ALVAREZ, F; COUTO, CG. Use of domperidone in the treatment of canine visceral leishmaniasis: A clinical trial. **Vet. J**., v. 179, n. 2, p. 259-263, 2009.

HERVAS J.; MÉNDEZ A.; CARRASCO L.; GÓMES-VILLAMANDOS J.C. Pathological study of visceral leishmaniasis in a Jackal. **Vet. Rec.**, n. 139, p. 293-295, 1996.

JUSTO, G. Z.; DURÁN, N.; QUEIROZ, M. L. S. Natural killer cell activity, lymphocyte proliferation, and cytokine profile in tumor-bearing mice treated with

MAPA, a magnesium aggregated polymer from Aspergillus oryzae. **Immunopharmacol. Immunotoxicol.**, v. 25, n. 3, p. 305-319, 2003.

KOUTINAS, A. F.; KOUTINAS, C. K. Pathologic mechanisms underlying the clinical findings in canine leishmaniosis due to Leishmania infantum/chagasi. **Vet. Pathol.,** v. 51, n. 2, p. 527-538, 2014.

LIMA, V.M.; FATTORI, K.R.; DE SOUZA, F.;EUGÊNIO, F.R.; SANTOS P.S.; ROZZA, D.B.; MACHADO, G.F. Apoptosis in T lymphocytes from spleen tissue and peripheral blood of *L. (L.) chagasi* naturally infected dogs. **Vet. Parasitol.** v. 184, n. 2, p. 147-153, 2012.

MELO, G. D.; GRANO, F. G.; SILVA, J. E. D.; SCHWEIGERT, A.; PEIRÓ, J. R.; LIMA, V. M.; AND MACHADO, G. F. TLR-2 but not TLR-9 is up-regulated in the Central Nervous System and Lymphoid Organs during Canine Visceral Leishmaniasis. In: 15th **International Congress of Immunology**, 2013, Milão - Itália. Frontiers Immunology, 2013.

MOGENSEN, TRINE H. Pathogen recognition and inflammatory signaling in innate immune defenses. **Clin. Microbiol. Rev.**, v. 22, n. 2, p. 240-273, 2009.

PANARO, MA; LISI, S; MITOLO, V; ACQUAFREDDA, A; FASANELLA, A; CARELLI, MG; BRANDONISIO, O. Evaluation of killing, superoxide anion and nitric oxide production by Leishmania infantum-infected dog monocytes. **Cytobios**, n. 95, p.151-60, 1998.

PINELLI, E.; GEBHARD, D.; MOMMAAS, A.M.; VAN HOEIJ, M.; LANGERMANS, J.A.; RUITENBERG, E.J.; RUTTEN, V.P. Infection of a canine macrophage cell line with *Leishmania infantum*: determination of nitric oxide production and anti-leishmanial activity. **Vet. Parasitol.,** n. 92, p.181-9, 2000.

REY, LUÍS. Leishmania e leishmaníases: Os parasitos. **Rey L. Parasitologia. 3rd ed. Rio de Janeiro: Guanabara Koogan**, p. 214-239, 2001.

ROBERTS, M. T. M. Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment. **Br. Med. Bull**., v. 75, n. 1, p. 115-130, 2006.

SACKS, D. L.; LAL, S. L.; SHRIVASTAVA, S. N.; BLACKWELL, J. E. N. E. F. E. R.; NEVA, F. A. An analysis of T cell responsiveness in Indian kala-azar. **J. Immunol.**, v.138, n.3, p.908-913, 1987.

SANTA ROSA I.C.A.; OLIVEIRA I.C.S. Leishmaniose visceral: breve revisão sobre uma zoonose reemergente. **R. Clin. Vet.**, ano II, n. 11, p. 24-28, 1997.

SANTIAGO, M. E. B.; NETO, L. S.; ALEXANDRE, E. C.; MUNARI, D. P.; ANDRADE, M. M. C.; SOMENZARI, M. A.; CIARLINI, P.C.; LIMA, V.M.F. Improvement in clinical signs and cellular immunity of dogs with visceral leishmaniasis using the immunomodulator P-MAPA. **Acta Trop.**, v. 127, n. 3, p. 174-180, 2013.

SLAPPENDEL R.J. Canine leishmaniasis: a review based on 95 cases in the Netherlands. **Vet. Quarterly**, v. 10, n. 1, p. 1-16, 1988.

SOLANO-GALLEGO L.; KOUTINAS A.; MIRO´ G.; CARDOSO L.; PENNISI M.G.; FERRER L.; BOURDEAU P.; OLIVA G.; BANETH G. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. **Vet. Parasitol.**, v. 165, n. 1, p. 1-18, 2009.

SOUZA-BRITO A.R.; ARCIFA S.; HAUN M.; NUNES O.D.; DURAN N. A new perspective in the treatment of AIDS: laboratorial and physiological parameters

of the immunomodulating compound SB-73. **Abstract. Seventh International Conference on AIDS, Florence, Italy**, p. 16-21, 1991.

VOULDOUKIS, I.; RIVEROS-MORENO, V.; DUGAS, B.; OUAAZ, F.; BECHEREL, P.; DEBRE, P.; MONCADA, S.; MOSSALAYI, M.D. The killing of Leishmania major by human macrophages is mediated by nitric oxide induced after ligation of the Fc epsilon RII/CD23 surface antigen. **Proc. Natl. Acad. Sci U S A**, v. 92, n. 17, p. 7804-7808, 1995.

WORLD HEALTH ORGANIZATION. Control of the leishmaniases: report of a meeting of the WHO Expert Commitee on the Control of Leishmaniases**, Geneva**, 22-26 March 2010. 2010.

Capítulo 2

CAPÍTULO 2 – EFFECTS OF P-MAPA IMMUNOMODULATOR ON TOLL-LIKE RECEPTOR 2, ROS, NITRIC OXIDE, MAPKp38 and IKK IN PBMC AND MACROPHAGES FROM DOGS WITH VISCERAL LEISHMANIASIS

Melo, L.M[a], Perosso, J.[a], Almeida, B.F.M.[a], Oliveira, K.L.S. de[a], Somenzari, M.A.[b], Lima, V.M.F. de[b]

[a] Pós-graduação em Ciência Animal Faculdade de Medicina Veterinária, Universidade Estadual Paulista - Araçatuba- SP, Brasil

[b] Departamento de Clínica, Cirurgia e Reprodução Animal, Faculdade de Medicina Veterinária-Universidade Estadual Paulista - Araçatuba, SP, Brasil.

Correspondence: Departamento de Clínica e Cirurgia e Reprodução Animal
Faculdade de Odontologia, UNESP, Campus de Araçatuba
Rua Clóvis Pestana, 793.
Bairro Jardim Dona Amélia.
CEP 16050-680 São Paulo Brazil
Email: vmflima@fmva.unesp.br

SUMMARY

Leishmania (L.) chagasi is the etiologic agent of visceral leishmaniasis (VL) that can be transmitted to humans and dogs. VL in Brazil represents a serious public health problem; therefore, it is important to study new alternatives to treat infected dogs. In dogs, the therapeutic arsenal against canine VL is limited. The immunomodulator protein aggregate magnesium-ammonium phospholinoleate-palmitoleate anhydride (P-MAPA) improves immunocompetence when the immune system is impaired, but its dependence on Toll-like receptors (TLRs) and the mechanisms involved in the immune response remain unclear. The *in vitro* action of P-MAPA on the expression of TLR2 and TLR4, reactive oxygen species (ROS), nitric oxide (NO) and p38 mitogen-activated protein kinase (p38MAPK) and IKK phosphorylation were studied in mononuclear cells from peripheral blood and macrophages from healthy and *Leishmania*-infected dogs. The PBMC or macrophages were isolated and cultured with different concentrations of P-MAPA (20,100 and 200µg/mL) in a humid environment at 37°C with 5% CO_2. Observation revealed that *Leishmania*-infected dogs showed a decrease in TLR2 in macrophages compared with healthy dogs and in induction with P-MAPA. ROS were increased in PBMCs from *Leishmania* sp-infected dogs compared with healthy dogs and P-MAPA improved ROS production. NO production was increased in culture supernatant from macrophages stimulated by P-MAPA in both healthy and *Leishmania* sp. infected dogs. Treatment of macrophages from healthy dogs with immunomodulatory P-MAPA induced p38 MAPK and IKK phosphorylation, suggesting signal transduction by this pathway. These findings

suggest that P-MAPA has potential as a therapeutic drug in the treatment of canine visceral leishmaniasis.

Keywords: *Leishmania* sp., P-MAPA, dog, immunomodulatory, TLR.

1. Introduction

Visceral leishmaniasis is an endemic disease that has spread over several continents, mainly in tropical and subtropical regions. It is caused by the *Leishmania infantum, L. chagasi and L. donovani* species and affects millions of people worldwide[1]. The parasite is transmitted by sand flies to mammals, including humans and dogs, via host blood-feeding. The dog is considered the most important urban reservoir of *L. chagasi* due to its high level of infection and its proximity to humans[2].

Treatment of canine visceral leishmaniasis (CVL) has certain limitations because the therapeutic arsenal against this disease is limited and the most commonly used drugs present high toxicity (nephrotoxicity, intestinal problems, muscle pain), are costly and can be ineffective in some cases[3,4] . Treatment failures have epidemiological implications since, following treatment the dogs become asymptomatic but remain a reservoir for transmission of the parasite to sand flies[2]. These factors indicate that new drugs should be evaluated for the treatment of CVL.

The suppression of cellular immunity is the most important aspect of the pathogenesis and progression of CVL. Dogs infected with *L. infantum* show a negative response to the cutaneous test with parasite antigens[5] and a reduction

in the number of T lymphocytes in peripheral blood[6] due to increased rates of T cell apoptosis[7]. Therefore, a drug that promptly reverses immunosuppression is desirable when treating infected dogs.

Cellular immune activation involves stimulation of receptors on macrophages, which are single chain glycoproteins that recognize conserved structures on the surface of pathogens. Binding pathogens to receptors on the host cell is achieved by activating antimicrobial multiple intracellular signals, including adapter molecules, kinases like p38 mitogen-activated protein kinase (p38 MAPK), and transcription factors. This results in signal transduction, gene expression and synthesis of various molecules, including cytokines, chemokines, adhesion molecules and immunoreceptors, which regulate the innate response, while simultaneously maintaining an important link with the adaptive immune response[8].

Since the failure of cellular immunity contributes to the progression of the disease, immunomodulatory substances have been studied to treat it[9]. Effective CVL immunotherapy requires the use of suitable antigens that stimulate cellular immunity and block negative regulatory mechanisms that prevent the immunotherapeutic effects[10]. Domperidone, a dopamine D2 receptor antagonist, has been effective at controlling and reducing clinical signs and antibody titers and increasing cellular immunity[11]; but the number of immunotherapeutic drugs available is limited.

The protein aggregate of magnesium-ammonium phospholinoleate-palmitoleate anhydride (P-MAPA) is a compound obtained by fermenting the

fungus *Aspergillus oryzae*. Its immunomodulatory activities include the induction of Toll-like receptor (TLR)-2 in human embryonic kidney (HEK) cells[12], stimulation of marrow myelopoiesis[13,14], antimicrobial[12,14,15] and antitumoral activity[13], increased spleen cell proliferation, the production of cytokines IL-2 and IFN-γ and NK cell activity[16], which all promote greater stimulation of cellular immunity. Dogs with visceral leishmaniasis were treated with P-MAPA and presented a significant reduction in clinical signs and improvement in cellular immune response[17]. Toxicological studies have determined that P-MAPA is safe in mice[12], dogs[17] and humans[12].

P-MAPA has a stimulatory effect on the immune response; however, its mode of action remains unknown, thus this study aimed to examine, *in vitro*, the drug's effect on TLR2 and TLR4 expression superficial, reactive oxygen species and nitric oxide production and p38 MAPK and IKK phosphorylation in mononuclear cells from healthy control dogs and dogs infected with *Leishmania* sp.

2. Materials and Methods

2.1. Study Area

The study was conducted in Araçatuba, São Paulo State, Brazil, an area endemic for both canine (CVL) and human visceral leishmaniasis.

2.2. Animals

Sixty (60) male and female dogs housed at the Araçatuba Zoonosis Control Center (CCZA) that were seropositive for *L. (L.) chagasi* by indirect ELISA[18] method were included in the study. They were symptomatic, i.e. they presented at least three clinical signs of CVL.

Sixty (60) healthy male and female dogs from private homes in Araçatuba were included in the study after the owners signed a term permitting the collection of samples.

Serology was performed on dogs using *Leishmania* spp specific antibodies, as determined by the indirect ELISA method[18], and normal blood tests were performed for those testing seronegative. Blood samples from both groups were taken from each dog, 4.5 mL of blood was collected from the cephalic vein and maintained in tubes with anticoagulant, and 1.0 mL was coagulated at room temperature and then centrifuged to extract the serum.

The local animal research ethics committee approved this study under protocol no. 322.

2.3. Treatment with P-MAPA

The product P-MAPA is an immunomodulator developed by Farmabrasilis[19] that is a proteinaceous aggregate of magnesium and ammonium phospholinoleate-palmitoleate anhydride (P-MAPA) derived from *A. oryzae*[20]. The P-MAPA immunomodulator contains 11.6±4.0% of total lipids, (22.7±5.0% of palmitoleic acid, 42.9±2.0% of linoleic acid, and 32.0±3.0% of oxidized linoleic acid), 20.1±0.9% of magnesium ions, 10.0±3.3% of ammonium ions, 45.2±2.7%

of phosphate and 0.49±0.01% of protein (Asp 7.19%, Thr 3.56%, Ser 7.56%, Glu 8.53%, Pro 0.5%, Gly 9.69%, Ala 7.46%, Val 1.0%, Met 4.38%, Isoleu 2.54%, Leu 3.03%, Tyr 0.5%, Phe 1.0%, His 2.83%, Lys 3.56%, Trp 1.3%, and Arg 35.2%). The compound is produced when the *A. oryzae* fungus is cultured in a medium consisting of an aqueous solution of oat and gelatin (10:1, wt/wt) for a period of 5 days in a bioreactor maintained between 20 and 35°C, with pH stabilized between 2 to 4, under low aeration (2 L/min) and slow agitation (5 rotations per h). The culture medium is then mechanically filtrated and the compound extracted with ethyl acetate and precipitated under pH 11 by a 20% aqueous solution of sodium carbonate. The resulting crystals are washed in ethyl acetate and ether and dried.

For *in vitro* use, P-MAPA was prepared in RPMI-1640 (Sigma) after hydration for 12 h at 4°C, the suspension was homogenized 3 times using a sonicator at 60W at 4°C. The suspension was divided into 1mL aliquots and stored at -20°C until use.

2.4. Purification and culture of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by density gradient using Ficoll-Paque Plus (GE Healthcare Bio-sciences), in accordance with the manufacturer's recommendations, immediately cultured ($5x10^6$ cells/mL) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin-G, 100 µg/mL streptomycin, and 2mmol/L L-glutamine (Life Technologies, Grand Island,

NY) and incubated at 37°C with 5% CO_2. Cell counts were performed in a Neubauer chamber. To obtain macrophages, the PBMCs were isolated by Histopaque gradient® 1077 and 1119 (Sigma-Aldrich, St. Louis, MO, USA), in accordance with the manufacturer's recommendations. Mononuclear cells were immediately cultured ($5x10^6$ cells/mL) in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin-G, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine (Life Technologies, Grand Island, NY) and incubated at 37°C with 5% CO_2. Macrophage acquisition was achieved as previously described with one modification[21] an incubation period of 10 days was used.

2.5. TLR2 and TLR4 quantification

To examine TLR2 and TLR4 expression in PBMCs and macrophages of *L. chagasi* infected and control dogs, the cells ($5x10^6$ cells/mL) were cultured with 20 µg/mL, 100 µg/mL or 200 µg/mL de P-MAPA for 3 h at 37°C with 5% CO_2, and then double-stained with specific fluorochrome-conjugated antibodies: monoclonal fluorescein isothiocyanate (FITC) conjugated anti-human TLR2 antibody (eBioscience, San Diego, USA), and anti-human TLR4 conjugated to phycoerythrin (PE) (eBioscience, San Diego, USA)[22] or control isotypes conjugated to FITC and PE (eBioscience, San Diego, USA). Following data acquisition in EasyCyte mini® (Guava, Hayward, CA), analysis of the data was performed using the software Guava Express® Plus.

2.6. Measurement of reactive oxygen species (ROS) levels

Intracellular ROS levels were measured in PBMCs (5x10^6 cells/mL) from infected and healthy dogs after the cells were cultured with 20µg/mL, 100µg/mL or 200µg/mL of P-MAPA for 90 min at 37°C with 5% CO_2 and incubated with 10µM H_2DCFDA (29,79-dichlorodihydroflurescein diacetate, Invitrogen Molecular Probes - Leiden, The Netherlands) for 30 min at 37ºC, in accordance with the manufacturer's recommendations. Fluorescence was measured by flow cytometry. Following data acquisition in EasyCyte mini® (Guava, Hayward, CA), analysis of the data was performed using the software Guava Express® Plus. Positive control was achieved by adding 10µL of PMA (1µM/mL) (Sigma-Aldrich, St. Louis, MO, EUA) to the cell culture, following the same protocol.

2.7. Determination of nitrite concentration (NO_2^-)

Macrophages derived from monocytes from the peripheral blood of *L. chagasi*-infected and control dogs (approx. 5x10^6 cells/mL) were stimulated with 20 µg/mL, 100 µg/mL or 200 µg/mL of P-MAPA or LPS (0.1µg/mL) (Sigma-Aldrich, St. Louis, MO, EUA) for 24 h at 37°C with 5% CO_2. Nitrite ion (NO_2^-) production in the supernatants was quantified using standard Griess reagent[23]. Briefly, 100mL of macrophage culture supernatant was mixed with an equal volume of Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) containing 1% sulfanilamide (Sigma-Aldrich, St. Louis MO, USA) diluted in 5% H_3PO_4 and 0.1% N-(1 naphthyl) ethylenediamine (Sigma-Aldrich Co, U.S.). Absorbance at 540 nm was determined using an automated ELISA plate reader (Packard Spectra Count, Packard Instrument Co., Downers Grove, IL, USA). Conversion of the

absorbance of micromolar concentrations of NO_2- was performed using a standard $NaNO_2$, curve with an initial concentration of 100 µM and a final of 0.75 µM. All the measurements were performed in triplicate and are expressed in micromolar concentrations of NO_2^-.

2.8. Determination of p38 MAPK and IKK

Macrophages derived from monocytes from the peripheral blood of *L. chagasi*-infected and control dogs (approx. $5x10^6$ cells/mL) were stimulated with 20 µg/mL, 100 µg/mL or 200 µg/mL of P-MAPA or LPS (0.1µg/mL) (Sigma-Aldrich, St. Louis, MO, EUA) for 1 h at 37°C with 5% CO_2. Following incubation, the cells were recovered and placed in 1.5 mL tubes and incubated with 1mL IC Fixation Buffer (Invitrogen, Leiden, The Netherlands) for 10 min at 4ºC, washed with PBS (pH 7.2), then washed again with HF Permeabilization Buffer (eBioscience, San Diego, USA) and incubated with specific antibodies: mouse monoclonal anti-TBK1 fluorochromes conjugated to phycoerythrin (BD Biosciences), monoclonal mouse anti-p38 MAPK conjugated to Alexa Fluor (BD Biosciences) for 30 min at 4ºC. Next, 300µL of fixation buffer (PBS with 10% formalin) was added. Subsequently, the samples were acquired in EasyCyte mini® cytometer (Guava, Hayward, CA), and analysis was performed using the software Guava Express® Plus.

2.9. Statistical Analysis

Statistical differences were analyzed using GraphPad PRISM 3 Software (San Diego, CA, USA). Considering the nonparametric nature of all data sets, the Mann-Whitney test was used to verify significant differences between the control and infected groups and the Wilcoxon test was used to verify significant differences between treatments. In all cases, the differences were considered significant when the probabilities of equality, P values, were $P<0.05$.

3. Results

3.1. TLR2 and TLR4 percentage in PBMCs and macrophages from infected and control dogs

The percentage of TLR2 in PBMCs from infected and controls dogs not show statistically significant difference ($P>0.05$; Table 1), while the percentage of TLR4 was higher in control dogs compared with infected dogs ($P<0.05$; Table 1). Treatment with P-MAPA in PBMC revealed no statistically significant differences in the percentages of TLR2 and TLR4 expression compared with baseline conditions (data not shown).

Under baseline macrophage conditions, the percentage of TLR2 expression of infected dogs was decreased compared with control dogs (Figure 1a, $P<0.05$). P-MAPA increased TLR2 expression in control (Figure 1b, $P<0.05$) and infected dogs (Figure 1c, $P<0.05$). No statistically significant difference was observed in the percentage of TLR4 expression between control and infected dogs, and no change occurred when macrophages were treated with the immunomodulator P-MAPA (data not shown).

3.2. Measurement of ROS levels in PBMCs

The baseline value of ROS production in mononuclear cells from infected dogs was four-fold greater than that observed in control dog cells (Figure 2a, $P<0.05$). Mononuclear cells from control dogs stimulated by P-MAPA at concentrations of 100 µg/mL and 200 µg/mL increased ROS production compared with that observed in cells cultured in medium alone (Figure 2b, $p<0.05$). In contrast, in mononuclear cells from infected dogs, all three concentrations of P-MAPA (20 µg/mL, 100 µg/mL and 200 µg/mL) promoted an increase in ROS production of compared with cells cultured in medium alone (Figure 2c, $P<0.05$). The PMA positive control showed increased ROS production compared with the baseline values of both groups (Figures 2a, 2b and 2c, $P<0.05$).

3.3 NO production by macrophages from infected and control dogs treated with P-MAPA

In macrophages from control dogs, P-MAPA at concentrations of 100 µg/mL and 200 µg/mL induced NO production ($P<0.05$) compared with cells cultured in medium alone (Figure 3a). In macrophages from infected dogs, only a P-MAPA concentration of 200 µg/mL increased NO production ($P<0.05$) compared with cells cultured in medium alone (Figure 3b). Macrophages from control and infected dogs stimulated with LPS did not produce NO (Figure 3a and 3b).

3.4 Determination of p38 MAPK and IKK

In macrophages from healthy dogs, P-MAPA increased the induction of IKK and p38 MAPK phosphorylation compared with baseline values ($P<0.05$, Wilcoxon test), indicating that the two signal transduction pathways are stimulated by the immunomodulator. Similarly, LPS induced increased IKK and p38 MAPK phosphorylation ($P<0.05$, Wilcoxon test; Table 2).

In infected dogs, a slight increase in p38 MAPK and IKK production was observed; however, this was not statistically significant (data not shown).

4. Discussion

The results of this study indicate that the use of P-MAPA increased TLR2 expression and induced ROS and NO production in macrophages from *L. chagasi*-infected dogs.

Toll-like receptor 2 is part of a family of highly conserved pattern recognition receptors (PRR) in mammals that participate in the innate and adaptative immune responses. The role of TLRs in the pathogenesis of CVL has not been fully addressed. This study confirms that TLRs can be detected on dog white cells, as previously reported by[22]. TLR4 and TLR2 expression in canine peripheral blood leukocytes has also been previously demonstrated[24,25].

The PBMCs from symptomatic dogs naturally infected with *L. chagasi* showed diminished TLR4 expression. Similar results were observed by[26] in peripheral blood monocyte-derived macrophages (MDMs) from patients with

visceral leishmaniasis. Prior studies demonstrated that TLR4 is required for efficient parasite control, probably due to the activity of inducible NO synthase. The activation of inducible NO synthase leads to NO synthase and *Leishmania* sp. death[27]. NO production by macrophages is correlated with the induction of anti-*Leishmania* activity[28,29]. In the absence of sufficient TLR4 expression, the enhanced activity of arginase increases the formation of urea and reduces NO[27]. The high parasite load observed in symptomatic dogs could be related to low TLR4 expression in PBMCs; future studies should clarify this hypothesis.

The decrease in TLR4 observed in symptomatic dogs suggests that *Leishmania* sp. uses this receptor to infect cells in dogs and the process should be regulated by cytokines. In humans, MDMs infected with *L. donovani* present suppression of TLR4 expression in late infection, when TGF-beta-1 attains high levels[26]. In symptomatic dogs, high levels of TGF-beta-1 have been observed[30,31], suggesting that this cytokine could downregulate TLR4 expression in CVL.

When treated with P-MAPA, TLR4 expression in PBMCs from infected dogs showed no increase or decrease under the experimental conditions described. The expression values obtained under basal conditions were similar to those observed in the P-MAPA treatments *in vitro*. The immunomodulatory effect was also assessed in mononuclear cells of healthy dogs and similar results were observed. The lack of alteration in TLR4 expression observed in presence or absence of P-MAPA could be related to the low concentration of molecule used. Unlike the results for TLR4, macrophages stimulated by higher concentrations of P-MAPA showed an increase in TLR2 expression for both

infected and control dogs. These results indicate an interaction between these molecules. Similar results were observed by[12], who showed that P-MAPA had a stimulating effect on TLR2 in HEK293 cells.

In macrophages from infected dogs showed significant reduction in TLR2 expression compared with controls, similar to that observed in the spleen of mice chronically infected with *L. chagasi*[32]. The increase in TLR2 expression following P-MAPA treatment seems to restore the immune balance, and given that the relationship between the presence of TLR2 and resistance to disease has been demonstrated, greater TLR2 expression may be a key point to initiating an effective immune response against this parasite. Higher levels of TLR2 mRNA were observed in mice resistant to *Leishmania* than those observed in susceptible mice[33].

Stimulation of TLRs leads to the activation of NF-kB, which can regulate the expression of cytokines[34] and the production of nitric oxide and oxygen radicals[27]. P-MAPA increased TLR2 expression, thus due to the relation observed between TLR and ROS and NO production, these molecules were investigated in PBMCs and macrophages from infected dogs.

The basal ROS production in infected dogs was higher compared with that observed in control dogs. It is known that phagocytosis of parasites by monocytes in the blood leads to ROS production, as demonstrated in human and murine macrophages[35]. However, the high level of ROS production observed in PBMCs from infected dogs suggests that the microbicidal effect generated is not sufficient to eliminate the parasite, because symptomatic dogs tend to have a

high parasitic load[36]. In fact, *L. infantum* possesses a mitochondrial and cytosolic enzyme that shows peroxidase activity[37]. The overexpression of this enzyme in *Leishmania* sp. has a protective action against oxidative stress[38] and could protect the parasite from ROS.

The immunomodulator P-MAPA increased ROS levels in PBMCs from infected and control dogs. Thus, it is possible that this output could effect parasite death by eliminating their antioxidant defenses, since there is a positive correlation between the ability of ROS production and the ability of monocytes to kill canine *Leishmania* sp. *in vitro*[39].

The observation that P-MAPA is a potent inducer of ROS facilitates its use as an immunomodulatory drug in visceral leishmaniasis, since no toxic effect has been observed in previous experimental models[40] or dogs[17]. This contrasts from that observed with therapeutic drugs like glucantime, ketoconazole and miltefosine, which induce ROS production[41,42], but produce different side effects in dogs[4].

Apart from ROS production, the immunomodulator P-MAPA induced NO production in MDMs from infected dogs and controls. NO is generated following the activation of macrophages and plays an important role in leishmanicidal activity in canine macrophages[28].

Following stimulation with IFN-gamma associated with LPS, canine macrophages express the enzyme inducible NO synthase[21], suggesting that NO production requires two signal activators. P-MAPA induced NO production suggesting that can trigger more than one intracellular activation signal. However,

there was no nitric oxide production in macrophages infected or healthy dogs only upon stimulation with LPS[28].

Due to its ability to induce NO, P-MAPA appears to have similar action to other therapeutic drugs. Mononuclear cells from patients infected with visceral leishmaniasis *in vitro* following miltefosine stimulation produce NO[43] and murine macrophages infected with *L. donovani* and treated with sodium antimony gluconate also produce NO[41].

Treatment of macrophages from healthy dogs with immunomodulatory P-MAPA induced p38 MAPK and IKK phosphorylation at the cellular level, which stems from the recognition molecules by Toll-like receptors. The standard molecular recognition molecule MyD88 is recruited to the TIR domain of TLR, where it encounters the IRAK1/IRAK4 complex. IRAK4 phosphorylates IRAK1, creating a binding site for TRAF6, the IRAK1-TRAF6 complex dissociates and activates the protein kinase complex TKA1, activated TAK1 phosphorylates MAP kinase and IKK, two distinct signals transduction. The phosphorylation of p38 MAPK and IKK under P-MAPA stimulation suggests that this immunomodulator binds to TLR2, since increased nitric oxide production was also observed in healthy dogs and the p38 MAPK pathway is involved in NO generation[44].

In macrophages from infected dogs treated with P-MAPA, a discrete increase in the phosphorylated proteins p38 MAPK and IKK was detected, but this was not statistically significant. It is possible that the increase in P-MAPA concentration leads to an increase in IKK and p38 MAPK, because the decrease in these molecules is a consequence of the infection, *Leishmania* promotes failure

in the MAPK signaling pathway, leading to macrophage dysfunction, due to the lack of response to IFN-gamma and the inhibition of iNOS gene expression[45]. *L. donovani* infection leads to deactivation of the signaling system, since the presence of the parasite increases the expression of phosphatases that inhibit the p38 MAPK signaling pathway[46].

Taken together, our findings indicate that P-MAPA increased TLR2 expression and induced ROS production and NO, which are related to resistance mechanisms in visceral leishmaniasis, suggesting that is has the potential to treat *Leishmania* diseases.

Acknowledgements

The authors would like to thank the São Paulo Research Support Foundation (FAPESP) for its financial support (2009/50426-9 and 2010/13166-6) and Farmabrasilis-Brazil.

References

[1] World Health Organization. Technical Report Series. Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases; March 2010 22-26 [Geneva].

[2] Alvar J, Cañavate C, Molina R, Moreno J, Nieto J. Canine leishmaniasis. Adv Parasitol 2004;57:1-88.

[3] Baneth G, Shaw SE. Chemotherapy of canine leishmaniosis. Vet Parasitol 2002;106:315-24

[4] Solano-Gallego L, Koutinas A, Miró G, Cardoso L, Pennisi MG, Ferrer L, Bourdeau P, Oliva G, Baneth G. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Vet Parasitol 2009;165:1-18.

[5] Dos-Santos WLC, Jesus EE, Paranhos-Silva M, Pereira AM, Santos JC, Baleeiro CO, Nascimento EG, Moreira ED, Oliveira GGS, Pontes-de-Carvalho LC. Associations among immunological, parasitological and clinical parameters in canine visceral leishmaniasis: Emaciation, spleen parasitism, specific antibodies and leishmanin skin test reaction. Vet Immunol Immunopathol 2008;123:251-259.

[6] Bourdoiseau G, Bonnefont C, Magnol JP, Saint-André I, Chabanne L. Lymphocyte subset abnormalities in canine leishmaniasis. Vet Immunol Immunopathol 1997;56:345-351.

[7] Lima VMF, Fattori KR, de Souza F, Eugênio FR, Santos PS, Rozza DB, Machado GF. Apoptosis in t lymphocytes from spleen tissue and peripheral blood of l. (l.) chagasi naturally infected dogs. Vet Parasitol 2012;184:147-153.

[8] Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 2009;22:240-273.

[9] Bhattacharya P, Bhattacharjee S, Gupta G, Majumder S, Adhikari A, Mukherjee A, Majumdar SB, Saha B, Majumdar S. Arabinosylated lipoarabinomannan-mediated protection in visceral leishmaniasis through up-regulation of toll-like receptor 2 signaling: An immunoprophylactic approach. J Infect Dis 2010; 202:145-155.

[10] Waldmann, TA. Immunotherapy: past, present and future 2003;9:269-267.

[11] Gomez-Ochoa P, Castillo JA, Gascón M, Zarate JJ, Alvarez F, Couto CG. Use of domperidone in the treatment of canine visceral leishmaniasis: A clinical trial. Vet J 2009;179:259-263.

[12] Fávaro WJ, Nunes OS, Seiva FR, Nunes IS, Woolhiser LK, Duran N, Lenaerts AJ. Effects of p-mapa immunomodulator on toll-like receptors and p53: Potential therapeutic strategies for infectious diseases and cancer. Infect Agent Cancer 2012;7:14.

[13] Justo GZ, Durán N, Queiroz ML. Myelopoietic response in tumour-bearing mice by an aggregated polymer isolated from aspergillus oryzae. Eur J Pharmacol 2000;388:219-226.

[14] Melo A, Justo GZ, de Souza Queiroz ML. Stimulation of myelopoiesis in listeria monocytogenes-infected mice by an aggregated polymer isolated from aspergillus oryzae. Hum Exp Toxicol 2001;20:38-45.

[15] Durán N, Gowen BB, Costa FTM, Justo GZ, Brocchi M, Nunes OS, Nunes IS. A biotechnological product and its potential as a new immunomodulator for

treatment of animal phlebovirus infection: Punta toro virus. Antiviral Res 2009;83:143-147.

[16] Justo GZ, Durán N, Queiroz ML. Natural killer cell activity, lymphocyte proliferation, and cytokine profile in tumor-bearing mice treated with mapa, a magnesium aggregated polymer from aspergillus oryzae. Immunopharmacol Immunotoxicol 2003;25:305-319.

[17] Santiago MEB, Silveira Neto L, Alexandre EC, Munari DP, Andrade MMC, Somenzari MA, Ciarlini PC, Lima VMF. Improvement in clinical signs and cellular immunity of dogs with visceral leishmaniasis using the immunomodulator p-mapa. Acta Trop 2013;127:174-180.

[18] Lima VMF, Gonçalves ME, Ikeda FA, Luvizotto MC, Feitosa MM. Anti-leishmania antibodies in cerebrospinal fluid from dogs with visceral leishmaniasis. Braz J Med Biol Res 2003;36:485-489.

[19] Nunes, I. Building a new model for pharmaceuticals—P-MAPA, a novel immunomodulator against virus, bacterial, and protozoan infections. In: Abstracts of the International Conference on Drug Design and Discovery in Developing Countries; Trieste, Italy, July 3–5, 2008; Abstract I-8. International Centre for Science and High Technology (ICS), United Nations Industrial Development Organization (UNIDO). Accessed at www.ics.trieste.it/portal/ActivityDocument.aspx?id=5711.

[20] Nunes, I.S., Justo, G.Z, Durán, N. Protein aggregate magnesium ammonium phospholinoleate palmitoleate anhydride immunomodulator, its production process and formulation. USA/PAT 10/978683; 2004.

[21] Sisto M, Brandonisio O, Panaro MA, Acquafredda A, Leogrande D, Fasanella A, Trotta T, Fumarola L, Mitolo V. Inducible nitric oxide synthase expression in leishmania-infected dog macrophages. Comp Immunol Microbiol Infect Dis 2001;24:247-254.

[22] Burgener IA, Jungi TW. Antibodies specific for human or murine toll-like receptors detect canine leukocytes by flow cytometry. Vet Immunol Immunopathol 2008;124:184-191.

[23] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}n] nitrate in biological fluids. Anal Biochem 1982;126:131-138.

[24] Asahina Y, Yoshioka N, Kano R, Moritomo T, Hasegawa A. Full-length cdna cloning of toll-like receptor 4 in dogs and cats. Vet Immunol Immunopathol 2003;96:159-167.

[25] Bazzocchi C, Mortarino M, Comazzi S, Bandi C, Franceschi A, Genchi C. Expression and function of toll-like receptor 2 in canine blood phagocytes. Vet Immunol Immunopathol 2005;104:15-19.

[26] Das S, Pandey K, Kumar A, Sardar AH, Purkait B, Kumar M, Kumar S, Ravidas VN, Roy S, Singh D, Das P. TGF-beta(1) re-programs TLR4 signaling in l. Donovani infection: Enhancement of shp-1 and ubiquitin-editing enzyme a20. Immunol Cell Biol 2011;90:640-654.

[27] Kropf P, Freudenberg N, Kalis C, Modolell M, Herath S, Galanos C, Freudenberg M, Muller I. Infection of C57BL/10ScCr and C57BL/10ScNCr mice

with leishmania major reveals a role for toll-like receptor 4 in the control of parasite replication. J Leukoc Biol 2004;76:48-57.

[28] Pinelli E, Gebhard D, Mommaas AM, Hoeij Mv, Langermans JAM, Ruitenberg EJ, Rutten VPMG. Infection of a canine macrophage cell line with leishmania infantum: Determination of nitric oxide production and anti-leishmanial activity. Vet Parasitol 2000;92:181-189.

[29] Vouldoukis I, Riveros-Moreno V, Dugas B, Ouaaz F, Becherel P, Debre P, Moncada S, Mossalayi MD. The killing of leishmania major by human macrophages is mediated by nitric oxide induced after ligation of the fc epsilon CD23 surface antigen. Proc Natl Acad Sci U S A 1995;92:7804-7808.

[30] Correa AP, Dossi AC, de Oliveira Vasconcelos R, Munari DP, de Lima VM. Evaluation of transformation growth factor beta1, interleukin-10, and interferon-gamma in male symptomatic and asymptomatic dogs naturally infected by leishmania (leishmania) chagasi. Vet Parasitol 2007;143:267-274.

[31]Alves CNF, de Amorim IFG, Moura EP, Ribeiro RR, Alves CF, Michalick MS, Kalapothakis E, Bruna-Romero O, Tafuri WL, Teixeira MM, Melo MN. Expression of IFN-gama, TNF-alpha, IL-10 and TGF-beta in lymph nodes associates with parasite load and clinical form of disease in dogs naturally infected with leishmania (leishmania) chagasi. Vet Immunol Immunopathol 2009;128:349-358.

[32] Cezario GA, de Oliveira LR, Peresi E, Nicolete VC, Polettini J, de Lima CR, Gatto M, Calvi SA. Analysis of the expression of toll-like receptors 2 and 4 and cytokine production during experimental leishmania chagasi infection. Mem Inst Oswaldo Cruz 2011;106:573-583.

[33] Charmoy M, Megnekou R, Allenbach C, Zweifel C, Perez C, Monnat K, Breton M, Ronet C, Launois P, Tacchini-Cottier F. Leishmania major induces distinct neutrophil phenotypes in mice that are resistant or susceptible to infection. J Leukoc Biol 2007;82:288-299.

[34] Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. Nat Immunol 2001;2:947-950.

[35] Gantt KR, Goldman TL, McCormick ML, Miller MA, Jeronimo SM, Nascimento ET, Britigan BE, Wilson ME. Oxidative responses of human and murine macrophages during phagocytosis of leishmania chagasi. J Immunol 2001;167:893-901.

[36] Michelin A F, Perri SH, Lima VM. Evaluation of TNF-alfa, IL-4, and IL-10 and parasite density in spleen and liver of L. (L.) chagasi naturally infected dogs. Ann Trop Med Parasitol 2011;105:373-383.

[37] Castro H, Sousa C, Santos M, Cordeiro-da-Silva A, Flohe L, Tomas AM. Complementary antioxidant defense by cytoplasmic and mitochondrial peroxiredoxins in leishmania infantum. Free Radic Biol Med 2002;33:1552-1562.

[38] Dolai S, Yadav RK, Pal S, Adak S. Leishmania major ascorbate peroxidase overexpression protects cells against reactive oxygen species-mediated cardiolipin oxidation. Free Radic Biol Med 2008;45:1520-1529.

[39] Panaro MA, Lisi S, Mitolo V, Acquafredda A, Fasanella A, Carelli MG, Brandonisio O. Evaluation of killing, superoxide anion and nitric oxide production by leishmania infantum-infected dog monocytes. Cytobios 1998;95:151-160.

[40] Farmabrasilis. Estudos de toxicidade e segurança de uso-Toxicidade in vitro e em animais. P-MAPA monografia do produto. Disponível em: http://www.farmabrasilis.org.br/todos_conteudos_interna. Acessado em: 29 de maio, 2011.

[41] Mookerjee Basu J, Mookerjee A, Sen P, Bhaumik S, Sen P, Banerjee S, Naskar K, Choudhuri SK, Saha B, Raha S, Roy S. Sodium antimony gluconate induces generation of reactive oxygen species and nitric oxide via phosphoinositide 3-kinase and mitogen-activated protein kinase activation in leishmania donovani-infected macrophages. Antimicrob Agents Chemother 2006; 50:1788-1797.

[42] Shakya N, Sane SA, Vishwakarma P, Bajpai P, Gupta S. Improved treatment of visceral leishmaniasis (kala-azar) by using combination of ketoconazole, miltefosine with an immunomodulator-picroliv. Acta Trop 2011;119:188-193.

[43] Mukherjee AK, Gupta G, Adhikari A, Majumder S, Kar Mahapatra S, Bhattacharyya Majumdar S, Majumdar S. Miltefosine triggers a strong proinflammatory cytokine response during visceral leishmaniasis: Role of TLR4 and TLR9. Int Immunopharmacol 2012;12:565-572.

[44] Kao S-J, Lei H-C, Kuo C-T, Chang M-S, Chen B-C, Chang Y-C, Chiu W-T, Lin C-H. Lipoteichoic acid induces nuclear factor-κb activation and nitric oxide synthase expression via phosphatidylinositol 3-kinase, akt, and p38 mapk in raw 264.7 macrophages. Immunol 2005;115:366-374.

[45] Olivier M, Gregory DJ, Forget G. Subversion mechanisms by which leishmania parasites can escape the host immune response: A signaling point of view. Clin Microbiol Rev 2005;18:293-305.

[46] Kar S, Ukil A, Sharma G, Das PK. Mapk-directed phosphatases preferentially regulate pro- and anti-inflammatory cytokines in experimental visceral leishmaniasis: Involvement of distinct protein kinase C isoforms. J Leukoc Biol 2010;88:9-20.

Table 1. TLR2/TLR4 expression data are represented as mean standard deviation in PBMCs from *Leishmania* infected dogs and healthy controls.

	CT (mean±sd)	Inf(mean±sd)	P value
TLR4	40,78±26,86	12,09±9,11	0,0229
TLR2	45,35±28,65	45,55±27,76	0,1094

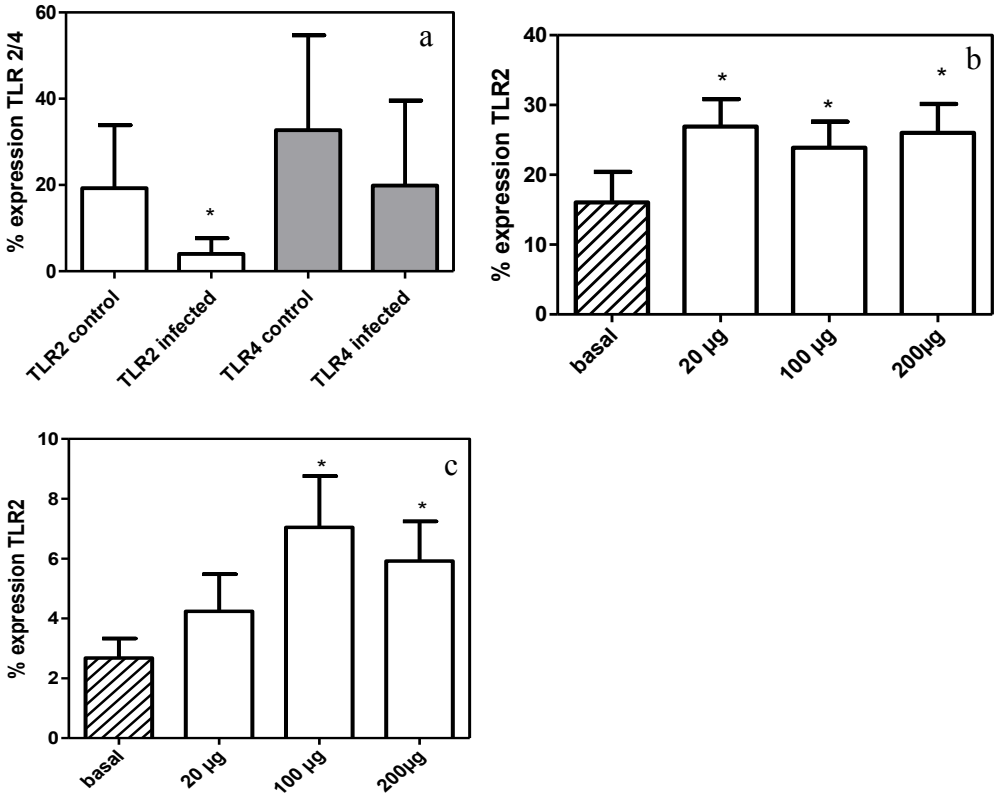

Figure 1. TLR2/ TLR4 expression in macrophages from mononuclear cells of infected dogs and controls. (a) The bars represent mean values per group. *P<0,05 significant differences between the mean baseline for dogs infected with *Leishmania* sp. and controls TLR2 and TLR4, (b) Expression of TLR2 in macrophages from control dogs was cultured in medium or with or P-MAPA (20 µg/mL, 100 µg/mL and 200 µg/mL) at 37 ° C, 5% CO_2. Cells were recovered after 24h of culture and the percentage of TLR2 expression was determined by flow cytometry. (c) Expression of TLR2 in macrophages from *Leishmania* sp. infected dogs was cultured in medium or with P-MAPA (20 µg/mL, 100 µg/mL and 200 µg/mL). Cells were stained after 24h of incubation, and the percentage of TLR2 expression was determined by flow cytometry. The bars represent the mean values of each group. The expression of TLR2 in macrophages of infected dogs *P<0,05.

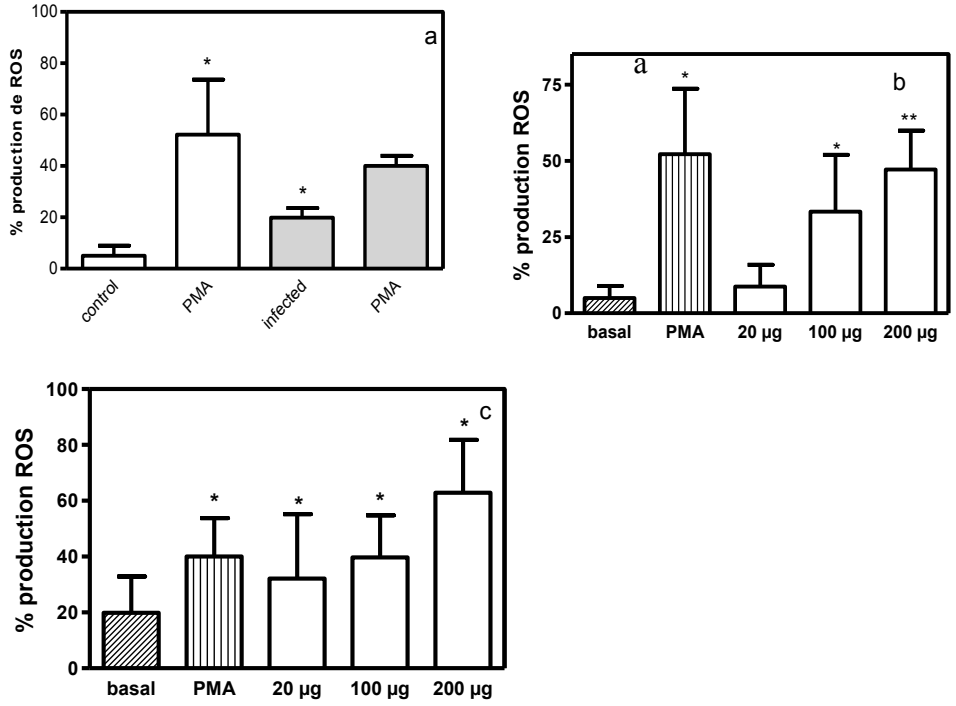

Figure 2. (a) ROS production in mononuclear cells from significant differences between the mean baseline for the groups studied of dogs infected with *Leishmania* sp. and controls. (b) ROS production in mononuclear cells of control dogs. (c) Production of ROS in infected dogs. Mononuclear cells were cultured in media or with PMA (1µM/mL) or P-MAPA (20 µg/mL, 100 µg/mL and 200 µg/mL) at 37°C with 5% CO_2. The cells were recovered after 24h of culture and production of ROS was determined by flow cytometry. The bars represent the mean values of each group. *P<0,05: significant differences between the averages of the values of the treatment and control.

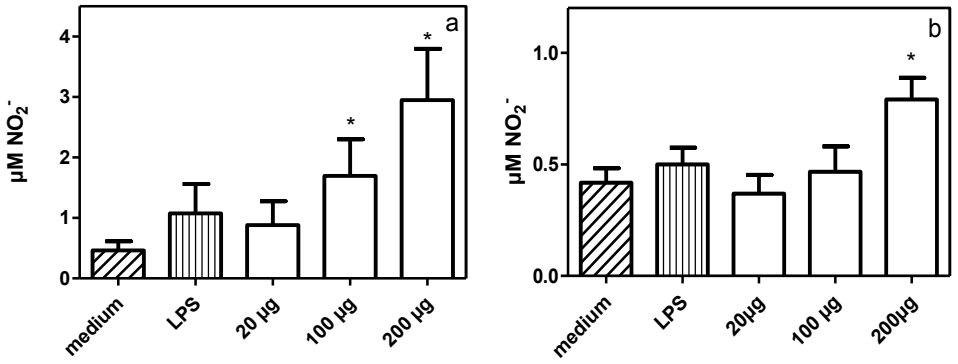

Figure 3. Production of NO by macrophages of controls dogs (a) and infected dogs (b). Macrophages were cultured in medium or with P-MAPA (20 µg/mL, 100 µg/mL and 200 µg/mL) or LPS (100 µg/mL). The culture supernatant was collected after 24h of incubation and the concentration of NO_{2^-} was determined using Griess reagent. The bars represent the average values of each group. *P<0,05 significant difference between the values of the mean treatment and control.

Table 2 – MAPKp38 protein expression (a) and IKK (b) phosphorylated in macrophages of control dogs (n = 12). The macrophages were cultured in medium or with LPS (100 µg/mL) or P-MAPA (20 µg/mL, 100 µg/mL and 200 µg/mL) at 37 ° C, 5% CO_2. After 24 h of culture the cells were recovered and detection was performed MAPKp38 and IKK-phosphorylated by flow cytometry.

	IKK (mean±sd)	P value	Mapk38(mean±sd)	P value
basal	1,53±2,52	-	0,84±1,21	-
LPS	2,30±2,84	0,0210	1,47±1,86	0,0108
20 µg/mL	2,77±2,70	0,0210	2,11±2,28	0,0029
100 µg/mL	3,28±3,83	0,0024	2,25±2,56	0,0005
200 µg/mL	3,55±4,17	0,0024	2,95±3,94	0,0010

CONCLUSÕES GERAIS

Os nossos resultados indicam que o P-MAPA aumenta a expressão de TLR2, que é a primeira linha de defesa contra leishmaniose, e induz a produção de ROS e NO, estes estão relacionados com os mecanismos de resistência na LV. Assim, sugerindo ser uma droga importante no tratamento da leishmaniose.

APÊNDICES

Apêndice A – Primeira página do artigo publicado.

International Immunopharmacology 18 (2014) 373–378

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Effects of P-MAPA immunomodulator on Toll-like receptor 2, ROS, nitric oxide, MAPKp38 and IKK in PBMC and macrophages from dogs with visceral leishmaniasis

L.M. Melo [a], J. Perosso [a], B.F.M. Almeida [a], K.L.O. Silva [a], M.A. Somenzari [b], V.M.F. de Lima [b,*]

[a] Pós-graduação em Ciência Animal, Faculdade de Medicina Veteinária, Universidade Estadual Paulista, Araçatuba, SP, Brazil
[b] Departamento de Clínica, Cirugia e Reprodução Animal, Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, SP, Brazil

ARTICLE INFO

Article history:
Received 19 November 2013
Accepted 9 December 2013
Available online 24 December 2013

Keywords:
Leishmania sp.
P-MAPA
Dog
Immunomodulatory
TLR

ABSTRACT

Leishmania (*L.*) *chagasi* is the etiologic agent of visceral leishmaniasis (VL) that can be transmitted to humans and dogs. VL in Brazil represents a serious public health problem; therefore, it is important to study new alternatives to treat infected dogs. In dogs, the therapeutic arsenal against canine VL is limited. The immunomodulator protein aggregate magnesium–ammonium phospholinoleate–palmitoleate anhydride (P-MAPA) improves immunocompetence when the immune system is impaired, but its dependence on Toll-like receptors (TLRs) and the mechanisms involved in immune response remain unclear. The in vitro action of P-MAPA on the expression of TLR2 and TLR4, reactive oxygen species (ROS), nitric oxide (NO) and p38 mitogen-activated protein kinase (p38 MAPK) and IKK phosphorylation was studied in mononuclear cells from peripheral blood and macrophages from healthy and *Leishmania*-infected dogs. The PBMC or macrophages were isolated and cultured with different concentrations of P-MAPA (20,100 and 200 μg/ml) in a humid environment at 37 °C with 5% CO_2. Observation revealed that *Leishmania*-infected dogs showed a decrease in TLR2 in macrophages compared with healthy dogs and in induction with P-MAPA. ROS were increased in PBMCs from *Leishmania* spp.-infected dogs compared with healthy dogs and P-MAPA improved ROS production. NO production was increased in culture supernatant from macrophages stimulated by P-MAPA in both healthy and *Leishmania* spp. infected dogs. Treatment of macrophages from healthy dogs with immunomodulatory P-MAPA induced p38 MAPK and IKK phosphorylation, suggesting signal transduction by this pathway. These findings suggest that P-MAPA has potential as a therapeutic drug in the treatment of canine visceral leishmaniasis.

1. Introduction

Visceral leishmaniasis is an endemic disease that has spread over several continents, principally in tropical and subtropical regions. It is caused by the *Leishmania infantum*, *Leishmania chagasi and Leishmania donovani* species and affects millions of people worldwide [1]. The parasite is transmitted by sand flies to mammals, including humans and dogs, via host blood-feeding. The dog is considered the most important urban reservoir of *L. chagasi* due to its high level of infection and its proximity to humans [2].

Treatment of canine visceral leishmaniasis (CVL) has certain limitations because the therapeutic arsenal against this disease is limited and the most commonly used drugs present high toxicity (nephrotoxicity, intestinal problems, muscle pain), are costly and can be ineffective in some cases [3,4]. Treatment failures have epidemiological implications since, following treatment, the dogs become asymptomatic but remain a reservoir for transmission of the parasite to sand flies [2]. These factors indicate that new drugs should be evaluated for the treatment of CVL.

The suppression of cellular immunity is the most important aspect of the pathogenesis and progression of CVL. Dogs infected with *L. infantum* show a negative response to the cutaneous test with parasite antigens [5] and a reduction in the number of T lymphocytes in peripheral blood [6] due to increased rates of T cell apoptosis [7]. Therefore, a drug that promptly reverses immunosuppression is desirable when treating infected dogs.

Cellular immune activation involves stimulation of receptors on macrophages, which are single chain glycoproteins that recognize conserved structures on the surface of pathogens. Binding pathogens to receptors on the host cell is achieved by activating antimicrobial multiple intracellular signals, including adapter molecules, kinases like p38 mitogen-activated protein kinase (p38 MAPK), and transcription factors. This results in signal transduction, gene expression and synthesis of various molecules, including cytokines, chemokines, adhesion molecules and immunoreceptors, which regulate the innate response, while simultaneously maintaining an important link with the adaptive immune response [8].

* Corresponding author at: Departamento de Clínica e Cirurgia e Reprodução Animal, Faculdade de Odontologia, UNESP, Campus de Araçatuba, Rua Clóvis Pestana, 793, Bairro Jardim Dona Amélia, CEP 16050-680 SP, Brazil.
E-mail address: vmflima@fmva.unesp.br (V.M.F. de Lima).

1567-5769/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.intimp.2013.12.012

Apêndice B – Normas da revista.

INTERNATIONAL IMMUNOPHARMACOLOGY

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

.
.

ISSN: 1567-5769

DESCRIPTION

.

International Immunopharmacology is the primary vehicle for the publication of original research papers pertinent to the overlapping areas of immunology, pharmacology, cytokine biology, immunotherapy, immunopathology and immunotoxicology. Review articles that encompass these subjects are also welcome.

The subject material appropriate for submission includes:
• Clinical studies employing immunotherapy of any type including the use of: bacterial and chemical agents; thymic hormones, interferon, lymphokines, etc., in transplantation and diseases such as cancer, immunodeficiency, chronic infection and allergic, inflammatory or autoimmune disorders.
• Studies on the mechanisms of action of these agents for specific parameters of immune competence as well as the overall clinical state.
• Pre-clinical animal studies and in vitro studies on mechanisms of action with immunopotentiators, immunomodulators, immunoadjuvants and other pharmacological agents active on cells participating in immune or allergic responses.
• Pharmacological compounds, microbial products and toxicological agents that affect the lymphoid system, and their mechanisms of action.
• Agents that activate genes or modify transcription and translation within the immune response.
• Substances activated, generated, or released through immunologic or related pathways that are pharmacologically active.
• Production, function and regulation of cytokines and their receptors.
• Classical pharmacological studies on the effects of chemokines and bioactive factors released during immunological reactions.
• Studies on the nature and function of drug and hormone receptors on lymphocytes and other cells in the immune system.• Studies of cell-derived or humoral factors that modify the immune system causing cytotoxicity, inducing antibody production and mediating inflammatory responses.
• The development of immunologically based assays and their application to disease, including assays for drugs, hormones, cyclic nucleotides, tumor antigens, etc.
• The Editors will be pleased to receive published books for possible independent review.

Please Bookmark this URL: http://www.elsevier.com/locate/intimp

Backfiles_Banner_sml.gifScienceDirect Backfiles
These Backfiles include Immunopharmacology and the International Journal of Immunopharmacology.

IMPACT FACTOR

.

2013: 2.711 © Thomson Reuters Journal Citation Reports 2014

ABSTRACTING AND INDEXING

.

Elsevier BIOBASE
Cambridge Scientific Abstracts
Chemical Abstracts MEDLINE® PASCAL/CNRS
Science Citation Index
Excerpta Medica
Current Awareness in Biological Sciences
Current Contents (Life Sciences, Clinical Medicine) SIIC Data Bases
Scopus

EDITORIAL BOARD

.

GUIDE FOR AUTHORS

Your Paper Your Way
ypyw-gfa-banner.gifyour paper your way

INTRODUCTION

International Immunopharmacology is an interdisciplinary journal devoted to the publication of original scientific papers interrelating immunology and pharmacology. Manuscripts will be considered for publication on the condition that the results reported are based on original research that has not been published elsewhere in any journal or language.

Types of Paper

• Full-length articles are full-length descriptions of original research. The scope may include basic science, clinical results, or applications. These manuscripts will undergo standard review. We emphasize that papers on natural products must meet the following specific criteria: a) any natural extract or drug should be fully characterized and information provided regarding origin - specifically, the "drug" under study must have been fractionated and the active substances within the natural product identified; b) isolation or purification techniques must be described in detail; and c) the authors must state in their paper that the material under study is endotoxin free, including not only natural products, but also all biologics and synthetics used for immunopharmacologic studies. The Editor-in-Chief requests that authors of Natural Product contributions read the guidelines as set out in the Editorial article of International Immunopharmacology volume 6, issue 8.
• Short Communications. These are short manuscripts that have important and generally conclusionary data on a specific issue. They should consist of no more than 3000 words with up to an additional 20 references and two tables and/or figures.
• Preliminary Studies/Reports. This section is for the publication of new observations that are promising but have not achieved the stature of a regular article, yet are judged by the reviewers or editorial staff to contribute a novel, important, or useful observation worthy of publication. These reports, including clinical studies on a limited patient population, will generally be brief reports of a single or a few observations or results comprising up to four printed pages including all references and display items; appropriate statistical analyses should be included.
• Reviews are comprehensive appraisals of research and clinical outcomes in a field of current interest. Potential authors are instructed to contact the Editor-in-Chief first regarding topic and suitability. All reviews are subject to the normal review process.
• Invited Commentary. Topical editorials, commentaries, prospective reviews and hypotheses demonstrating acceptable standards of originality. Such articles will only be published following an invitation to the author(s) from an Editor. Other prospective authors should obtain editorial permission before submitting a

manuscript. Commentaries (3000 words) will serve as the means for expressing controversial, critical and often personal evaluations of research trends. Commentaries in the form of prospective reviews or hypotheses will be composite presentations of wide-ranging observations from the literature, which should indicate, and possibly stimulate, new directions for potentially rewarding immunopharmacological research.
• Letters to the Editor may be considered at the discretion of the Editors.
• Book Reviews. Books for review may be sent to the Editor-in-Chief. Authors interested in reviewing a particular book should communicate directly with him.
• Meeting/Symposia reports are synopses of meetings and symposia, generally shorter than five journal pages. Please contact the Editor-in-Chief prior to submitting. Reports must be submitted within two weeks of the end of the meeting.
• Special Issues or Thematic Reports are collections of full-length articles and/or reviews on a specific topic. These require approval by the Editor-in-Chief; however, recommendations or suggestions are encouraged.

BEFORE YOU BEGIN
Ethics in publishing
For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/journal-authors/ethics.

Policy and ethics
Upon acceptance for publication of an article in International Immunopharmacology, the author tacitly agrees to make available any materials used in the published experiments, or novel or natural products disclosed in the article that are not commercially available, so that qualified investigators may confirm the observations.

Conflict of interest
All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest. Further information and an example of a Conflict of Interest form can be found at: http://help.elsevier.com/app/answers/detail/a_id/286/p/7923.

Submission declaration and verification
Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see http://www.elsevier.com/postingpolicy), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck http://www.elsevier.com/editors/plagdetect.

Authorship
All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Changes to authorship
This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:
Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.
After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Article transfer service
This journal is part of our Article Transfer Service. This means that if the Editor feels your article is more suitable in one of our other participating journals, then you may be asked to consider transferring the article to one of those. If you agree, your article will be transferred automatically on your behalf with no need to reformat. More information about this can be found here: http://www.elsevier.com/authors/article-transfer-service.

Copyright
This journal offers authors a choice in publishing their research: Open access and Subscription.

For subscription articles
Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright, see http://www.elsevier.com/copyright). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are

included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions.

For open access articles
Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (for more information see http://www.elsevier.com/OAauthoragreement). Permitted reuse of open access articles is determined by the author's choice of user license (see http://www.elsevier.com/openaccesslicenses).

Retained author rights
As an author you (or your employer or institution) retain certain rights. For more information on author rights for:
Subscription articles please see http://www.elsevier.com/journal-authors/author-rights-and-responsibilities.
Open access articles please see http://www.elsevier.com/OAauthoragreement.

Role of the funding source
You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies
Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies.

Open access
This journal offers authors a choice in publishing their research:

Open access
• Articles are freely available to both subscribers and the wider public with permitted reuse
• An open access publication fee is payable by authors or their research funder
Subscription
• Articles are made available to subscribers as well as developing countries and patient groups through our access programs (http://www.elsevier.com/access)
• No open access publication fee

All articles published open access will be immediately and permanently free for everyone to read and download. Permitted reuse is defined by your choice of one of the following Creative Commons user licenses:

Creative Commons Attribution (CC BY): lets others distribute and copy the article, to create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.
Creative Commons Attribution-NonCommercial-ShareAlike (CC BY-NC-SA): for non- commercial purposes, lets others distribute and copy the article, to create extracts, abstracts and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text and data mine the article, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, do not modify the article in such a way as to damage the author's honor or reputation, and license their new adaptations or creations under identical terms (CC BY-NC-SA).

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND): for non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

To provide open access, this journal has a publication fee which needs to be met by the authors or their research funders for each article published open access.
Your publication choice will have no effect on the peer review process or acceptance of submitted articles.

The open access publication fee for this journal is $3000, excluding taxes. Learn more about Elsevier's pricing policy: http://www.elsevier.com/openaccesspricing.

Language (usage and editing services)
Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop (http://webshop.elsevier.com/languageediting/) or visit our customer support site (http://support.elsevier.com) for more information.

Submission
Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts source files to a single PDF file of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF files at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail removing the need for a paper trail.

For online submission to this journal please visit: http://ees.elsevier.com/intimp.

Submit your article
Please submit your article via http://ees.elsevier.com/intimp

Referees
Authors are encouraged to provide the names, addresses, e-mail addresses and fax numbers of up to four researchers qualified to review the manuscript.

PREPARATION
NEW SUBMISSIONS
Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process.
As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or lay- out that can be used by referees to evaluate your manuscript. It should contain high enough quality figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

References
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements
There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.

If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes.
Divide the article into clearly defined sections.

Please ensure the text of your paper is double-spaced- this is an essential peer review requirement.

Figures and tables embedded in text
Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file.

REVISED SUBMISSIONS

Use of word processing software
Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure
Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered
1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
An Introduction should first "set the scene" for a non-specialist and then continue with the specific reasons for undertaking the investigation. Exhaustive reviews of the literature should be avoided and no attempt should be made to indicate the results obtained.

Materials and Methods. Procedures used should be given in sufficient detail to permit the repetition of the work by others. However, published procedures should be briefly summarized and only described in detail if they have been substantially modified. Special chemicals, drugs, etc. with their sources of supply should be grouped under a separate subheading, Materials.

Results. In this section the findings should be described without discussion of their significance. Subsections should be used to clarify the expression of the results.

Discussion. In this section the authors' interpretations of their findings should be accompanied by an assessment of their significance in relation to previous work. Subsections should be used wherever possible.

Essential title page information
• Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• Author names and affiliations. Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.
• Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract
Each paper must begin with an Abstract that summarizes the results obtained and the conclusions drawn. It should not exceed 250 words.

Graphical abstract
A Graphical abstract is optional and should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 ×
13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.
Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images also in accordance with all technical requirements: Illustration Service.

Highlights
Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords
Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations
The excessive use of abbreviations in the text is strongly discouraged. In particular, awkward and unfamiliar abbreviations and those intended to express concepts or experimental techniques will not be permitted.

Nomenclature and Units
• Drug names should be the official or approved names; trade names or common names may be given in brackets where the drug is first mentioned. The manufacturer's name and address must be given. The doses of the drugs should be given as unit weight/unit body weight, e.g. mmol/kg or mg/kg. Concentrations should be given in terms of molarity (eg. nM or uM), or as unit weight/unit volume solution (eg. mg/l) stating whether the weight refers to the salt or the active component of the drug. The molecular weight, inclusive of water of crystallization, should be stated if doses are given as unit weight.

• Isotopic specifications should conform to the IUPAC system (Biochem. J. (1975) 145, 1-20).

• The IUB Enzyme Commission (EC) number must be quoted with the full name of the enzyme when it is first mentioned in the text. Subsequently the accepted trivial name shall be used, e.g. Full Name: Acetyl-CoA:choline O-acetyltransferase (EC 2.3.1.6.) Trivial Name: Choline acetyltransferase not choline acetylase. For this information the author should refer to Enzyme Nomenclature (1973), Elsevier, Amsterdam.

• Authors are requested to follow the IUPHAR Guidelines for Classification of New Receptor Subtypes
(see Immunopharmacology, Vol. 30, p. 189 or p. 255).

• Symbols for physical units should be restricted to the Systeme Internationale (S.I.) Units. Examples of commonly used symbols can be found in Biochemical Journal 145, 1-20 (1975) and more detailed description, in Quantities, Units and Symbols (1971) The Royal Society, London.

Database linking
Elsevier encourages authors to connect articles with external databases, giving their readers one- click access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See http://www.elsevier.com/databaselinking for more information and a full list of supported databases.

Footnotes
Footnotes should be used sparingly. Number them consecutively throughout the article. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.
Table footnotes
Indicate each footnote in a table with a superscript lowercase letter.

Artwork Electronic artwork General points
• Make sure you use uniform lettering and sizing of your original artwork.

• Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Indicate per figure if it is a single, 1.5 or 2-column fitting image.
• For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
• Please note that individual figure files larger than 10 MB must be provided in separate source files. A detailed guide on electronic artwork is available on our website: http://www.elsevier.com/artworkinstructions.
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
Regardless of the application used, when your electronic artwork is finalized, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'.
TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi. TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF (or JPG): Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low.
• Supply files that are too low in resolution.
• Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Please note: Because of technical complications that can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions
Ensure that each illustration has a caption. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables
Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References
Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or
'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software
This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference formatting
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[1] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun
2010;163:51–9. Reference to a book:
[2] Strunk Jr W, White EB. The elements of style. 4th ed. New York: Longman; 2000.
Reference to a chapter in an edited book:
[3] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith
RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 2009, p. 281–304.
Note shortened form for last page number. e.g., 51–9, and that for more than 6 authors the first
6 should be listed followed by 'et al.' For further details you are referred to 'Uniform Requirements for Manuscripts submitted to Biomedical Journals' (J Am Med Assoc 1997;277:927–34) (see also http://www.nlm.nih.gov/bsd/uniform_requirements.html).

Video data
Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version

of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

AudioSlides
The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at http://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary data
Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high- resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist
The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.
Ensure that the following items are present:
One author has been designated as the corresponding author with contact details:
• E-mail address
• Full postal address
• Telephone
All necessary files have been uploaded, and contain:
• Keywords
• All figure captions
• All tables (including title, description, footnotes) Further considerations
• Manuscript has been 'spell-checked' and 'grammar-checked'
• All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Web)
• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge)
and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print
• If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes
For any further information please visit our customer support site at http://support.elsevier.com.

AFTER ACCEPTANCE
Use of the Digital Object Identifier
The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. Example of a correctly given DOI (in URL format; here an article in the journal Physics Letters B):
http://dx.doi.org/10.1016/j.physletb.2010.09.059
When you use a DOI to create links to documents on the web, the DOIs are guaranteed never to change.

Online proof correction
Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.
If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.
We will do everything possible to get your article published quickly and accurately - please upload all of your corrections within 48 hours. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility. Note that Elsevier may proceed with the publication of your article if no response is received.

Offprints
The corresponding author, at no cost, will be provided with a personalized link providing 50 days free access to the final published version of the article on ScienceDirect. This link can also be used for sharing via email and social networks. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's WebShop (http://webshop.elsevier.com/myarticleservices/offprints). Authors requiring printed

copies of multiple articles may use Elsevier WebShop's 'Create Your Own Book' service to collate multiple articles within a single cover (http://webshop.elsevier.com/myarticleservices/booklets).

AUTHOR INQUIRIES
You can track your submitted article at http://help.elsevier.com/app/answers/detail/a_id/89/p/8045/. You can track your accepted article at http://www.elsevier.com/trackarticle. You are also welcome to contact Customer Support via http://support.elsevier.com.